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Elevated levels of serum MiR-152 and miR-24 in uterine sarcoma: potential for inducing autophagy via SIRT1 and deacetylated LC3

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ABSTRACT

Background: MiRNAs may be associated with the risk of uterine sarcoma and related molecular mechanism remains unclear.

Methods: A total of 101 patients with uterine sarcoma (cases) and 54 healthy subjects (controls) were enrolled. The levels of serum miR-152, miR-205, miR-222, miR-24, miR-150 and sirtuin 1 (SIRT1, an NAD +-dependent class III histone deacetylase) were measured by qRT-PCR. HeLa cells were transfected with the mimics of miR-152 and miR-24. The autophagic rates, and the levels of SIRT1 and acetylation of microtubule-associated protein 1A/1B-light chain 3 (LC3) were measured.

Results: Levels of miR-152, miR-24 and SIRT1 decreased while the levels of miR-205, miR-222 and miR-150 increased in cases vs. controls (all $P < 0.05$). All miRNAs were linked with stage of the cases' sarcoma (all $P = 0.001$). Kaplan–Meier analysis demonstrated uterine sarcoma patients have better survival rates with high-level miR-152 and miR-24, with a five-year overall survival of 21.8% and 67.5%, respectively ($P = 0.003$ and 0.004). The mimics of miR-152 and miR-24 induced autophagy by increasing the level of SIRT1, which deacetylated LC3.

Conclusion: Present findings demonstrate altered miRNA species in uterine sarcoma that are linked to disease stage, and a new molecular mechanism, by which miR-152 and miR-24 promote autophagy by activating SIRT1 and deacetylating LC3.

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Introduction

Although uterine sarcomas comprise 3% of all uterine cancers and 7% of new cases, effective treatment strategies are unclear and biomarkers undeveloped [1,2]. Uterine sarcomas are categorised into three histological types: leiomyosarcomas (LMS), endometrial stromal sarcomas (ESS) and mixed tumors [3]. Most LMS belong to stage I, ESS belong to stage II and malignant Mullerian mixed tumors (carcinosarcomas, CS) are allocated to stage III according to the last International Federation of Gynecology and Obstetrics (FIGO) classification system [4]. Uterine sarcomas are rapidly aggressive and patients have a poor prognosis with two-year survival rate <50% [5].

Biomarkers are associated with the risk and progression of uterine cancers [6]. For example, serum amyloid A may be important in the diagnosis and monitoring of uterine serous papillary carcinoma [7]. MiRNAs, a class of small non-coding RNAs of 19–25 nucleotides, have been found to be associated with the risks of various cancers. For instance, the low level of miR-124 is associated with the risk of pancreatic ductal adenocarcinoma [8]; MiR-21 has been reported to play a critical role in osteosarcoma development [9]. MiR-146a has been found to be related to the progression of gastric cancer [10]. The reduction of MiR-199a-3p has been demonstrated to affect the invasion and metastasis of papillary thyroid carcinoma [11]. However, miRNAs are seldom reported in the development of uterine sarcomas and the molecular mechanisms remain widely unknown.

Microtubule-associated protein 1A/1B-light chain 3 (LC3) is an autophagosome that plays an important role in autophagic activities [12]. Autophagy has been reported to induce cell death that can be targeted for preventive and intervention strategies for inhibiting the progression of human uterine leiomyoma [13]. Sirtuin 1 (SIRT1) is a histone deacetylase and mediates autophagy via LC3. Further findings demonstrate the deacetylation of endogenous LC3 by Sirt1-induced autophagy [14].

We hypothesised altered serum levels of miR-152, miR-24, miR-205, miR-222 and miR-150 in uterine sarcoma predict disease stage. In addition, we sought a molecular mechanism for the function of miRNAs in the progression of uterine sarcoma which is demonstrated by investigating autophagy rates, the level of SIRT1 and acetylation of LC3.

Patients and methods

All protocols were approved by the Ethical Committee of Liaoning Cancer Hospital & Institute (Shenyang, China)

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and informed consent was obtained from all subjects. From February 2008 to October 2010, a total of 248 patients with uterine sarcoma were subject to the following inclusion criteria: a histologic diagnosis of uterine sarcoma, surgical stages defined by histopathology, at least one progressive target lesion according to computed tomography or magnetic resonance imaging, neutrophil count >1000/μL, platelet count >100,000/μL, total bilirubin <1.5-fold the institutional upper limit of normal, an Eastern Cooperative Oncology Group performance status score ≤2 [15] and finished any previous therapy more than one month before the enrolment. Fifty-four healthy subjects were collected as a control group. Exclusion criteria for all subjects were pregnancy, lactation, a history of malignancy, neuropathy, or nervous system metastases and that the patient did not receive follow-up care.

Demographic data were obtained from each patient's medical record, including age, blood pressure, tumour stage, presenting symptom, histological subtype, operative procedure, adjuvant radiotherapy and chemotherapy and overall survival and current disease status. Patients were diagnosed by the FIGO 2009 staging system [16]. Overall survival was defined as the time from the date of diagnosis to the date of death at five-year follow-up. The serum triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured by an Olympus AU 2700 analyser (Beckman Coulter, Brea, CA, USA).

Blood samples were obtained and serum was isolated within two hours. Total RNA was extracted using a miRNeasy Serum/Plasma Kit (QIAGEN Sciences, Germantown, MD, USA). Two-μg RNA was obtained from 2 ml of serum. qRT-PCR was conducted using an Applied Biosystems 7300 Real-Time PCR System. One-ul RT products were added to 20-μl reaction volume including 0.5-μl sense primer and reverse primer, 1-μl SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, Waltham, MA, USA), and one-unit Taq (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China). The reaction was carried out as follows: 94 °C for 5 min, followed by 45 cycles of 94 °C for 20 s and 65 °C for one min. After the reaction, the C_T was calculated via threshold settings. The ratio miRNA in cases vs. healthy subjects was presented using $2^{-\Delta G}$, in which $\Delta G = C_{T\ cancer} - C_{T\ normal}$. The levels of MiR-152, miR-205, miR-222, miR-24, miR-150 and SIRT1 were normalised to total RNA. The miRNA expression levels were classified into high or low groups according to the median value (cut-off value = 28). To evaluate the relationship between the levels of miR-152 or miR-24 and survival of uterine sarcoma patients, the overall survival was analysed by a log-rank test, and survival rates by Kaplan–Meier.

Therapy of uterine sarcoma was as follows. Among 101 patients, 98 (97%) were treated with surgery, and hysterectomy was performed in 11. Total abdominal hysterectomy (TAH) and bilateral salpingo-oophorectomy were performed in 47 patients, and extensive or sub-extensive abdominal hysterectomy was performed in 14. Nodal dissection was performed in 14 patients and cytoreduction was performed in 12. No additional treatment was recommended for 12 patients. Adjuvant external beam radiotherapy (EBRT) for the whole pelvis was performed in 25 patients with a dose range of 40–45 Gy within 4 weeks. Both radical radiotherapy and chemotherapy were performed in 16 patients. During the 5 years (2010–2015), 47 patients received four or eight courses of vincristine + actinomycin-D + Cyclophosphamide, 14 patients received four or eight courses of cisplatin + etoposide + bleomycin and 16 patients received four or eight courses of cisplatin actinomycin-D + cyclophosphamide.

Cell culture and transient transfection were as follows. The fusion protein GFP-LC3 (green fluorescent protein and LC3) is a simple tool to observe LC3 dots or puncta, by which the autophagy can be evaluated. Cervical cancer cell line Hela was purchased from the Cell Bank of CAS (Shanghai, China) and cultured in DMEM with 10% FBS at 37 °C and 5% CO_2. A monolayer was digested by trypsin when cells reached 80% confluency. The cells were washed three times with fresh DMEM and cell concentration adjusted to 1×10^9 cells/L. The cells were transfected with plasmid ptfLC3 (Addgene, Cambridge, MA, USA), miR-152 or miR-24 (10 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Western Blot and co-immunoprecipitation analysis were as follows. Hela cells were lysed by freezing and thawing. Ten-μg proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane, and blocked by non-fat dry milk for 30 min. The membranes were incubated with the antibodies SIRT1 and LC3 (diluted 1:1000) 10 h. With *X*-ray film exposure, the expression of SIRT1 and LC3 was detected via Quantity One software. Hela lysates were mixed with antibodies and PureProteome Protein A/G Mix Magnetic Beads. The proteins were separated by SDS-PAGE and transferred to a PVDF membrane as above mentioned. The acetylation LC3 was determined via Quantity One software.

Categorical variables were expressed as frequencies and analysed by chi-squared continuous data as mean with standard deviation (analysed by *t* test) or median with interquartile range (Mann–Whitney *U* test). Survival rates were calculated using the Kaplan–Meier method and survival curves were compared using log-rank test. Univariate analysis was performed using SPSS version 20.0. The difference across the four stages of uterine sarcoma was determined by linear trend for ordered groups.

Results

One hundred and one patients passed inclusion and exclusion criteria. Clinical characteristics of all participants were presented in Table 1. There were no

Table 1. Demographics and baseline clinical characters between patients and healthy subjects.

	Patients ($n = 101$)	Control ($n = 54$)	P value
Mean age, years	47.1 ± 15.8	47.5 ± 15.1	0.37
BMI, kg/m^2	23.2 ± 3.5	23.8 ± 4.1	0.24
Smoking n (%)	53 (53)	29 (53.7)	0.87
Use of alcohol, n (%)	51 (51)	30 (55.6)	0.74
SBP, mmHg	141 ± 17.2	144 ± 16.8	0.81
DBP, mmHg	77.5 ± 9.2	78.5 ± 10.1	0.62
Total cholesterol, mmol/L	4.6 (3.7, 4.9)	4.5 (3.7, 4.9)	0.49
LDL-c, mmol/L	2.5 (1.9, 2.9)	2.6 (2.1, 3.1)	0.83
HDL-c, mmol/L	1.3 (1.2, 1.6)	1.4 (1.1, 1.6)	0.75
Triglyceride, mmol/L	1.3 (0.8, 1.6)	1.4 (0.9, 1.7)	0.58

Note: Data presented as mean (SD), median (IQR) or number (%).

significantly statistical differences between patients and healthy subjects. The histologic diagnoses were 35 patients with LMS, 42 with ESS and 24 with CS. The patients were staged using the FIGO classification, and cases with stage I, II and III/IV uterine sarcoma were 54, 33 and 14 cases, respectively. The main symptoms were abnormal uterine bleeding, postmenopausal bleeding and abdominal mass (including abdominal pain or discomfort, urinary symptoms and infertility).

qRT-PCR analysis shows that serum levels of miR-152 and miR-24 were lower in cases than in controls, and that levels decreased with uterine sarcoma stage I to III/IV. In contrast, miR-205, -222 and -150 were higher in cases than healthy subjects, and increased from stage I to III/IV. Levels of SIRT1 were lower in cases vs. controls, and decreased from stage I to III/ IV disease (Table 2).

Follow-up examination was performed every 3 months during the first 2 years, every 6 months during the third to fifth year and annually thereafter. The mean follow-up was 46 months. Five-year survival rate was 46.5% overall, 73.8% for ESS, 34.3% for LMS and 16.7% for CS patients. Kaplan–Meier analysis demonstrates that patients have better survival rates if they have high levels of miR-152 (Figure 1(A)) and miR-24 (Figure 1(B)), with a five-year overall survival rate of 21.8 and 67.5% ($P = 0.003$ and 0.004), respectively.

There was no link between chemotherapy and improved prognosis. On the contrary: patients treated with chemotherapy survived for a shorter time than patients with matching stages, although the difference in survival was not statistically significant. Characteristics of the patients were shown in Table 3. Univariate analyses of the relationships between prognostic variables and survival showed no significant difference for the age of diagnosis, the stage of uterine sarcoma, histological type and uterine size among all the characteristics reviewed, including the age of diagnosis, stage, grade, treatment, uterine size and histological type.

Figure 2 showed that miR-152 and miR-24 mimics induced autophagy vacuoles in Hela cells (Figure 2(A), arrow), while green fluorescence increased with the expression of GFP-LC3 (Figure 2(B)). The results suggest miR-152 and miR-24 mimics induce autophagy by increasing the expression of LC3 and SIRT1 (Figure 2(C)). Co-immunoprecipitation (IP) was carried out to measure the binding of SIRT1 and LC3. The results demonstrated that miR-152 promoted the binding between SIRT1 and LC3, which deacetylated LC3 (Figure 2(D)). Similarly, miR-24 promoted the binding between SIRT1 and LC3, which deacetylated LC3 (Figure 2(E)).

Table 2. Relative serum levels of miRNA and SIRT1 among different groups.

				Uterine sarcoma patients			
	Controls ($n = 54$)	All patients ($n = 101$)	P1	Stage I ($n = 54$)	Stage II ($n = 33$)	Stage III/IV ($n = 14$)	P2
MiR-152	98 (66–126)	41 (10–62)	0.005	50 (39–62)	30 (21–39)	16 (14–20)	0.001
MiR-24	62 (43–125)	46 (10–65)	0.016	59 (42–65)	34 (21–58)	19.5 (13–26)	0.001
MiR-205	8 (5–14)	16 (8–65)	0.024	12 (8–16)	14 (10–20)	38 (22–57)	0.001
MiR-222	12 (9–17)	33 (11–150)	0.001	18 (11–22)	32 (20–49)	93.5 (65–129)	0.001
MiR-150	6 (5–9)	18 (5–74)	0.031	9 (5–15)	24 (15–35)	44 (28–64)	0.001
SIRT1	23 (16–31)	13 (4–22)	0.022	15 (9–22)	12 (6–19)	8 (6–14)	0.001

Notes: Data presented as median (interquartile range). P1 = difference between uterine sarcoma patients and healthy controls via a Mann–Whitney test. P2 = difference across the four disease stages by linear trend for ordered groups. SIRT1, sirtuin 1.

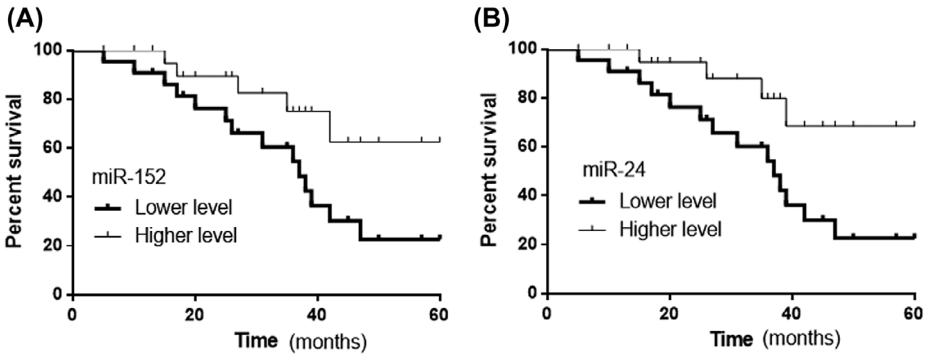

Figure 1. Kaplan–Meier survival curves showed the relationship between serum levels of microRNA and survival rates. Notes: A, the relationship between serum levels of miR-152 and survival rates. B, the relationship between serum levels of miR-152 and survival rates.

Table 3. Patient characters and treatment.

	LMS ($n = 35$)	ESS ($n = 42$)	CS ($n = 24$)	*P* values
Stages (n, %)				
I	20 (57.1)	26 (61.9)	8 (33.3)	0.007
II	11 (31.4)	12 (28.6)	10 (41.7)	0.541
III	3 (8.6)	3 (7.1)	5 (20.8)	0.197
IV	1 (2.9)	1 (2.4)	1 (4.2)	0.918
Symptoms				
Postmenopausal bleeding	4 (11.4)	7 (16.7)	11 (45.8)	0.004
Abnormal uterine bleeding	17 (48.6)	30 (71.4)	7 (29.2)	0.003
Mass effect	14 (40)	5 (11.9)	6 (25)	0.017
Surgery				
No surgery	1	1	1	1.000
HT	7	0	4	0.006
HT-BSO/USO	18	16	13	0.551
E or SE HT+ lymphadenectomy	3	10	1	0.001
E or SE HT	1	9	4	0.026
Cytoreductive surgery	5	6	1	0.072
Adjuvant therapy				
No therapy	4	7	1	0.034
Chemotherapy (CT)	20	29	12	0.005
Radiotherapy (RT)	5	3	1	0.135
Sequential CT+RT	16	52	9	0.001
Chemotherapeutic regimens				
VAC	12	22	13	0.055
PVB	6	5	3	0.472
PAC	7	4	5	0.519

Notes: HT, hysterectomy; BSO, bilateral salpingo-oophorectomy; USO, unilateralsalpingo-oophorectomy, E or SE-HT, Extensive or sub-extensive abdominal hysterectomy. VAC = vincristin + actinomycin-D + Cyclophosphamide, PVB = cisplatin + etoposide + bleomycin, PAC = cisplatin actinomycin-D + cyclophosphamide. *P* value by chi-squared test.

Discussion

Serum biomarkers provide a convenient tool for the diagnosis of uterine sarcoma and may be developed as a simple reliable method in cancer research. Present findings show that certain serum miRNAs are linked to the presence of, and stage of, uterine sarcoma. Furthermore, the miRNAs can be directly obtained from serum, and serve as simple biomarkers for uterine sarcoma diagnosis.

MiR-152, miR-205, miR-222, miR-24 and miR-150 have been reported to be associated with many types of cancers. For an example, miR-152 functions as a tumour suppressor in colorectal cancers (CRC) by targeting PIK3R3 [17] and miR-152 suppression in non-small cell lung cancer cells promotes the metastasis of neuropilin-1-mediated cancer [18]. Up-regulation of miR-205 is consistently reported in the development of carcinoma [19,20]. Mucinous adenocarcinoma is the leading pathology of CRC, and is associated with cancer progression and poor prognoses. miR-205 contributes to the aggression of mucinous adenocarcinoma in CRC. Lower levels of miR-222 greatly increase the expression of PTEN and p27 kip1, and reduce the level of phospho-Akt. Low levels of miR-222 increase cellular apoptosis and decrease IC50 of cancer cells [21]. miR-24 is a potential tumour repressor linked to risk of prostate and gastric cancers, and miR-150 is positively related to the development of prostate and lung cancers [22,23].

Survival rates for uterine sarcoma patients are uniformly poor. Most series report a five-year survival rate of 14–48% [24], which is in agreement with our data of rates of 47.5% overall, and of ESS, LMS and CS (66.7, 40.0 and 25%, respectively). The histopathological distribution of our patients demonstrates that ESS is most frequent (41.6%) followed by LMS (34.6%) and CS (23.8%). In previous studies, the percentage of LMS and CS in all uterine sarcomas was reported as 40 and 40% [25], 22 and 48% [26] and 33 and 30% [27], respectively.

Patients with uterine sarcomas are usually diagnosed at older ages. In other studies, similar findings have been reported [28]. In our study, most patients were diagnosed at early stages (stage I and II, 86.1%), as reported in other studies [29]. Histological subtype's effect on survival is still in debate. Our multivariate analysis shows that histological types of uterine sarcomas have a significant effect on survival rate. Present findings demonstrate that the serum levels of miR-152 and miR-24 are reduced while the levels of miR-205, miR-222 and miR-150 are increased with the stage of uterine sarcoma. The results suggest that theses miRNAs may be potential biomarkers for evaluating survival rate of uterine sarcoma. The elevated serum miR-152 and miR-24 are associated with increased survival rates.

SIRT1 is a NAD-dependent histone deacetylase and its level is higher in patients group than in a control group. In the cells, CL3 was deacetylated by SIRT1 and increased cellular autophagy. Present results demonstrated that miR-152 and miR-24 mimics increased the levels of SIRT1 and LC3, resulting in an increase of cellular autophagy. Further study demonstrates that autophagy may be caused by the deacetylation of LC3 since LC3 is associated with autophagysome [30].

In conclusion, serum levels of MiR-152, miR-205, miR-222, miR-24 and miR-150 can be developed as non-invasive biomarkers for uterine sarcoma diagnosis. A new molecular mechanism is reported: miR-152 and miR-24 mimics increase the levels of SIRT1 and LC3, resulting in an increase of cellular autophagy, which may be important in

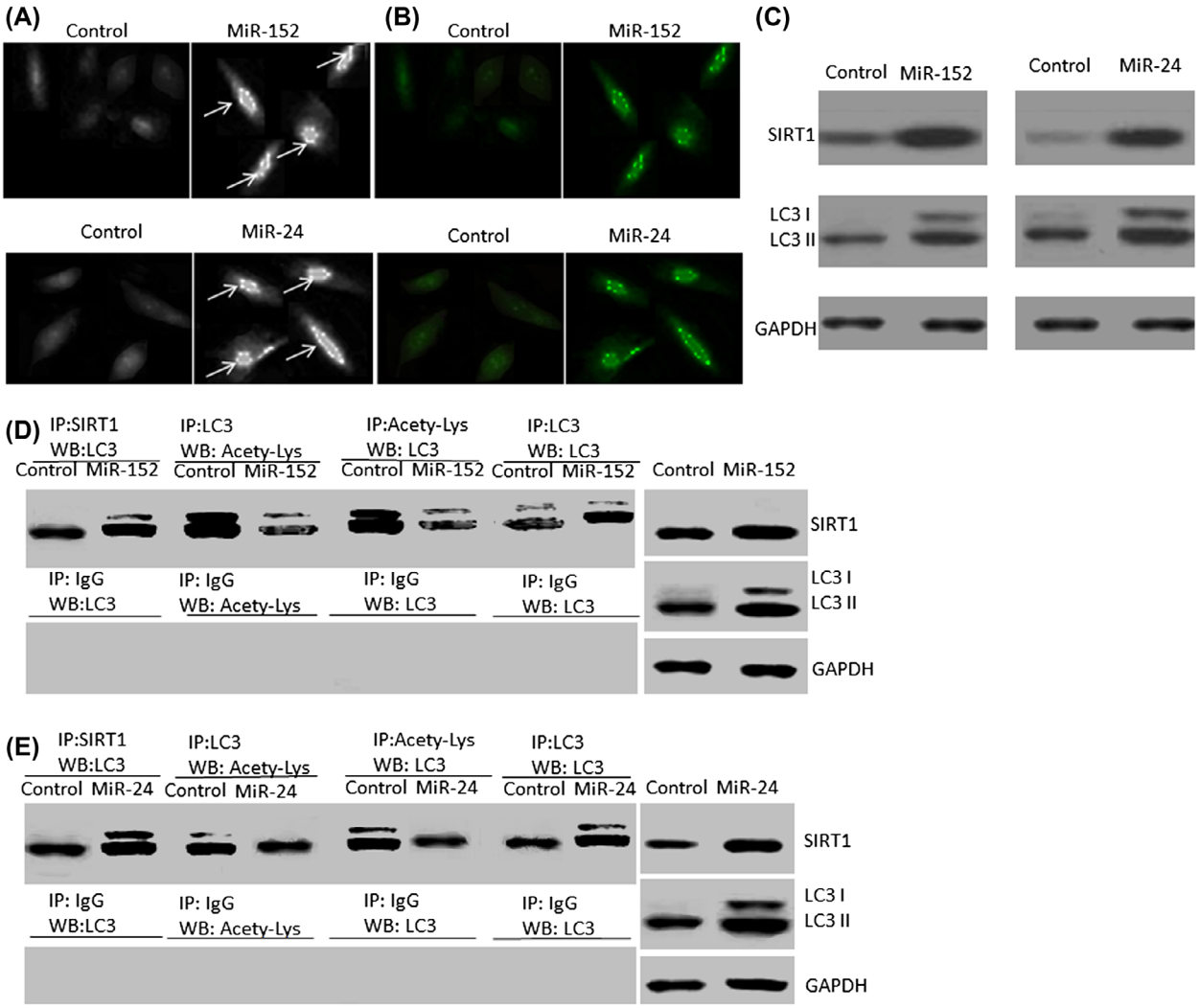

Figure 2. miRs mimic increased the level of SIRT1, deacetylation of endogenous LC3 and LC3-induced autophagy. Notes: A, Autophagic vacuolisation (arrows) in Hela cells confected with miR-152 and miR-24 under a phase contrast microscope (×200). B, Distribution of GFP-LC3 in Hela cells confected with miR-152 and miR-24 under a fluorescent microscope (×200). C, SIRT1 and LC3 expression. D, miR-152 promoted the binding of SIRT1 to LC3 and acetylated LC3, which was detected via an IP test and Western Blot. E, miR-24 promoted the binding of SIRT1 to LC3 and acetylated LC3, which was detected via an IP test and Western Blot.

preventing the development of uterine sarcoma. To confirm the results, much work needs to be done in a larger population in the future. This work represents an advance in biomedical science because elevated serum MiR-152 and miR-24 improve the five-year survival rates of uterine sarcoma patients and fight against uterine sarcoma by inducing autophagy via SIRT1 and deacetylated LC3.

Summary table

What is known about this subject:

- There are still many difficulties for the therapy of uterine sarcomas and effective treatment is difficult to be found.
- MiRNAs have been found to be associated with the risks of various cancers and may be associated with the risk of uterine sarcoma.
- Autophagy has been reported to induce cell death that can be targeted for preventive and intervention strategies for uterine sarcoma.

What this paper adds:

- miR-152, -24, -205, -222 and -150 are potential biomarkers for the diagnosis of uterine sarcoma.
- Serum levels of miR-152 or miR-24 are positively related to five-year survival rates of uterine sarcoma patients.
- Elevated serum MiR-152 and miR-24 fight against uterine sarcoma by inducing autophagy via SIRT1 and deacetylated LC3.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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