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miR-199a-5p regulates *HIF-1α* and *OSGIN2* and its expression is correlated to soft-tissue sarcoma patients' outcome

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Abstract. Soft tissue sarcomas are a heterogeneous group of malignant neoplasms of mesenchymal origin. Partly due to hypoxia, an aggressive and radioresistant phenotype frequently develops, resulting in poorer patient outcome. microRNAs (miRNAs) are tiny, non-coding regulators of gene expression and in situations of cellular stress situations may predict clinical progression and patient outcome. In the present study, hypoxia-associated miR-199a-5p expression in 96 soft tissue sarcoma samples was analysed by reverse transcription-quantitative polymerase chain reaction and associations between miR-199a-5p expression and patient clinicopathological characteristics and survival were measured. Additionally, luciferase reporter assays analyzed the post-transcriptional regulation of hypoxia-associated genes hypoxia-inducible factor 1α (*HIF-1α*), oxidative stress induced growth inhibitor 2 (*OSGIN2*) and vascular endothelial growth factor (*VEGF*) by miR-199a-5p. Survival analyses indicated that low expression of miR-199a-5p was significantly correlated with poorer tumor-specific survival (univariate Cox's-Regression analyses; relative risk=1.92, P=0.029). Furthermore, it was demonstrated that the 3'UTR of *HIF-1α* and *OSGIN2* genes were regulated by miR-199a-5p *in-vitro*, although the 3'UTR of *VEGF* was not. To the best of our knowledge, this is the first report demonstrating the regulation of the 3'untranslated region of the *OSGIN2* gene by miR-199a-5p and a significant correlation between low miR-199a-5p expression and a poor outcome of patients with soft tissue sarcoma.

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Abbreviations: HIF-1α, hypoxia-inducible factor 1α; OSGIN2, oxidative stress induced growth inhibitor 2; VEGF, vascular endothelial growth factor; RR, relative risk of tumor-related death; p, probability; CI, 95% confidence interval; r_S, Spearman's rank correlation coefficient; h, hour; UTR, untranslated region

Key words: miR-199a-5p, hypoxia, soft tissue sarcoma, HIF-1α, OSGIN2, survival

Introduction

MicroRNAs (miRNAs) are small (18-25 nt), non-coding RNAs of endogenous origin. To date, >2,500 different mature miRNA species have been detected in humans (1). Following maturation, double-stranded miRNAs are immediately bound to Argonaute proteins and unwound. In the single-stranded conformation, they build up the active component of the RNA-induced silencing complex (RISC) (2,3). miRNAs bind to complementary sequences in the 3'untranslated region (UTR) of their target mRNAs, thus acting as translational repressors. This action is primarily performed by a hexamer sequence at the 5'UTRs called the seed sequence and the subsequent translocation to the P-bodies (processing bodies), which are specialized cellular components for RNA silencing and decay (4,5). Due to the simple target recognition sequence and the vast number of different species of miRNA, it is estimated that ~60% of the human protein-coding genes are regulated post-transcriptionally by miRNAs (6). Due to their high abundance and immense impact on cellular gene expression profiles, miRNAs serve an important role in cellular proliferation and differentiation processes. Furthermore, they contribute to the adaptation to cellular stress, including stress caused by an acidic or hypoxic environment (7,8).

Hypoxia, the state of decreased oxygen saturation in a tissue, is a major problem in the treatment of solid tumors. Due to rapid growth, regions in solid tumors tend to be cut off from oxygen maintenance by blood vessels. The tumor cells must adapt to this new environmental challenge, leading to the clonal selection of successful tumor cells, increased aggressiveness and decreased chemo- and radiosensitivity of the tumor.

There are several studies describing the effect of hypoxia on miRNA expression. Kulshreshthra *et al* (9,10) have demonstrated that the miRNAs miR-210, miR-23a, miR-24-1, miR-26b and miR-107 are overexpressed in hypoxic conditions,

while the miRNAs miR-19a, miR-122a, miR-141, miR-101, miR-186, miR-374, miR-424, miR-422b, miR-320, miR-29b and miR-197 are all downregulated.

The most prominent hypoxia-induced miRNA is the ubiquitously expressed miR-210 (11). In the majority of cell lines studied so far, hypoxia triggers the upregulation of miR-210 (12). Furthermore, high expression of miR-210 is associated with a poorer outcome for patients with mammary carcinoma, head and neck carcinoma, pancreatic adenocarcinoma or soft tissue sarcoma (13-16). Thus, under hypoxic conditions, miR-210 may act as an oncomir, promoting cell survival and adaptation to the changed environment and leading to increased tumor aggressiveness. Hypoxia-inducible miRNAs, including miR-210, may be antagonized by hypoxia-downregulated microRNAs, such as miR-199a. miR-199a is located within a cluster together with miR-214, which is regulated concordantly by diverse transcription factors, including *TWIST1* (17). miR-199a-5p regulates hypoxia-inducible factor 1α (*HIF-1α)* and *Sirt* mRNA, thus suppressing adaptation to persistent hypoxic conditions (18). Furthermore, expression of the homologous miR-199b is inversely correlated to HIF-1α protein expression in hepatocellular carcinoma (HCC) and its elevated expression predicts an improved outcome for patients with HCC (19). Recently, a self-regulatory network in testicular germ cell tumors was investigated and it was demonstrated that *p53* is activated and aids in the positive regulation of miR-199a-2/miR-214 by repressing *DNMT1* (20).

In the current study, the impact of miR-199a-5p expression on the clinical parameters and outcome of patients with soft tissue sarcoma was evaluated. Several studies have highlighted the association of miR-199a-5p downregulation with the cellular hypoxia response (18,21-23). Furthermore, hypoxia is an important factor in sarcomagenesis and the increasing aggressiveness of sarcoma; therefore, the present study evaluated the impact of miR-199a-5p on hypoxia-related genes by retesting the regulation of *HIF-1α* and *VEGF* through miR-199a-5p and additionally proposing oxidative stress induced growth inhibitor 2 (*OSGIN2)* as a novel miR-199a-5p target gene.

Materials and methods

Patients. The cohort consisted of 96 patients with soft tissue sarcoma who underwent surgical tumor resection between March 1998 and April 2001 in the Department of Surgery, University of Leipzig (Leipzig, Germany). None of the patients had received adjuvant treatment prior to resection. Patients and tissue samples have been described in previous studies (24,25). Tissues were snap-frozen in liquid nitrogen immediately following resection and stored at -80˚C. The present study was approved by the local ethics committee of the Martin Luther University Halle-Wittenberg. All patients gave written informed consent.

RNA isolation. RNA was isolated using TriZol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA USA) according to the manufacturer's protocol. Briefly, the tissue was cut in a cryotome and 20-30 slices (30-μm thick) were lysed in TriZol reagent. The tissue protein and DNA were precipitated by centrifugation. DNAse I (Qiagen GmbH, Hilden, Germany) digestion was performed on the flow-through for 30 min to eliminate remaining traces of DNA. RNA was precipitated by isopropanol (Sigma-Aldrich, St. Louis, Mo, USA) overnight at -20˚C and washed twice with ice-cold ethanol prior to elution in RNAse-free water.

miRNA synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). A total of 10 ng RNA was used for cDNA synthesis of miR-199a-5p and U18 small nucleolar (sno) RNA (reference gene) using stem-loop primers of the TaqMan® microRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). cDNA synthesis was performed by MuLV Reverse Transcriptase kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was performed with the HotStartTaq DNA Polymerase kit (Qiagen GmbH) on a Rotor-gene Cycler (LTF Labortechnik, Wasserburg, Germany). U18 snoRNA values were uniform and served as an internal reference. C_q values were obtained and transformed via the ΔΔCq method (26) and the lowest C_q value served as a calibrator (27).

Luciferase reporter assay. 3'UTR regions of *HIF-1α, OSGIN2* and *VEGF* were amplified using the following primers: *HIF-1α*, forward, 5'-AAACTCGAGTGGCATGTAGACTGCTGGGGCAA-3' and reverse, 5'-AAACTCGAGTGGCTACCACGTACTGCTGGCAA-3'; *OSGIN2*: forward, 5'-AAACTCGAGTGGGGTTTTGCAGTGTACTGGCT-3' and reverse, 5'-AAACTCGATGGACCCCACCCCCAGTTATACA-3'; *VEGF* forward, 5'-AAACTCGAGTGGACCACACCATCACCATCGAC-3' and reverse, 5'-AAACTCGAGTGGCGTCTGACCTGGGGTAGAGA-3'. The 3'UTR sequences with the putative miRNA binding sites were cloned into a psiCheck™-2 vector carrying a renilla luciferase and a constitutively expressed firefly luciferase gene (Promega Corporation, Madison, WI, USA) by applying restriction digestion with *Xho*I (Fermentas; Thermo Fisher Scientific, Inc.) and ligation with Ligase I (Fermentas; Thermo Fisher Scientific, Inc.). Cells from the human osteosarcoma SAOS-2 cell line were cultivated for 24 h in Dulbecco's modified Eagle's medium (DMEM) in 5% CO_2, transfected with psiCheck2-3'UTR-constructs and mimics for miR-199a-5p (Ambion; Thermo Fisher Scientific, Inc.). miR-199a-5p mimics were transfected as double-stranded RNA according to the manufacturer's protocol, with the complementary miR-199a-3p strand inactivated by appropriate chemical modifications. Luciferase activity measurements with the Dual-Glo® Luciferase assay (Promega Corporation) were performed after 24 h in a Tecan X50 reader (Tecan Austria GmbH, Grödig, Austria), to determine relative chemiluminescence. Firefly luciferase activity served as an internal reference. Relative values were standardized on the luminescence values of the non-mimic transfected control.

Western blot analyses. For the analysis of miR-199a-5p-mediated translation inhibition of the selected target genes, two sarcoma cell lines were chosen as representative sarcoma systems. Rhabdomyosarcoma (RD) and SAOS-2 cells were transfected with miR-199a-5p mimics or scrambled RNA (Ambion; Thermo Fisher Scientific, Inc.), respectively and cultivated for 24 h under hypoxic or normoxic conditions in DMEM in 5% CO_2. Cells were harvested on ice immediately

following reoxygenation using a cell scraper and protein was isolated according to the RIPA method. For western blot analyses, 20 ng of whole protein was separated on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel (4-12%; Thermo Fisher Scientific, Inc.), blotted on a polyvinylidene fluoride membrane and blocked with 5% dry milk/phosphate-buffered saline (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Membranes were incubated with murine anti-human OSGIN2 (dilution 1:500; #ab88829; Abcam, Cambridge, UK), murine anti-human HIF-1α (dilution 1:1,000; #610958; BD Transduction Laboratories™; BD Biosciences) or murine anti-β-actin antibody (dilution 1:2,000; #A2228; Sigma-Aldrich) and tagged with a secondary horseradish peroxidase-coupled antibody (dilution 1:10,000; #P0260; Dako, Glostrup, Denmark). Detection was performed with enhanced chemiluminescent Detection substrate (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Statistical analysis. Statistical analyses were performed using SPSS 19.0 (IBM SPSS, Armonk, NY, USA). In detail, bivariate correlations (Spearman-Rho), Kaplan-Meier analyses, uni- and multivariate Cox's regression analyses (the latter was adjusted to tumor entity, tumor localisation, resection type and tumor stage) were performed to evaluate possible correlations between miR-199a-5p and clinical parameters and outcome. To evaluate the luciferase reporter assay, unpaired Student's *t*-tests were performed. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-199a-5p is associated with clinical and molecular factors in soft tissue sarcoma. miR-199a-5p expression was quantified in 96 patients with soft tissue sarcoma: 37 of these patients experienced ≥1 relapses, 29 experienced metastasis and 50 died of the tumor (Table I). Bivariate correlation analyses according to Spearman-Rho were performed to detect significant associations between miR-199a-5p expression and relevant clinical or molecular factors (Table II). Intriguingly, it was demonstrated that miR-199a-5p expression is associated with the number and interval of relapses (r_s=0.39, P=0.00009 and r_s=0.52, P=3.6x10^{-8}, respectively) as well as the tumor-specific survival time (r_s=0.36, P=0.00024). There was an association between miR-199a-5p expression and *OSGIN2* mRNA expression, although this was not statistically significant (r_s=-0.23; P=0.09). Furthermore, there was a correlatiojn between miR-199a-5p and *HIF-1α* mRNA expression (r=0.27, P=0.018). These findings may suggest the existence of a feedback-loop, induced by increased HIF-1α levels, leading to a downregulation through miR-199a.

Survival analyses. miR-199a-5p expression was transformed according to a tercentile distribution (low expression, <2.24; medium expression, 2.24-10.3; elevated expression, >10.4). A Kaplan-Meier analysis indicated that low miR-199a-5p expression was associated with decreased patient survival time, although this association was not significant (P=0.07, mean survival times: Low expression, 38.3 months; medium expression, 72.2 months; elevated expression, 105.9 months; Fig. 1A). To investigate the effect of lower levels of miR-199a-5p expression in detail, the groups of patients exhibiting medium and elevated expression were combined. It was demonstrated that lower miR-199a-5p expression was associated with a significant decrease in survival time (P=0.026; median survival time for low expression, 38.3 months; for elevated expression, 95.6 months; Fig. 1B).

Furthermore, a univariate Cox's regression analysis demonstrated that patients with low miR-199a expression exhibited significantly poorer outcomes and were 1.92 times more likely to experience tumor-associated mortality [P=0.029; 95% confidence interval (CI), 1.07-3.44]. However, in a multivariate Cox's regression analysis (adjusted to resection type, tumor stage, entity and localisation), no correlation was detected between miR-199a-5p expression and tumor-specific survival (P=0.66; relative risk=1.16; 95% CI, 0.6-2.22).

OSGIN2 and HIF-1α are target-genes of miR-199a-5p in vitro. To analyse possible linkages between miR-199a expression and hypoxia-related target genes, an *in silico* database search for putative 3'-UTR mRNA interactions of miR-199a was performed. Beside others, TargetScan (www.targetscan.org) suggested *HIF-1α*, *VEGF* and *OSGIN2* mRNA as putative target genes of miR-199a-5p (Fig. 2). The 3'UTR sequences of these genes, including the predicted miR-199a-5p target binding site, were cloned into psiCheck2 vectors and luciferase reporter assays were performed to validate regulation by miR-199a-5p. Co-transfection of SAOS-2 cells with 20 or 50 nM miR-199a-5p mimics induced a significant downregulation of *HIF-1α* and *OSGIN2* 3'UTR coupled luciferase expression (Fig. 3A and B; 35.4 and 26.2% respectively, compared to controls treated with scrambled mimics; P=0.008 and P=0.01, respectively; Student's *t*-test). However, no significant change in *VEGF*-3'UTR coupled luciferase expression (105.6% compared to control, Fig. 3C) was detected.

Furthermore, western blot analyses of HIF-1α and OSGIN2 protein expression following miR-199a-5p transfection were performed in RD and SAOS-2 cells under hypoxic conditions. HIF-1α and OSGIN2 proteins were expressed in hypoxic conditions, whereas no visible bands were detectable under normoxic conditions (data not shown). In hypoxic conditions, transfection of miR-199a-5p mimics resulted in the downregulation of HIF-1α and OSGIN2 protein in comparison to untreated control cells or cells treated with negative mimics only (Fig. 3D). Taken together, these results suggest that miR-199a-5p downregulation contributes to the adaptation of cells to hypoxia.

Discussion

The present study evaluated the impact of miR-199a-5p on the survival of patients with soft tissue sarcoma and validated *HIF-1α* and *OSGIN2* as miR-199a-5p target genes. Interestingly, *OSGIN2* was linked for the first time to miR-199a-5p regulation and may be an interesting target for analyses of sarcomagenesis and progression, tumor hypoxia and patient outcome.

The effect of the expression of several well-known cancer genes on the mRNA and protein level is well known, however, the data about the impact of most types of miRNA on the outcome of patients with soft tissue sarcoma remains scarce. To the best of our knowledge, the present study is first to report a

Table I. Clinical and histopathological parameters and miR-199a-5p expression level.

Patient characteristic	No. cases	miR-199a-5p ≤2.24	miR-199a-5p >2.24	χ^2 test (P-value)
Total	96			
Gender				
Male	47	12	35	
Female	49	21	28	n.s.
Histological subtype				
LS	18	7	11	
FS	7	1	6	
RMS	7	3	4	
LMS	17	9	8	
NS	11	4	7	
Syn	7	0	7	
NOS	22	7	15	
Other	7	1	6	n.s.
Tumor size				
T1	21	3	18	
T2	75	29	46	n.s.
Tumor grade[a]				
I	13	6	7	
II	41	9	32	
III	42	17	25	n.s.
Tumor stage[b]				
I	10	4	6	
II	42	10	32	
III	34	12	22	
IV	10	6	4	n.s.
Complete resection				
Radical (R0)	62	20	42	
Not radical (R1)	34	12	22	n.s.
Location				
Extremities	61	18	43	
Trunk wall	11	3	8	
Head/neck	3	1	2	
Abdomen/retro-peritoneum	19	9	10	
Multiple locations	2	1	1	n.s.
Number of relapses				
0	59	22	37	
1	17	5	12	
>2	20	5	15	n.s.
Metastasis				
Yes	29	10	19	
No	67	22	45	n.s.
Patient status				
Alive	46	11	35	
Succumbed	50	21	29	n.s. (P=0.06)

[a]According to van Unnik *et al*, 1993 (50). [b]According to the Union for International Cancer Control guidelines (51). LS, liposarcoma; FS, fibrosarcoma; RMS, rhabdomyosarcoma; LMS, leiomyosarcoma; NS, neuronal sarcoma; Syn, synovial sarcoma; NOS, not other specified; tumor size, T1 ≤5 cm in diameter, T2 >5 cm in diameter; n.s., not significant.

1.92-fold increased risk of tumor-associated mortality for soft tissue sarcoma patients (33% percentile) with low miR-199a-5p expression (univariate Cox's regression analysis, P=0.029; 95% CI: 1.07-3.44). Following multivariate Cox's Regression

Table II. Bivariate correlations (Spearman's Rho test) between miR-199a-5p expression in tumor tissues and different clinical and molecular factors of soft tissue sarcoma patients.

Parameter	rs	P-value	n
Age	-0.21	0.04[a]	98
Tumor-specific survival time	0.36	0.00024[a]	98
Number of relapses	0.39	0.00009[a]	98
Time interval of relapse	0.52	($3.6x10^{-8}$)[a]	98
HIF-1α mRNA	0.266	0.018[a]	85

[a]P<0.05, indicating a statistically significant value. *HIF-1α*, hypoxia-inducible factor 1α.

analysis, no effect of miR-199a-5p expression on patients' survival was detected, however, non-parametric correlation analyses demonstrated a correlation with tumor entity (P=0.014, Kruskal-Wallis test). This is concordant with the results of a previous study by Guled *et al* (28), describing a diagnostic role of miR-199-5p for the identification of undifferentiated pleomorphic sarcoma compared to leiomyosarcoma. It has also been demonstrated that low miR-199a-5p expression is associated with a poorer outcome in serous ovarian carcinoma (29), non-small cell lung carcinoma measured in patients' sera (30) and renal cell carcinoma (31). Concordantly, decreased expression of the complementary miRNA sequence miR-199a-3p is associated with a shorter time to recurrence in hepatocellular carcinoma (32). Furthermore, multivariate Cox's regression analyses demonstrated that low miR-199a-3p expression was significantly associated with poorer overall survival in patients with osteosarcoma (33). However, previous studies have linked the elevated expression of miR-199a-3p with poorer overall patient survival in colorectal cancer (34) and esophageal adenocarcinoma (35), pointing towards a differential expression signature and possibly altered regulation mechanisms of miR-199a processing and RISC incorporation in tumor cells.

Several studies have described miR-199a as a type of miRNA associated with hypoxia-adaptation (36-39). It was demonstrated that miR-199a-5p downregulation is *AKT*-dependent (39,40) and may be antagonized by the β-adrenergic receptor (40). In the current study, in order to facilitate luciferase reporter assays, the interactions between miR-199a-5p and the 3'UTRs of three putative hypoxia-related target genes, namely *HIF-1α, OSGIN2* and *VEGF,* were investigated. These target genes were chosen following a literature search demonstrating the potential link between miR-199a-5p expression and hypoxia. The results of the present study demonstrated that miR-199a-5p post-transcriptionally regulated *HIF-1α* and *OSGIN2*, but not *VEGF*. Downregulation of miR-199a-5p in cardiac myocytes mimics hypoxia preconditioning by lowering the miR-199a-5p-mediated suppression of *HIF-1α* mRNA translation (18). Furthermore, ethanol downregulates miR-199a expression in liver-sinusoidal endothelial liver cells independently of hypoxic stress, and this downregulation coincides with an increase in *HIF-1α* and Endothelin-1 mRNA (41). By contrast, overexpression of miR-199a-5p and miR-199a-3p has been detected in advanced liver fibrosis in samples from mouse and human tissue (42). On a molecular level miR-199a-5p targets the prominent oncogenes *ERBB2* and *ERBB3* directly in ovarian and lung carcinoma cell lines (43). Additionally, it was observed that miR-199a-5p overexpression in ovarian carcinoma cells significantly decreased their ability to induce angiogenesis, an effect mediated via *ERBB2* and *ERBB3* signalling (21). Moreover, overexpression of miR-199a-5p in multiple myeloma cells significantly impaired migration by downregulating the expression of adhesion molecules, including vascular cell adhesion molecule-1 (*VCAM-1)* and intracellular adhesion molecule 1 (*ICAM-1)* (39). By contrast, the complementary miR-199a-3p transcript regulates the tumor-associated mammalian target of rapamycin (*mTOR*) and *c-Met* in hepatocellular carcinoma cells and its overexpression restores hypoxia and doxorubicin sensitivity (32). Recently, Kinose *et al* (44) demonstrated that miR-199a-3p serves as tumor suppressor gene in ovarian cancer by directly repressing its target gene *c-Met* and subsequently inhibiting proliferation, adhesion and invasiveness (44). The present study validated miR-199a-5p as regulator of *HIF-1α*, but not *VEGF*. These results are concordant with those from previous studies demonstrating that *VEGF* is an indirect target gene of miR-199a, which is regulated by *HIF-1α* and *ERBB2/3* downregulation rather than by direct miRNA-medicated *VEGF* translation inhibition and mRNA destabilization (21).

Furthermore, the results of the present study suggested that *OSGIN2* is a putative target of miR-199a-5p and suggested a possible functional link between a hypoxic tumor environment and the miRNA-induced derepression of stress genes protecting tumor as well as normal cells from stress-induced cell death. *OSGIN2* is localized on chromosome 8q21.3 in the neighbourhood of Nijmegen breakage syndrome gene 1 (45). The transcript is translated into a protein 505 amino acids long and there is an additional shorter transcript variant translated to a protein with a distinct N-terminus (45). *OSGIN2* is a still poorly characterized homolog of *OSGIN1,* with 49% identity and 62% similarity (46). A previous study demonstrated that *OSGIN2* was upregulated in the liver biopsies of transplants exhibiting initial poor graft function (47). Furthermore, in a high-throughput assessment for known and novel breast cancer candidate genes, *OSGIN2* was mapped to a chromosomal region demonstrating a significant amplification gain in breast cancer cell lines and tumor tissue (48). Additionally, it has been identified that *OSGIN2* is overexpressed following activation of *PGC-1* related coactivator, a protein maintaining mitochondrial homeostasis and linking mitochondrial status to cell cycle (49). However, the physiological function of *OSGIN2*, and its potential association with cancer genesis and progression remain elusive and further studies are necessary to clarify the impact of this gene on tumor hypoxia.

In conclusion, the present study identified a correlation between low miR-199a-5p expression and a poorer outcome of patients with soft tissue sarcoma. Low miR-199a-5p expression increased the risk of tumor-associated mortality by 1.92-fold. Furthermore, *HIF-1α* and *OSGIN2* may be target genes of miR-199a-5p. However, the precise role of miR-199a-5p in cancer remains ambiguous, due to its diverging functions in different cell types and stress situations. Thus, further studies, particularly on the regulation and impact of this hypoxia-associated miRNA are required.

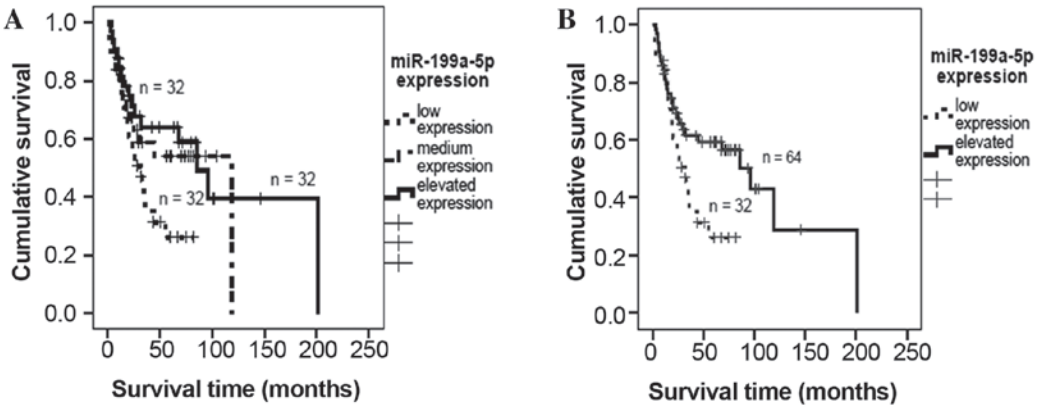

Figure 1. Survival analyses for miR-199a-5p expression in patients with soft tissue sarcoma. Kaplan-Meier analysis demonstrated decreased survival rates of patients with low miR-199a-5p expression compared to patients with elevated miR-199a-5p expression. Univariate Cox's regression analyses confirmed the negative effect of low miR-199a-5p expression on disease-specific survival (RR=1.9; P=0.029). miR-199a-5p, microRNA-199a-5p; RR, relative risk.

Figure 2. *In silico* analyses of miR-199a-5p target sequence binding. The overlapping sequences of mi-R-199a-5p and (A) *HIF-1α*, (B) *OSGIN2* and (C) *VEGF* are presented according to TargetScan annotation (www.targetscan.org). The complimentary seed sequences are indicated by boxes. HIF-1α, hypoxia-inducible factor 1α OSGIN2, oxidative stress induced growth inhibitor 2; VEGF, vascular endothelial growth factor.

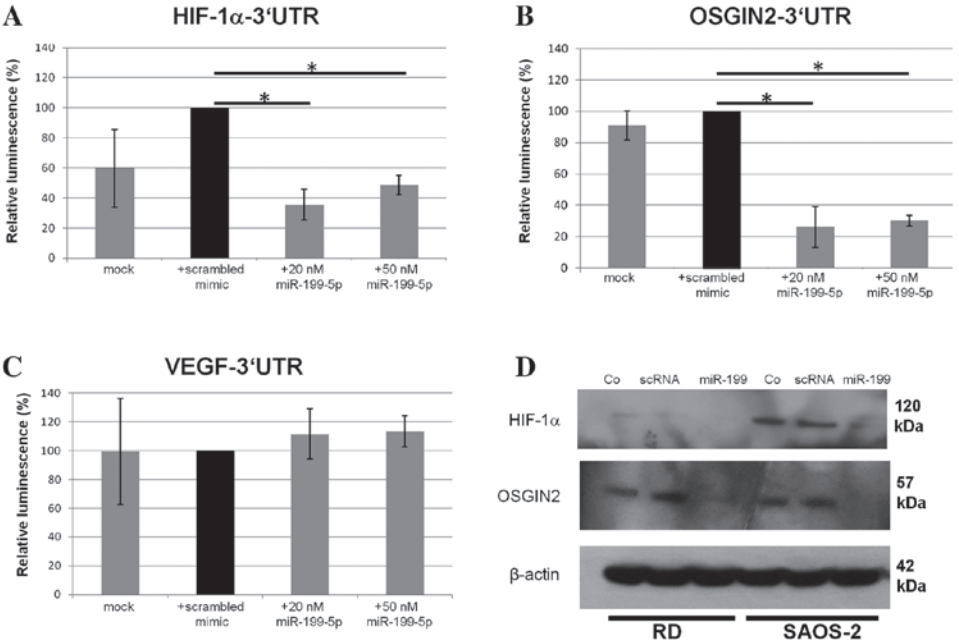

Figure 3. Luciferase reporter assays with reporter constructs containing (A) *HIF-1α* (B) *OSGIN2* and (C) *VEGF* 3'UTRs. Relative luminescence coefficients were standardized on a scrambled mimic transfected control. Co-transfection with 20 nM miR-199a-5p yielded a significant downregulation of the luciferase-coupled *HIF-1α*-3'UTR construct (35.4%, P=0.041, Student's *t*-test) and the *OSGIN2*-3'UTR construct (26.2%; P=0.029, Student's *t*-test, error bars defining standard derivation) compared with controls (n=3; *P<0.05). No such downregulation was observed in VEGF 3'UTRs. (D) Western blot analyses presented a downregulation of HIF-1α and OSGIN2 expression following miR-199a-5p (20 nM) overexpression. HIF-1α, hypoxia-inducible factor 1α OSGIN2, oxidative stress induced growth inhibitor 2; VEGF, vascular endothelial growth factor; UTR, untranslated region; scRNA, scrambled RNA; RD, Rhabdomyosarcoma cells.

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