MicroRNA-335 and its target Rock1 synergistically influence tumor progression and prognosis in osteosarcoma

YONG WANG[1*], NINGNING WANG[2*], XIANDONG ZENG[3], JIE SUN[4],
GUANGBIN WANG[5], HUIMIAN XU[6] and WEI ZHAO[1]

Departments of [1]Orthopedics, [2]Cardiology, [3]Surgical Oncology and [4]Pathology,
Central Hospital Affiliated to Shenyang Medical College, Shenyang, Liaoning 110024;
[5]Department of Orthopedics, Shengjing Hospital of China Medical University, Shenyang, Liaoning 110072;
[6]Department of Surgical Oncology, The First Hospital of China Medical University, Shenyang, Liaoning 110001, P.R. China

Abstract. MicroRNA (miR)-335 and Rho-associated serine-threonine protein kinase 1 (Rock1) is ectopically expressed in multiple malignant tumors including osteosarcoma. The present study aimed to clarify whether the combined ectopically expressed miR-335 and Rock1 was correlated with clinicopathological features and prognosis in patients with osteosarcoma. The expression of miR-335 and Rock1 in 91 osteosarcoma tissue samples and 47 noncancerous bone tissues were determined respectively by *in situ* hybridization and immunohistochemistry. The association between miR-335 and Rock1 expression with the clinicopathological features of osteosarcoma was calculated using the Pearson's χ^2 test. Spearman's correlation analysis was used to study the association between the miR-335 and Rock1 expression. Survival curves were drawn using the Kaplan-Meier method. Univariate and multivariate analysis was performed using the Cox's proportional hazard regression model to allow the prognostic values to be assessed. Expression levels of miR-335 were significantly reduced in osteosarcoma tissues (P<0.001), compared with that in noncancerous bone tissues, while Rock1 expression was significantly increased in osteosarcoma tissues (P<0.001). A strong correlation between miR-335 and Rock1 expression was also shown (P<0.001). Decreased miR-335 expression was identified to be positively associated with higher clinical stage (P=0.004) and distant metastasis (P=0.016), while elevated expression levels of Rock1 was positively associated with a larger tumor size (P=0.013), higher clinical stage (P=0.027) and distant metastasis (P=0.022). The combined high expression of Rock1 and low expression of miR-335 was clearly associated with distant metastasis (P=0.010) and a higher clinical stage (P=0.010). Patients with elevated Rock1 or decreased miR-335 expression exhibited a worse overall survival (OS) and disease-free survival (DFS) compared with patients with decreased Rock1 or increased miR-335 (P<0.001 for the two). In addition, patients with decreased miR-335 and increased Rock1 had the worst OS and DFS (P<0.001 for the two). In multivariate survival analysis, clinical stage (P=0.002 for DFS, P=0.015 for OS), distant metastasis (P=0.024 for DFS, P=0.002 for OS), low expression of miR-335 (P<0.001 for DFS, P=0.002 for OS) and combined depressed miR-335 and elevated Rock1 (P=0.021 for DFS, P=0.050 for OS) expression remained as the independent prognostic factors for DFS and OS. The present findings suggest that there may be an association between the combined downregulation of miR-335 and upregulation of Rock1 with tumor progression and adverse prognosis in patients with osteosarcoma.

Correspondence to: Dr Wei Zhao, Department of Orthopedics, Central Hospital Affiliated to Shenyang Medical College, 5 South Seven West Road, Tiexi, Shenyang, Liaoning 110024, P.R. China
E-mail: zhaowei332@126.com

Dr Xiandong Zeng, Department of Surgical Oncology, Central Hospital Affiliated to Shenyang Medical College, 5 South Seven West Road, Tiexi, Shenyang, Liaoning 110024, P.R. China
E-mail: ZXD_landy1116@163.com

*Contributed equally

Introduction

Osteosarcoma is the most common type of primary malignant tumor originating from bone in young adolescents (1). Although progress has been made in surgical technology and combined therapeutic strategies, the total survival rate of osteosarcoma remains poor, particularly in patients with lung metastasis (2). Several molecules, including cluster of differentiation 44, tumor protein p53, PLA2G16, vascular endothelial growth factor and periostin have been identified for their use in the prognosis of osteosarcoma (3-7). However, it was reported that p53 only predicted a decreased short-term survival rate, but not 3- or 5-year survival, and cluster of differentiation 44 expression level is not associated with overall survival rate and metastasis (8,9). Therefore, it is important to identify novel and effective predictors of tumor progression and survival rate for patients with osteosarcoma.

MicroRNAs (miRNAs) are transcribed from non-protein coding genes or introns, and mediate the translational suppression or cleavage of their target mRNAs by binding to the complementary sites of their 3'-untranslated region. The aberrant expression of miRNA, as oncogenes or anti-oncogenes, is observed in various types of cancer (10,11). In osteosarcoma, certain miRNAs including miR-1247 and miR-27 have been identified as oncogenes (12,13), while others, including miR-15a, miR-646 and miR-218, are regarded as anti-oncogenes (14-16). These previous findings prove that miRNAs are a key element in the tumorigenesis of osteosarcoma.

Previously, miR-335 has been identified as a tumor suppressor in multiple types of cancer, including pancreatic, ovarian, breast, small cell lung and renal cancer (17-21). Furthermore, our former study identified that miR-335 could inhibit the metastasis of osteosarcoma by directly targeting the regulation of Rho-associated serine-threonine protein kinase 1 (Rock1), in order to mediate Rho signaling (22). Until recently, there was little research focusing on the prognostic values of miR-335 and Rock1 in osteosarcoma. An increasing number of studies have revealed that combined miRNA and target gene expression profiling may provide vital information for the diagnosis and prognosis of numerous types of human cancer (23,24). However, there have been a limited number of findings on the clinical significance of the combined expression of miR-335 and Rock1 in human osteosarcoma. Considering the aforementioned results, the present study hypothesized that there would be a connection between the combined abnormal expression of miR-335 and its target Rock1 with tumor progression and prognosis in patients with osteosarcoma.

The present study, examined miR-335 and Rock1 expression levels in osteosarcoma tissues and noncancerous bone tissues using *in situ* hybridization and immunohistochemistry, respectively. The clinical significance of abnormal miR-335 and Rock1 expression in osteosarcomas was explored.

Materials and methods

Patients and tissue samples. Approval for the present study was obtained from the Medical Ethics Committee of China Medical University (Liaoning, China). The need for written informed consents by the patients was waived due to the retrospective nature of the present study. A total of 91 osteosarcoma specimens were collected from patients with osteosarcoma who underwent curative tumor resection at the First Affiliated Hospital of China Medical University (Liaoning, China) between January 2003 and January 2008. During surgery, a total of 47 adjacent non-tumorous bone tissues were collected as controls. No patients underwent chemotherapy or radiotherapy prior to surgery. The clinical stage of the patients was classified according to the 6th edition of the Tumor Node Metastases Classification of Malignant Tumors, International Union against Cancer (25). The clinicopathological information for all patients is presented in Table I. All 91 patients with osteosarcoma were monitored during follow-up appointments, which lasted between 72 and 132 months. Mortality occurred in 9 patients during the follow-up period. The median overall survival (OS) and disease-free survival (DFS) of patients was 68 and 55 months, respectively.

In situ hybridization. miR-335 expression and subcellular localization in osteosarcoma tissues and matched noncancerous bone tissues were measured using *in situ* hybridization. Briefly, following the manufacturer's protocol, the tissue slides were mixed with 5'-digoxigenin LNA-modified-miR-335 (Exiqon A/S, Vedbaek, Denmark) using the IsHyb *in situ* Hybridization kit (BioChain Institute Inc., Eureka Drive, Newark, CA, USA).

Immunohistochemistry analysis. Rock1 protein expression and subcellular localization in osteosarcoma tissues and matched noncancerous bone tissues was detected using immunohistochemical staining. Firstly, tissue sections (4 μm thick) were incubated with the rabbit monoclonal Rock1 antibody (dilution, 1:100; cat. no. ab134181; Abcam, Cambridge, UK) at 4°C overnight and subsequently incubated with biotinylated secondary antibodies (dilution, 1:1,000; cat. no. E043201; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) at 37°C for 30 min. Samples were subsequently incubated with streptavidin horseradish peroxidase for another 30 min (LSAB kit; Dako; Agilent Technologies, Inc.) and stained with 3,3-diaminobenzidine. Finally, the slides were counterstained with hematoxylin, dehydrated in a graded ethanol series (absolute ethyl alcohol for 3 min, 95% ethanol for 3 min and 85% ethanol for 3 min), then mounted. Negative control sections were performed under the same conditions, except without primary antibodies.

Evaluation of in situ hybridization and immunostaining. The immunoreactivity intensity was scored according to four values: 0) Negative staining; i) weak positive staining; ii) moderate positive staining; and iii) strong positive staining. The proportion of stained cells for that intensity over the total number of tumor cells on the slide was recorded in 5% increments from a range of 0-100. The final immunoreactive score was obtained by multiplying the intensity score with the percentage of positively stained cells, and ranged between 0 and 300% and assigned with 5% increments (0, 5, 10, ...300%). These scores were used to determine the cutoff value in receiver operating characteristic (ROC) curves to discriminate between tumors of high miR-335 or Rock1 expression and those of low expression. The sensitivity and specificity was plotted on ROC curves to investigate the survival status of patients with osteosarcoma.

Statistical analysis. All data were analyzed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). The Student's *t*-test was used to compare the difference for data with normal distribution, while Wilcoxon rank-sum tests or Kruskal-Wallis tests were used to compare the difference for data with unequal variance. The association between miR-335 and Rock1 expression and clinicopathological characteristics in patients with osteosarcoma was evaluated using the Pearson's χ^2 test or Fisher's exact probability test. The association between miR-335 and Rock1 expression was assessed by Spearman's rank correlation analysis. The Kaplan-Meier curves were plotted to demonstrate the survival difference, and the survival probabilities were assessed using a log-rank test. Univariate and multivariate Cox's proportional hazards regression models were used to assess the association of potential confounding variables with

the OS or DFS. P≤0.05 was considered to indicate a statistically significant difference.

Results

Subcellular localizations and expression patterns of miR-335 and Rock1 in osteosarcoma tissues. In situ hybridization and immunohistochemistry analysis were used to confirm the subcellular localizations and the expression patterns of miR-335 and Rock1 protein in 91 osteosarcoma samples and 47 noncancerous bone samples respectively. As demonstrated in Fig. 1, miR-335 and Rock1 immunopositive staining were mainly localized in the cytoplasm. It was also observed that compared to noncancerous bone tissues, the miR-335 expression levels were considerably decreased in osteosarcoma tissues (P<0.001), while Rock1 expression levels increased significantly in osteosarcoma tissues (P<0.001).

Selection of the cutoff value for miR-335 and Rock1. ROC curve analysis was applied to determine an optimal cutoff value for the expression of miR-335 and ROCK1 in osteosarcoma samples. Considering the OS status, cutoff values of 115 and 87.5% were selected for the expression of miR-335 and Rock1, respectively (Fig. 2A and B). Tumors in which the immunohistological scores were ≥115 and <115% were defined as those of high and low expression of miR-335, respectively. Tumors with immunohistological scores ≥87.5 and <87.5% were defined as those with high and low expression of Rock1, respectively.

miR-335 expression associates with Rock1 expression in osteosarcoma tissues. In Table II, it can be observed how miR-335 and Rock1 were expressed in 91 osteosarcoma samples. As shown in the cutoff value used in the present study, 11/91 (12.09%) samples were of high miR-335 and high Rock1 expression, 11/91 (12.09%) were of low miR-335 and low Rock1 expression, 31/91 (34.07%) were of high miR-335 expression, but low Rock1 expression, and 38/91 (41.76%) were of low miR-335 expression but high Rock1 expression. There was a significant negative correlation between miR-335 and Rock1 expression in osteosarcoma tissues (r=-0.378, P<0.001, Table II), according to Spearman's correlation analysis.

Association between combined low-expression of miR-335 and high-expression of Rock1 and the aggressive clinicopathological features of patients with osteosarcoma. As demonstrated in Table III, the low expression of miR-335 was significantly associated with distant metastasis (P=0.016) and grade IIB/III osteosarcoma (P=0.004). The high expression of Rock1 was also significantly associated with distant metastasis (P=0.022), grade IIB/III osteosarcoma (P=0.027) and higher tumor size (P=0.013). Table IV presents the association of combined miR-335 and Rock1 expression with distant metastasis and clinical stage. Out of all comparisons investigated in the present study, tumors with a high expression of Rock1 but low expression of miR-335 were the most strongly associated with distant metastasis (P=0.010) and a higher clinical stage (P=0.010).

Prediction of poor prognosis of osteosarcoma patients using combined downregulated miR-335 and upregulated Rock1. Kaplan-Meier analysis and log-rank test were used to evaluate

Table I. Clinicopathological characteristics of patients with osteosarcoma.

Characteristics	No. of cases	%
Age at diagnosis, years	91	
<18	45	49.5
≥18	46	50.5
Gender	91	
Female	50	54.9
Male	41	45.1
Tumor size, cm	91	
<5	45	49.5
≥5	46	50.5
Anatomic location	91	
Tibia/femur	56	61.5
Elsewhere	35	38.5
Histological subtype	90	
Osteoblastic	30	33.3
Chondroblastic	30	33.3
Fibroblastic	12	20.0
Mixed	18	13.4
Clinical stage	87	
I+IIA	54	62.1
IIB/III	33	37.9
Distant metastasis	91	45.1
Absent	55	60.4
Present	36	39.6

Table II. Expression of miR-335 and Rock1 proteins in 91 patients with osteosarcoma.

miR-335	Rock1 expression		P-value
	High (n=55)	Low (n=36)	
High (n=42)	11	31	<0.001
Low (n=49)	38	11	

P-value obtained from Spearman's rank correlation test. miR, microRNA; Rock1, Rho-associated serine-threonine protein kinase 1.

the association of different miR-335 and Rock1 expression level with the OS or DFS in osteosarcoma patients. The data demonstrates that a high expression of Rock1 was significantly associated with shorter OS (P<0.001, Fig. 3A) and DFS (P<0.001, Fig. 3B), and the low expression of miR-335 was also evidently associated with shorter OS (P<0.001, Fig. 3C) and DFS (P<0.001, Fig. 3D). Furthermore, univariate and multivariate Cox's proportional hazards regression models were used to evaluate the association of potential confounding variables with the OS or DFS.

Univariate results identified that gender (P=0.020 for OS), higher tumor size (P=0.008 for DFS; P=0.001 for OS), higher
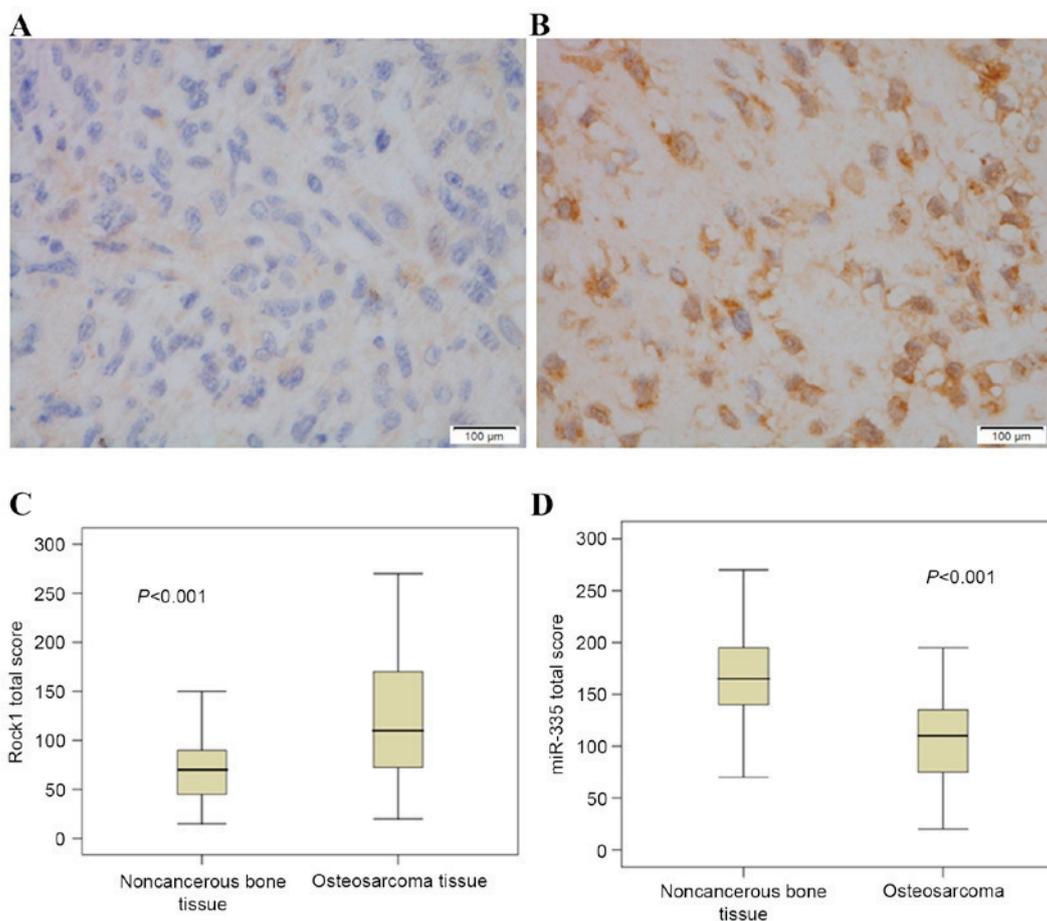
Figure 1. Comparison of protein expression in osteosarcoma and adjacent noncancerous bone tissues. Representative *in situ* hybridization images for miR-335 expression in (A) osteosarcoma tissues and (B) adjacent noncancerous bone tissues (original magnification, x400). Compared with the adjacent noncancerous bone tissues, the expression of (C) Rock1 protein was significantly increased, while (D) miR-335 was significantly decreased in osteosarcoma tissues. miR, microRNA; Rock1, Rho-associated serine-threonine protein kinase 1.
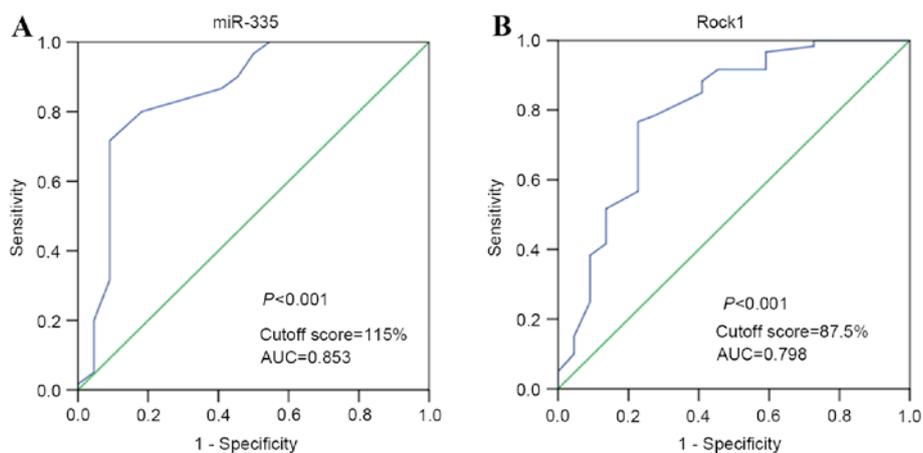

Figure 2. Receiver operating characteristic curves were used to determine the cutoff score for (A) miR-335 and (B) Rock1 expression based on the overall survival status of patients with osteosarcoma. The sensitivity and specificity for each outcome were plotted and the AUCs and P-value are indicated. miR, microRNA; Rock1, Rho-associated serine-threonine protein kinase 1; AUC, areas under curve.

clinical stage (P<0.001 for DFS; P<0.001 for OS), distant metastasis (P=0.006 for DFS; P<0.001 for OS), anatomic location (P<0.001 for DFS), miR-335 low expression (P<0.001 for DFS; P<0.001 for OS) and high Rock1 expression (P<0.001 for DFS; P=0.001 for OS), were associated with the OS and DFS in

patients with osteosarcoma (Table V). In addition, multivariate Cox regression analysis identified that higher clinical stage (P=0.002 for DFS; P=0.015 for OS), distant metastasis (P=0.024 for DFS; P=0.002 for OS) and low expression of miR-335 (P<0.001 for DFS; P=0.002 for OS) remained independent

Table III. Association of miR-335 and Rock1 expression with the clinicopathological features of patients with osteosarcoma.

Characteristics	No. of cases	miR-335 low, n (%)	P-value	Rock1 high, n (%)	P-value
Age at diagnosis, years			0.746		0.346
<18	45	25 (55.6)		25 (55.6)	
≥18	46	24 (52.2)		30 (65.2)	
Gender			0.845		0.722
Female	40	22 (55.0)		25 (62.5)	
Male	51	27 (52.9)		30 (58.8)	
Tumor size, cm			0.113		0.013
<5	46	21 (45.7)		22 (47.8)	
≥5	45	28 (62.2)		33 (73.3)	
Anatomic location			0.618		0.090
Tibia/femur	56	29 (51.8)		30 (53.6)	
Elsewhere	35	20 (57.1)		25 (71.4)	
Histological subtype			0.067		0.510
Osteoblastic	30	11 (36.7)		16 (53.3)	
Chondroblastic	30	19 (63.3)		19 (63.3)	
Fibroblastic	12	6 (50.0)		6 (50.0)	
Mixed	18	13 (72.2)		5 (27.8)	
Clinical stage			0.004		0.027
I+IIA	54	22 (40.7)		28 (51.9)	
IIB/III	33	24 (72.7)		25 (75.8)	
Distant metastasis			0.016		0.022
Absent	55	24 (43.6)		28 (50.9)	
Present	36	25 (69.4)		27 (75.0)	

P-value obtained from Pearson's χ^2 or Fisher's exact test. miR, microRNA; Rock1, Rho-associated serine-threonine protein kinase 1.

Table IV. Association of the combination of miR-335 and Rock1 expression with clinical stage and distant metastasis of osteosarcoma.

		miR-335 and Rock1 expression			
Variables	No. of cases	miR-335-low/ Rock1-high, n (%)	Both high or low	miR-335-high/ Rock1-low, n (%)	P-value
Clinical stage					0.010
I+IIA	54	17 (31.5)	16 (29.6)	21 (38.9)	
IIB/III	33	20 (60.6)	9 (27.3)	4 (12.1)	
Distant metastasis					0.010
Absent	55	16 (29.1)	20 (36.4)	19 (34.5)	
Present	33	22 (61.1)	8 (22.2)	6 (16.7)	

P-value obtained from Pearson's χ^2 test or Fisher's exact text. miR, microRNA; Rock1, Rho-associated serine-threonine protein kinase.

prognostic factors for OS in patients with osteosarcoma. Subsequently, the present study combined miR-335 and Rock1 expression into three groups: High expression of miR-335 and Rock1, either high expression of miR-335 or Rock1, and low expression miR-335 and Rock1 to investigate whether the combined expression of miR-335 and Rock1 exhibited an effect on the progression and prognosis of osteosarcoma.

Kaplan-Meier analysis and log-rank tests (Fig. 3E and F) revealed that the OS and DFS of patients with high expression of Rock1 and low expression of miR-335 was considerably shorter compared with those with high or low expression of miR-335 and Rock1, and those with low Rock1 expression and high miR-335 expression (P<0.001 for the two). Results of univariate and multivariate Cox proportional hazards

Table V. Univariate Cox regression analysis of OS and DFS in patients with osteosarcoma.

Category	Disease-free survival		Overall survival	
	RR (95% CI)	P-value	RR (95% CI)	P-value
Age, years (≥18/<18)	1.151 (0.714-1.855)	0.564	1.172 (0.705-1.946)	0.541
Gender (male/female)	1.105 (0.681-1.793)	0.686	0.951 (0.570-1.588)	0.020
Tumor size, cm (≥5.0/<5.0)	1.912 (1.184-3.090)	0.008	2.326 (1.388-3.897)	0.001
Clinical stage (IB/III/I+IIA)	4.031 (2.377-6.834)	<0.001	4.098 (2.396-7.011)	<0.001
Histological subtype (mixed/fibroblastic/ (Mixed/fibroblastic/chondroblastic/osteoblastic)	1.052 (0.870-1.272)	0.603	1.051 (0.858-1.288)	0.630
Distant metastasis (present/absent)	1.991 (1.216-3.259)	0.006	3.450 (1.999-5.954)	<0.001
Anatomic location (elsewhere/tibia/femur)	2.513 (1.63-3.87)	<0.001	1.539 (0.919-2.578)	0.101
miR-335 expression (negative/positive)	0.310 (0.184-0.523)	<0.001	0.287 (0.163-0.507)	<0.001
Rock1 expression (positive/negative)	2.633 (1.528-4.535)	<0.001	2.887 (1.579-5.276)	0.001
miR-335/Rock1 expression (miR-335-low and Rock1-high/others)	2.542 (1.553-4.161)	<0.001	2.931 (1.735-4.950)	<0.001

OS, overall survival; DFS, disease free survival; RR, relative risk; CI, confidence interval; miR, microRNA; Rock1, Rho-associated serine-threonine protein kinase.

Table VI. Multivariate cox regression analysis of OS and DFS in osteosarcoma patients.

Category	Disease-free survival		Overall survival	
	RR (95% CI)	P-value	RR (95% CI)	P-value
Gender (male/female)	-	-	0.748 (0.428-1.306)	0.307
Tumor size, cm (≥5.0/<5.0)	1.324 (0.776-2.287)	0.314	1.810 (0.964-3.398)	0.065
Clinical stage (IB/III/I+IIA)	2.607 (1.427-4.762)	0.002	2.243 (1.169-4.305)	0.015
Distant metastasis (present/absent)	1.956 (1.091-3.506)	0.024	3.056 (1.514-6.167)	0.002
Anatomic location (elsewhere/tibia/femur)	1.443 (0.855-2.434)	0.169	1.119 (0.637-1.965)	0.696
miR-335 expression (negative/positive)	0.173 (0.065-0.459)	<0.001	0.170 (0.056-0.519)	0.002
Rock1 expression (positive/negative)	2.182 (0.917-5.190)	0.078	2.469 (0.903-6.755)	0.078
miR-335/Rock1 expression (miR-335-low and Rock1-high/others)	0.243 (0.073-0.807)	0.021	0.253 (0.064-0.998)	0.050

OS, overall survival; DFS, disease free survival; RR, relative risk; CI, confidence interval; miR, microRNA; Rock1, Rho-associated serine-threonine protein kinase 1.

regression analysis are presented in Table VI. The findings demonstrate that combined high Rock1 expression and low miR-335 expression was considered as an independent prognostic factor for shorter OS (P=0.050) and DFS (P=0.021) in patients with osteosarcoma.

Discussion

The present study used a number of clinical samples in order to attempt to detect the combined expression of miR-335 and its target gene Rock1 in osteosarcoma tissues, to analyze its association with osteosarcoma clinicopathological characteristic and to assess the correlation of combined miR-335 and Rock1 expression with the prognosis of patients with osteosarcoma. The present findings provide evidence that the combined

downregulation of miR-335 and upregulation of Rock1 may be associated with the progression and undesirable prognosis in osteosarcoma.

Recently, numerous studies have reported that miRNAs, which target various oncogenes or anti-oncogenes, may perform a diverse role in tumorigenesis (26-28). Therefore, miRNAs are suggested as a type of prognostic biomarker and potential therapeutic site for tumor treatment. As a transcript of genomic region chromosome 7q32.2, the function of miR-335 has been widely reported (29). It was revealed that miR-335 functions as a tumor suppressor in malignant tumors and that the loss of miR-335 expression may lead to the progression of aggressive tumor phenotypes. Wang *et al* (30) reported that miR-335 could directly suppress B-cell lymphoma 2 and lead to inhibition of the proliferation and invasion in renal
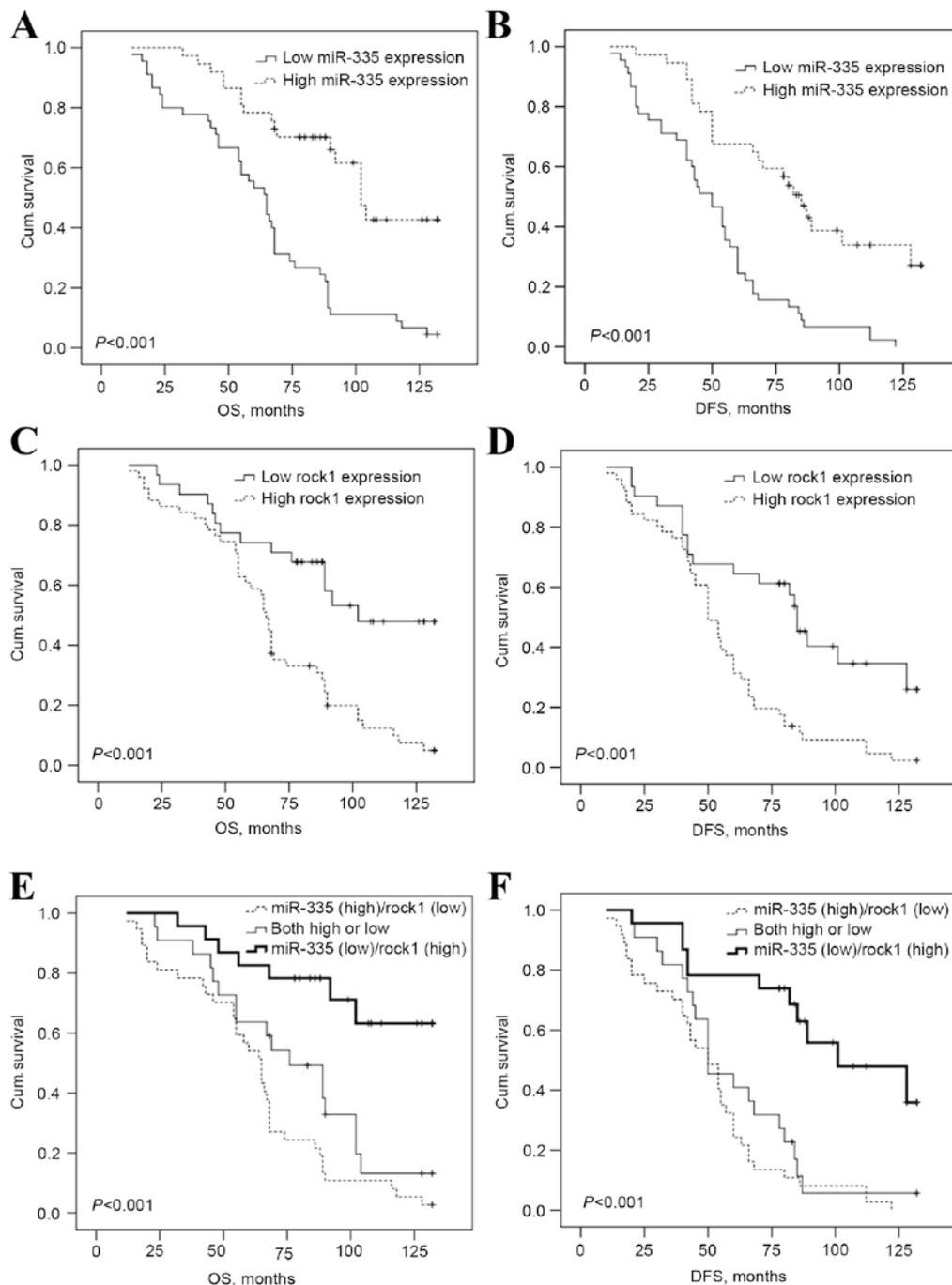
Figure 3. Kaplan-Meier survival analysis of miR-335 and Rock1 expression in patients with osteosarcoma. Survival curves show the association between miR-335 expression and (A) OS or (B) DFS in patients with osteosarcoma. Survival curves show the association between Rock1 expression and (C) OS and (D) DFS in 91 patients with osteosarcoma. Survival curves show the association between the combined expression of miR-335 and Rock1 and (E) OS or (F) DFS in patients with osteosarcoma. miR, microRNA; Rock1, Rho-associated serine-threonine protein kinase 1; OS, overall survival; DFS, disease free survival.

cell carcinoma cells. Gao *et al* (18) reported that miR-335 could target the regulatory oncoprotein c-Met and suppress the migration of breast cancer cells. However, miR-335 also functions as a tumor oncogene in certain types of cancer. Shu *et al* (31) identified that targeting oncogenic miR-335 suppressed the growth and invasion of malignant astrocytoma cells. Shi *et al* (32) reported that miR-335 directly targeted retinoblastoma 1 and promoted meningiomas cellular proliferation. In a previous study, we revealed that miR-335 could target Rock1 and cause the suppression of migration and invasion in osteosarcoma cells (22). Our data proved that there is a

marked decline in the expression levels of miR-335 in human osteosarcoma tissues compared with noncancerous bone tissues, and that there is also a close correlation of downregulated miR-335 expression with osteosarcoma progression, and even adverse outcomes in patients with osteosarcoma, which increases evidence that miR-335 may be a tumor suppressor in osteosarcoma.

Rock consists of two isoforms, Rock1 and Rock2 (23), and is the key kinase of Rho signaling (22). Activated Rocks promote the reassembly of focal contacts and the formation of stress-fibers while interacting with the actin

cytoskeleton (33). Subsequently, this regulates various cellular processes, including cellular proliferation, assembly of focal adhesion, fomation of invadopodium and acto-myosin contractility (34). An increasing number of evidence demonstrates the key role Rocks perform in oncogenesis, particularly regarding Rock1. Oellers *et al* (35) identified that Rock1 was essential for glioma cell migration on myelinated axons. Kamai *et al* (36) revealed a prominent association of the Rho/Rock pathway with the invasion/migration of bladder cancer. Akagi *et al* (37) demonstrated that Rock1 could be a novel prognostic marker in vulvar cancer. In osteosarcoma, Liu *et al* (38) revealed that the downregulation of Rock1 could suppress proliferation but promote apoptosis in osteosarcoma cells. Cai *et al* (23) identified that the high expression of Rock1 was significantly associated with the metastasis of osteosarcoma and the response to preoperative chemotherapy. The present study identified a significant elevation of Rock1 but decreased expression of miR-335 in human osteosarcoma tissues. Evidence also demonstrated that there was a close correlation of the upregulated Rock1 expression with osteo-sarcoma progression and adverse outcomes in patients with osteosarcoma, which additionally suggests that Rock1 may be an oncogene in osteosarcoma.

However, whether the combined expression of miR-335 and Rock1 is applicable for use in the clinical setting remains unknown in osteosarcoma. The findings of the present study demonstrate that the combined high expression of Rock1 and low expression of miR-335 was positively associated with distant metastasis, higher clinical stage and worse OS and DFS. Therefore, the prediction of advanced tumor progression and unfavorable prognosis can be determined by the down-regulation of miR-335 and the upregulation of its target gene, Rock1, for patients with osteosarcoma.

In summary, miR-335 was identified to be downregulated, while Rock1 was upregulated in patients with osteosarcoma. Additionally, the combined high expression of Rock1 and low expression of miR-335 was positively correlated with distant metastasis, higher clinical stage, and poor OS and DFS. In addition, multivariate survival analysis demonstrated that the combined low expression of miR-335 and high expression of Rock1 were independent prognostic factors for OS and DFS. Finally, data in the present study provided evidence that the combined downregulation of miR-335 and upregulation of Rock1 may be associated with tumor progression and poor prognosis in patients with osteosarcoma.

Acknowledgements

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