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miRNAs 144-3p, 34a-5p, and *206* are a useful signature for distinguishing uterine leiomyosarcoma from other smooth muscle tumors

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Abstract

Background: Leiomyosarcoma (LMS) is a rare uterine neoplasm that has a high mortality rate and no specific treatment. The origin of LMS remains unknown; although, it is hypothesized that LMS arises from the malignant transformation of a degenerated uterine leiomyoma (LM). LMs are the most common benign tumors diagnosed and rare variants of LM (unconventional LM) morphologically resemble LMS, thereby making an early and precise diagnosis of LMS difficult. Various molecular features may influence the malignancy risk of LMS tumors, including microRNAs (miRNAs). However, the role of miRNAs in uterine mesenchymal tumors remains poorly understood. Here, our aim was to assess the miRNA expression profiles of LMS, LM, and LM variants (ULM) to identify a specific signature that may facilitate differentiation among these tumor types. Possible associations between these profiles and patients' clinical and pathological features were also analyzed.

Methods: Total RNA was extracted from formalin-fixed paraffin-embedded tissue samples of uterine LMS ($n = 37$), LM (n = 3), ULM ($n = 8$), and myometrium (MM) ($n = 2$) to perform real-time PCR analyses and detect expression levels of a panel of 84 miRNA sequences related to cancer.

Results: Between the LMS and LM samples, 16 miRNAs were found to be differentially expressed, with *miR-372* and *miR-34a-5p* exhibiting the highest and lowest levels of expression, respectively. When LMS and ULM were compared, 5 differentially expressed miRNAs were identified, with *miR-34a-5p* downregulated and *miR-144-3p* upregulated. Between ULM and LM, all of the differentially expressed miRNAs were upregulated, and *miR122-5p* exhibited 10-fold higher expression. In addition, significant correlations were found between various miRNAs and tumor relapse (*miR-148a-3p*), metastasis (*miR-27b-3p*), and patient death (*miR-124-3p* and *miR-183-5p*). Downregulation of *miR135b-5p* was associated with disease-free survival.

Conclusion: Expression profiling of miRNAs *144-3p*, *34a-5p,* and *206* may be useful in characterizing uterine LMS and distinguishing it from benign tumors. Furthermore, deregulation of *miRNAs 148a-3p*, *27b-3p*, *124-3p, 183-5p*, and *135b-5p* appear to indicate a poor prognosis for LMS patients.

Keywords: miRNA, qRT-PCR, Uterine mesenchymal tumor, Prognosis

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Background

Uterine leiomyosarcoma (LMS) is a rare and extremely aggressive tumor that is characterized by poor prognosis, early dissemination, and high metastatic potential, even when LMS is confined to the uterine corpus at the time of diagnosis (Baiocchi et al., 2016; Serrano & George, 2013). Obtaining an early diagnosis of LMS is difficult because the symptoms and radiologic parameters of patients with these tumors are similar to those of patients with leiomyoma (LM). Moreover, LM is the most common benign gynecologic neoplasm that currently affects reproductive age women. Since the benefits of chemotherapy or radiotherapy for LM remain controversial, most patients are diagnosed with LMS after undergoing surgery for suspected LM (Baiocchi et al., 2016; Serrano & George, 2013; Gockley et al., 2014).

There are also smooth muscle neoplasms which exhibit morphological characteristics that are similar to those of LM and LMS. These neoplasms are labeled as: unusual leiomyoma (ULM) (which includes mitotically active LM and cellular LM) and smooth muscle tumors of uncertain malignant potential (STUMP) (Garcia et al., 2016; Arleo et al., 2015; Kurman et al., 2014).

It has been hypothesized that LM and LMS represent distinct entities with independent cellular and/or molecular origins (D'Angelo & Prat, 2010). Accumulating evidence suggests that development of LMS is due to chromosomal instability and multiple genetic errors (Gockley et al., 2014). In addition, several studies have shown that microRNAs (miRNAs) contribute to the development and prognosis of these cancers (Danielson et al., 2010; Starega-Roslan et al., 2011).

miRNAs are endogenous, single strand non-coding RNAs that are 19–25 nucleotides in length and they post-transcriptionally regulate gene expression. Accordingly, miRNAs have been found to regulate many physiological processes such as cell proliferation, differentiation, apoptosis, and development (Starega-Roslan et al., 2011; Guled et al., 2014; Ravid et al., 2016). Conversely, deregulation or aberrant expression of miRNAs has been linked to many disorders. In some cancers, alterations in levels of oncogenes and tumor suppressors have been identified (Danielson et al., 2010; Starega-Roslan et al., 2011). Moreover, miRNA expression profiles or 'signatures' have been used to successfully classify many tumor types (Danielson et al., 2010). The aims of this study were to: 1) identify miRNAs that differentiate LMS from its benign counterparts, LM and ULM, and 2) identify a miRNA profile which can help predict prognosis for LMS patients.

Methods

Tissue samples

A total of 37 formalin-fixed paraffin-embedded (FFPE) tissue samples of uterine LMS were selected from the files of two different institutions in Sao Paulo, Brazil: the A.C.Camargo Cancer Center and the Obstetrics and Gynecology Department of the Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo. The samples were originally collected between 1982 and 2010 and between 2000 and 2010, respectively. In addition, we selected LM variants ($n = 8$), conventional LMs ($n = 3$), and myometrium (MM) ($n = 2$).

Two expert pathologists (IWC, LDB) reviewed all of the selected cases to consistently classify the cases according to criteria of the World Health Organization (2014) and to prepare the samples for molecular studies. The institutional review boards of both participating institutes approved this study (Registration no. 1816/13 and 1,517,306, respectively).

miRNA RT-PCR array

Total RNA was isolated from each sample by using the ReliaPrep™ FFPE Total RNA Miniprep System (Promega, Madison, WI, USA). A total of 250 ng of total RNA from each sample was then subjected to reverse transcription with a miScript II RT kit (Qiagen, Germantown, MD, USA), as described previously (de Almeida et al., 2017). DNA was quantified with a NanoDrop 2000 Spectrophotometer (ThermoScientific, Fremont, CA, USA).

From each sample, 20 μl of cDNA was prepared with a miScript II RT kit with miScript Hispec Buffer and diluted with RNase-free water. To perform miRNA expression profiling, real-time PCR was performed using the miScript miRNA PCR Array Human Cancer PathwayFinder (MIHS-102Z, Qiagen). This array provides rapid profiling of 84 of the most relevant cancer-related miRNAs and 12 control assays (Calvano Filho et al., 2014). The data obtained with 7500 System SDS Software (Thermoscientific) were analyzed with the threshold-cycle method ($\Delta\Delta C_T$). miRNA expression levels were subsequently normalized based on the expression levels detected for reference small nucleolar RNAs, *SNORD95* and *SNORD96A*, which were determined to be the most stable RNAs according to the NormFinder tool (https://moma.dk/normfinder-software/normfinder-faq). MM samples were used to calibrate the analyses performed. All of the expressed miRNAs were analyzed with a Ct value up to 35 cycles (de Almeida et al., 2017; Calvano Filho et al., 2014).

Expression levels of miRNAs were compared with Student's *t*-test and the Wilcoxon rank sum test. miRNA signals were normalized and log-transformed prior to calculations of fold-change and applications of statistical tests. *P*-values were corrected according to the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995). MiRNAs with a corrected *p*-value less than 0.1 were considered to be differentially expressed. Package pheatmap in R language was used to cluster the miRNA expression data (https://CRAN.R-project.org/package=pheatmap).

Statistical analysis

Statistical analyses were performed with GraphPad Prism (version 5.01, San Diego, CA, USA) and SPSS (version 13.0). Significant associations between miRNA expression data and histological features and clinical data (i.e., tumor relapse, patient age, tumor size, metastasis events, and death) were analyzed with Fisher's exact test with $p < 0.05$ set as the significance level. Kaplan-Meier analyses with the log-rank test were performed to obtain survival curves from the date of diagnosis until death or last follow-up.

Results

miRNA expression profiles in the tumor samples examined

For our analyses of miRNA expression profiles, MM samples were included to provide reference levels of miRNAs from a normal uterine mesenchymal tissue. Their expression values were used to normalize miRNA levels and were not included in the statistical analyses.

A hierarchical cluster analysis of the miRNA data obtained shows that 57 miRNAs exhibited differential regulation in the tissues evaluated (Fig. 1; Additional file 1: Table S1). When a 2-fold change in expression was used as a cutoff, 13 miRNAs were found to be downregulated and 7 miRNAs were found to be upregulated in the LM tissues. In the ULM tissues, 27 miRNAs were downregulated and 11 miRNAs were upregulated. In the LMS tissues, 19 miRNAs were downregulated and 25 miRNAs were upregulated (Additional file 1: Table S1).

When the LMS and LM samples were compared, 16 miRNAs exhibited differential expression (with 7 miRNAs upregulated and 9 miRNAs downregulated) (Table 1; Fig. 2). In LMS, higher levels of *miRNA-372-3p* (a 5.69-fold increase) and lower levels of *miRNA-34a-5p* (a 3.94-fold decrease) were detected.

When LMS and ULM data were compared, two miRNAs exhibited an increase in expression, while three miRNAs exhibited a decrease in expression (Table 1; Fig. 2). *MiR-34a-5p* showed the greatest down-regulation (3 times negative fold change), while *miR-144-3p* showed the greatest increase in expression (a 2.37 fold change).

When ULM and LM miRNA data were compared, five miRNAs exhibited differential expression, with all of them being upregulated. In particular, *miR-122-5p* exhibited a 10.3-fold increase in expression, while *miR-372-3p* exhibited a 7.5-fold increase in expression (Table 1; Fig. 2). Furthermore, the latter increase was greater than that detected for the same miRNA between LMS and LM tissues.

Subsequently, we compared uterine LMS to both ULM and LM, and four miRNAs were found to be differentially expressed, with all of them upregulated (Table 1). In particular, *miR-206* exhibited more than a 3-fold change in expression.

Correlation between the miRNA profile of LMS and patients' clinicopathological features

Clinical and pathological data for the patients corresponding to the tissue samples analyzed were obtained as previously described (Garcia et al., 2016; de Almeida et al., 2017). To investigate possible associations between miRNA expression levels and LMS prognosis, we analyzed the following clinicopathological characteristics: patient age, tumor size, clinical stage, relapse, metastasis, treatment, and patient status (Table 2).

Among the miRNAs listed in Table 1, when a 4-fold change in expression level was used as the cutoff to identify significant associations with LMS patients' features, only four miRNAs were identified. Downregulation of *miR-27b-3p* was found to be associated with occurrence of metastasis ($p = 0.029$), upregulation of *miR*-124-3p and miR-183-5p were associated with patient death ($p = 0.024$ and $p = 0.001$, respectively), tumor relapse was associated with downregulation of *miR-148a-3p* n ($p = 0.021$), and upregulation of *miR-135b-5p* exhibited a significant association with poor disease-free survival (DFS) (Fig. 3). None of the other miRNAs exhibited an association with overall survival or DFS. Furthermore, when the cut-off value was reduced to a 2-fold change in expression level, the results remained unchanged.

Discussion

In this study, 84 miRNAs previously associated with human cancers were profiled in FFPE tissue samples of uterine LMS. It has previously been demonstrated that RNA can be successfully isolated from FFPE tissues to provide large amounts of sample (Bovell et al., 2013; Nagy et al., 2016). Moreover, these RNA samples include small RNA fragments, including miRNAs, which can be detected by qRT-PCR (Nagy et al., 2016).

We compared malignant LMS tumor samples with their benign counterpart, LM tumor samples, in order to compare the spectra of uterine muscle neoplasms that LMS and LM represent. Our aim was to identify miRNAs involved in the process of tumorigenesis and those that affect the biological behavior of tumors. miRNAs involved in cell proliferation, tumorigenesis, and tumor aggressiveness were identified, such as *miR-372-3p*. In addition, *miR-34a-5p* was identified as being differentially expressed, and this miRNA was previously characterized as having roles in multi-drug resistance and DNA damage and repair. However, neither miRNA was previously described in association with uterine LMS.

Moreover, to date, very few studies have discussed the role of miRNAs in uterine LMS tumors. Thus, it remains for a more accurate diagnosis and efficient treatment strategy to be identified for this rare malignancy, and the present work demonstrates that miRNAs represent a promising tool in these efforts.

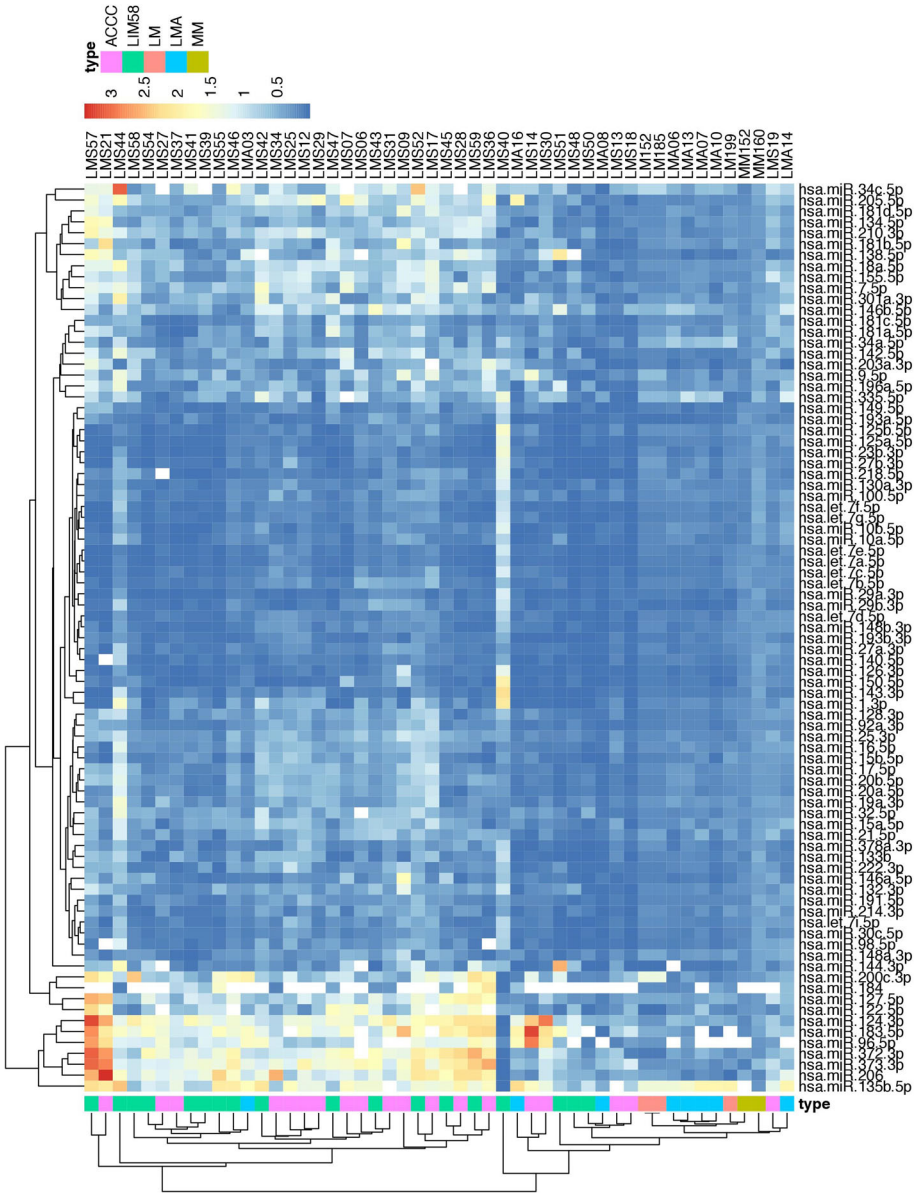

Fig. 1 Hierarchical clustering of miRNAs according to tissue. In this heatmap, hierarchical data of miRNA expression profiling in LM, ULM, and LMS tumors are represented, with MM samples used as a normal reference. Blue coloring indicates miRNAs that are downregulated and red coloring indicates miRNAs that are upregulated. Type color bar indicates institutional origin of the LMS samples (purple: ACCC – A.C.Camargo Cancer Center; green: LIM58 – Hospital das Clinicas da Universidade de Sao Paulo) and histological classification of non-malignant tissues (LM - red color, LMA - blue and MM – ochre)

In 2016, Ravid et al. (Ravid et al., 2016) reported a unique signature of seven differentially expressed miRNAs that differentiate primary uterine LMS from stromal sarcoma. In the present study, these same miRNAs exhibited low expression in our LMS samples compared to our LM samples, suggesting that these miRNAs may be related to cellular differentiation in addition to cell behavior.

miR-34a-5p has been well studied and is associated with many cancers, including Ewing's sarcoma (Nakatani et al., 2012), colorectal cancer (Gao et al., 2014), breast cancer (Si et al., 2016), and osteosarcoma (Pu et al., 2017). In many of these cancers, it has been observed that *miR-34a* is either lost or is present at lower levels, and this prevents a possible tumor suppressor role for this miRNA (Misso et al., 2014). It has also been proposed that *miR-34a* represents an ideal therapeutic molecule for targeting tumor metastasis and recurrence (Hart et al., 2016). In the present study, *miR-34a-5p* was found to be downregulated in our comparisons of LMS

Table 1 Comparison of differentially expressed miRNAs among the tumor types examined

miRNA expression profile (between tumors)				
Samples Compared	Upregulated		Downregulated	
	miRNA	Fold-change	miRNA	Fold-change
LMS x LM	*miR.372.3p*[a]	5.698754	*miR.23b.3p*	−2.137800
	miR.122.5p	4.837798	*miR.21.5p*	−2.163121
	miR.206	3.360921	*miR.218.5p*	−2.335078
	miR.144.3p	3.069912	*miR.181a.5p*	−2.539156
	miR.373.3p	2.935396	*miR.9.5p*	−2.626620
	miR.138.5p	2.694513	*miR.135b.5p*	−2.744331
	miR.142.5p	2.358521	*miR.148a.3p*	−2.927199
			miR.27b.3p	−3.096370
			miR.34a.5p[a]	−3.948848
LMS x ULM	*miR.144.3p*[a]	2.371905	*miR.135b.5p*	−2.388273
	miR.183.5p	2.147752	*miR.335.5p*	−2.916416
			miR.34a.5p[a]	−3.004453
ULM x LM[b]	*miR-122-5p*[a]	10.309624		
	miR-372-3p	7.464555		
	miR-124-3p	3.119064		
	miR-210-3p	3.038778		
	miR-373-3p	2.107957		
LMS x ULM + LM[b]	*miR-206*[a]	3.1593382		
	miR-373-3p	2.7755556		
	miR-372-3p	2.6455628		
	miR-124-3p	2.2583386		

[a]miRNAs with the greatest changes in expression (up or downregulation)
[b]There were no miRNAs that were found to be downregulated

and LM, and LMS and ULM. Therefore, we hypothesize that its suppression plays a role in tumorigenesis, and possibly in metastasis events as well, since ULM does not exhibit metastatic potential. In 2016, Pu et al. (Pu et al., 2017) demonstrated that *miR-34a-5p* promotes multi-drug resistance in osteosarcoma cell lines via *CD117*. Correspondingly, it has been observed that LMS is not effectively responsive to chemotherapy (Novetsky & Powell, 2013). Thus, future studies should investigate whether increased expression of *miR-34a-5p* would improve chemotherapy responsiveness for LMS.

When comparing miRNA levels between LMS and LM, *miR-372-3p* was identified as the most upregulated miRNA in LMS. Both *miR-372-3p* and *miR-373-3p* were previously shown to have roles in regulating the proliferation and invasion of cancer cells via neutralization of inhibition of p53-mediated cyclin-dependent kinase (Syring et al., 2015). These miRNAs also mediate activation of Wnt/β-catenin (Syring et al., 2015). Furthermore, in 2006, Voorhoeve et al. (Voorhoeve et al., 2006) hypothesized that *miR-372* and *miR-373* participate in the tumorigenesis process of some tumors by blocking wild-type p53 and they can induce sensitivity to treatments that promote DNA damage. Thus, deregulated expression of both of these miRNAs could predispose cells to an accumulation of carcinogenic events. *miR-372-3p* was also identified as the second most upregulated miRNA in ULM compared with LM, and its increased expression level in the latter comparison was greater than the increase in expression observed between ULM and LM. This finding is consistent with the observation that benign LM tumors exhibit extensive local growth. Moreover, given the role that *miR-372* has in cell proliferation, this would justify a higher cellularity or higher mitotic index, but would not necessarily enhance malignant potential.

Our comparison of tumor samples also identified lower levels of *miR135b-5p* in LMS compared to ULM and LM. Over the last decade, involvement of *miR-135b-5p* has been demonstrated in gastric carcinoma (Vidal et al., 2016), ovarian epithelial cancer (Wang et al., 2014), and endometrial tumors (Brany et al., 2015). However, since ULM and LM do not exhibit aggressive or metastatic potential behavior, the role of *miR135b-5p* in these types of cancer may be limited to cell differentiation, and may not extend to mechanisms of invasion. Nonetheless, the LMS patients in the present study with lower expression levels of *miR-135p-5p* had poor survival rates.

The observation that *miR-144-3p* is upregulated in LMS compared to ULM suggests that *miR-144-3p* is involved in cell proliferation and mechanisms of invasion and metastasis. Previously, *miR-144-3p* was described as an erythrosine-specific miRNA (Fu et al., 2009). More recently, Zhang et al. (Zhang et al., 2013) demonstrated in vitro and in vivo a potential oncogenic role for this miRNA in nasopharyngeal carcinoma (NPC). For example, upregulation of this miRNA enhanced the development and progression of NPC. Conversely, when this miRNA was present at lower levels, cell proliferation and tissue invasion in mice were inhibited. However, in a study of squamous cell carcinoma of the larynx, overexpression of *miR-144-3p* inhibited both cell cycle progression and tissue invasion (Zhang et al., 2016). Considering these previous data, upregulation of *miR-144-3p* in LMS, compared with ULM, suggests a role for this miRNA in mediating cell cycle progression and metastasis.

Previously, reduced expression of miR-122 in osteosarcoma compared to normal tissue was associated with regulation of tumor necrosis factor-related ligand-inducing (TRAIL) (Xiao et al., 2015). Thus, miR-122 was suspected of having a tumor suppressor role (Xiao et al., 2015). In the present study, *miR-122* was upregulated in ULM compared to LM. These results are consistent with a role for *miR-122*

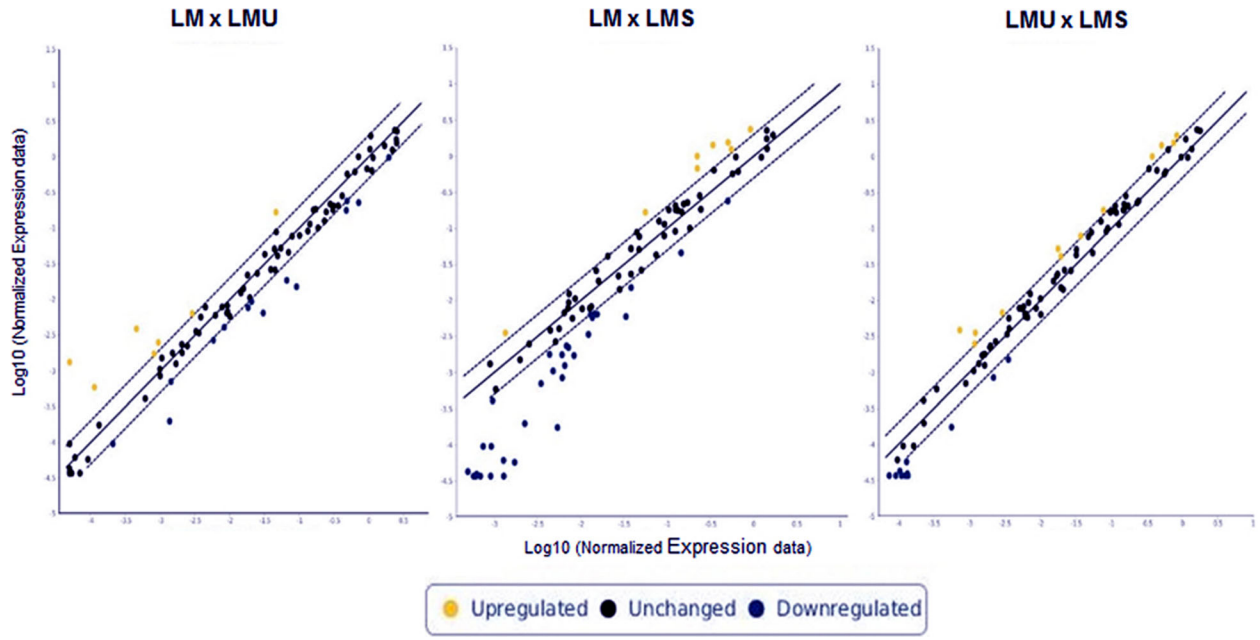

Fig. 2 Diferential miRNA expression between tumor types. Scatter plots present a global analysis of the miRNAs that were found to be differentially expressed among the tumors examined

in inhibiting cell proliferation and ULM development, without promoting invasion and metastasis.

When our LMS samples were compared with our LM and ULM samples, upregulation of *miR-206* was detected in the LMS samples. However, differential expression was observed in comparison with the LM samples, and was not observed in comparison with the ULM samples. In a study of uterine sarcomas, Kowalewska et al. (Kowalewska et al., 2013) reported that *miR-206* was also downregulated in LMS in relation to normal uterine fragments. However, the authors observed an unexpected (although not significant) trend in relation to the overexpression of *miR-206* in endometrial sarcomas and mixed epithelial-mesenchymal tumors compared to normal uterine tissue. In the present study, a contradictory result was observed: *miR-206* was upregulated in the LMS samples and not in the LM samples. Thus, additional studies of uterine LMS are needed to understand this result, since *miR-206* appears to serve as a tumor suppressor. Nevertheless, *miR-206* represents an important component of the differentiation signature identified in the present study for differentiating LMS from LM, since it was the most differentially expressed miRNA identified in the comparisons of LMS and ULM and LM. Furthermore, *miR-206* was found to be upregulated in LMS compared with LM, thereby indicating its involvement in the development of LMS.

Table 2 Clinicopathological features of the patients with LMS (n = 37)

Feature	Categories	No. cases (%)
Patient age	≤ 50 years	19 (51.0)
	> 50 years	18 (49.0)
Clinical stage	I	10 (27.0)
	II	10 (27.0)
	III	5 (13.5)
	IV	12 (32.5)
Relapse	No	6 (16.0)
	Yes	27 (73.0)
	NA[a]	4 (11.0)
Metastasis	Locoregional	12 (32.0)
	Distant	15 (41.0)
	Absent/NA	10 (27.0)
Treatment	Surgery alone	10 (27.0)
	Radiotherapy	11 (29.7)
	Chemotherapy	11 (29.7)
Patient status	Surviving	7 (18.9)
	Dead	26 (70.3)
	Loss of follow-up/NA	4 (10.8)

[a]NA: not available

Conclusion

The analyses performed in the present study demonstrate the potential for miRNAs to constitute a signature for distinguishing uterine LMS from LM. The miRNAs identified include: *34a-5p, 144-3p*, and *206*. Moreover, upregulation of *miR-122-5p* appears to characterize

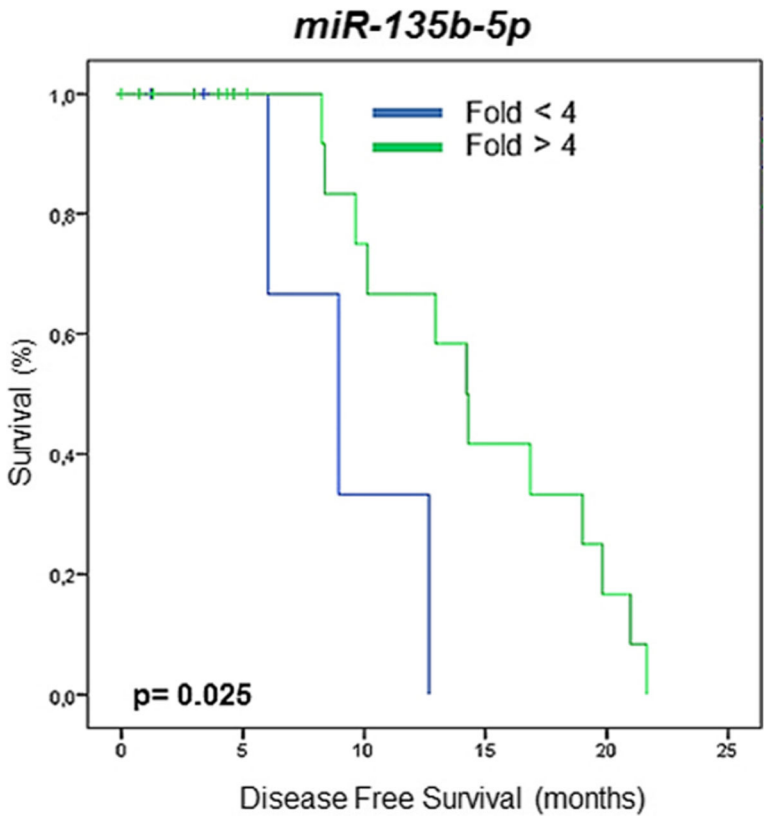

Fig. 3 Survival rates associated with *miR-135b-5p* expression. A Kaplan-Meier survival analysis shows DFS rates that are associated with positive expression values of *miR-135b-5p*. Data associated with less than a 4-fold change are shown in blue, while data associated with greater than a 4-fold change are shown in blue

ULM, thereby providing valuable insight into ULM tumors that share morphological characteristics with both LM and LMS.

Concerning prognosis, downregulation of miRNAs *27b-3p* and *135b-5p*, and upregulation of miRNAs *124-3p* and *183-5p*, appear to indicate a poor prognosis for LMS patients. It remains for future studies to confirm these findings.

Additional file

Additional file 1: Table S1. Global list of miRNAs exhibiting differential expression among LM, ULM, and LMS neoplasms with MM used as a reference. (DOCX 23 kb)

Abbreviations

cDNA: Complementary deoxyribonucleic acid; Ct: Threshold-cycle; LM: Leiomyoma; LMS: Leiomyosarcoma; MiRNA: MicroRNA; MM: Myometrium; NPC: Nasopharyngeal carcinoma; qRT-PCR: Quantitative real-time polymerase chain reaction; RNA: Ribonucleic acid; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; ULM: Unconventional leiomyoma; WHO: World Health Organization

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Availability of data and materials

All of the data for the present study are presented in the Tables and Figures provided, except patients' private information and raw data.

Authors' contributions

BNS – MiRNA analysis and manuscript preparation. KCC – Support of miRNA analysis and manuscript preparation. CMCC – Supervisor of miRNA experiments and manuscript preparation. GB – Collection and organization of patients' data. RV and RD– Statistical analysis of miRNA and patient data. ITS – Statistical analysis of miRNA and patient data. LDB – Case selection. FAS – Institutional infrastructure and intellectual support. IWC – Original conception of research idea, case selection, supervision of studies, and intellectual support. All of the authors have read and approved the manuscript in its present form.

Ethics approval and consent to participate

The present work was approved by institutional review boards of the A.C.Camargo Cancer Center and the Faculdade de Medicina da Universidade de Sao Paulo (Registration no. 1816/13 and 1,517,306, respectively).

Consent for publication

All of the authors have approved the manuscript in its present form and have signed the consent form.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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