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Uterine leiomyosarcoma and endometrial stromal sarcoma have unique miRNA signatures

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HIGHLIGHTS

- microRNA array analysis differentiates uterine endometrial stromal sarcoma from leiomyosarcoma.
- microRNAs are predominantly underexpressed in metastatic compared to primary leiomyosarcoma.
- Frizzled-6 silencing suppresses invasion in leiomyosarcoma cells in vitro.

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ABSTRACT

Objective. To compare the microRNA (miRNA) profiles of uterine endometrial stromal sarcoma (ESS) and leiomyosarcoma (LMS), and to compare the miRNA signatures of primary and metastatic uterine LMS.

Methods. Eight primary LMS, 9 primary ESS and 8 metastatic LMS were analyzed for miRNA profiles using TaqMan Human miRNA Array Cards. Findings for 20 differentially expressed miRNAs were validated in a series of 44 uterine sarcomas (9 primary uterine ESS, 17 primary uterine LMS, 18 metastatic LMS) using qPCR. Frizzled-6 protein expression was analyzed in 30 LMS (15 primary, 15 metastases). Frizzled-6 was silenced in SK-LMS-1 uterine LMS cells using siRNA and the effect on invasion, wound healing and matrix metalloproteinase-2 (MMP2) activity was assessed.

Results. Ninety-four miRNAs were significantly differentially expressed in ESS and LMS, of which 76 were overexpressed in ESS and 18 overexpressed in LMS. Forty-nine miRNAs were differentially expressed in primary and metastatic LMS, of which 45 were overexpressed in primary LMS and 4 in metastases. Differential expression was confirmed for 10/20 miRNA analyzed using qPCR. Frizzled-6 silencing in SK-LMS-1 cells significantly inhibited cellular invasion, wound healing and MMP-2 activity.

Conclusions. Differential miRNA signatures of ESS and LMS provide novel data regarding transcriptional regulation in these cancers, based on which new potential diagnostic markers, prognostic biomarkers and therapeutic targets may be explored. Differences in miRNA profiles of primary and metastatic LMS may improve our understanding of disease progression in this aggressive malignancy.

1. Introduction

Uterine sarcomas are rare tumors, comprising 7% of all soft tissue sarcomas and about 3% of uterine malignancies [1–3]. Leiomyosarcoma (LMS) and endometrial stromal sarcoma (ESS) are the most common histological types [2,3]. Adenosarcoma and carcinosarcoma are both mixed epithelial–mesenchymal tumors, but only the former have a true sarcomatous component, whereas carcinosarcomas are currently regarded as metaplastic carcinomas. ESS has been previously classified as low-grade or high-grade, later regarded as a single entity, and recently re-divided into low-grade and high-grade categories, although the latter group constitutes rare tumors [4].

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MicroRNAs (miRNAs) are small (19–25 nucleotides), non-coding RNAs that post-transcriptionally regulate gene expression [5]. miRNAs are synthesized as a long double-stranded precursor called pri-miRNA by DNA polymerase II in the nucleus. Pri-miRNA is cleaved at specific sites by the RNAse Drosha inside the nucleus, producing a pre-miRNA which is exported to the cytoplasm by the exportin 5 protein, where it is processed by Dicer into mature miRNA. Mature miRNAs are subsequently activated through binding to the RNA-induced silencing complex (RISC) [6,7]. Through the RISC, miRNAs can regulate their targets, mediating translational repression or degradation. The sequence at the 5′ end of the mature miRNA, called the "seed region", binds its complementary sequence within the 3′ untranslated regions (UTR) of the target mRNA [8]. Perfect or near-perfect complementarity between the miRNA and its mRNA target results in mRNA degradation, whereas lesser complementarity leads to translational inhibition.

Data regarding the miRNA profile of uterine sarcomas is limited to date. An inverse association was reported for the miRNA Let-7 and its target high-mobility group AT-hook-2 (HMGA2) in uterine LMS [9]. miRNA profiles which differentiate uterine LMS from leiomyoma [10] or from different types of leiomyoma as well as smooth muscle tumors of uncertain malignant potential (STUMP) [11] have been described. In another report, the miRNA profiles of uterine sarcomas and carcinosarcomas were compared to those of patient-matched benign tissue [12].

Our group has previously reported on gene expression profiles which differentiate LMS from ESS [13] and primary LMS from metastatic LMS [14]. Similar data with respect to miRNA profiles are unavailable to date to the best of our knowledge. The present study compared the miRNA profiles of primary ESS, primary LMS and metastatic LMS.

2. Material and methods

2.1. Patients and material

Specimens consisted of 44 uterine sarcomas, including 9 primary uterine ESS, 17 primary uterine LMS and 18 metastatic LMS, submitted for routine diagnostic purposes to the Department of Pathology at the Norwegian Radium Hospital during the period 1993–2009. Tumors were snap-frozen and kept at −70 °C. Frozen sections were evaluated for the presence of a >80% tumor component and absence of necrosis. Diagnoses were established by an experienced gynecologic pathologist (BD) based on morphology and immunohistochemistry. The series studied consisted of the same 35 tumors analyzed in our previous reports [13,14], to which 9 new tumors were added (2 high-grade ESS, 4 primary LMS, 3 metastatic LMS). As previously detailed [14], the majority of primary and metastatic LMS were not patient-matched. Clinicopathologic data are detailed in Table 1.

The study was approved by the Regional Committee for Medical Research Ethics in Norway.

2.2. Isolation of total RNA and miRNA

Total RNA, including miRNA, was isolated from the uterine biopsies using the miRVana miRNA Isolation Kit (Ambion-Applied Biosystems, Austin TX). RNA concentration and integrity were determined using a NanoDrop-1000 spectrophotometer (Thermo Scientific, Wilmington DE). RNA samples were stored at −80 °C before TaqMan miRNA array studies.

2.3. TaqMan miRNA arrays cards

miRNA expression profiling was performed using the TaqMan Human miRNA Array Card A (Applied Biosystems) containing 381 mature human miRNAs in miRBase 10.1 (http://microRNA.sanger.ac.uk). Briefly, miRNA was reverse-transcribed to cDNA using the Megaplex™ RT Human Primers Pool and the TaqMan MiRNA Reverse Transcription Kit (Applied Biosystems). Quantitative 384-well TaqMan low-density array real-time PCR was run on the ABI PRISM 7900 System using the TaqMan Universal PCR Master Mix (Applied Biosystems). Raw miRNA array data were analyzed by using the RQ manager and Data Assist software of the ABI system (Applied Biosystems). Normalized C_T (ΔC_T) was calculated by comparing each miRNA value to the geometric mean of three endogenous controls, RNU 44, RNU 46 and small nuclear U6 RNA. One assay not related to human miRNA (ath-miR159a) was included as negative control.

Eight primary and 8 metastatic LMS, as well as all 9 ESS were analyzed for miRNA profile and served as training group of our study. For validation, the entire material (17 primary LMS, 18 metastatic LMS, 9 primary ESS) was analyzed. Conversion of miRNA and mRNA into cDNA and quantitative PCR (qPCR) detection of miRNAs was carried out according to the manufacturer's protocols using the miScript Reverse Transcription Kit and miScript SYBR Green PCR Kit (Qiagen GmbH). qRT-PCR was performed on Mx3000P® QPCR System (Stratagene, La Jolla CA). Bioinformatics tools were used to identify their predicted targets and the molecular networks they may affect.

2.4. Western blot analysis

Thirty LMS (15 primary, 15 metastatic) were analyzed using Western blotting. Tumors were lysed in lysis buffer (1% NP40, 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 1× protease inhibitors cocktail and 0.1% SDS (Sigma, St. Louis MO). Fifteen micrograms of protein from each sample were loaded under reducing conditions on 8% SDS-PAGE gel. Following electrophoresis, the proteins were transferred to PVDF transfer membranes (Millipore, Bedford MA). Membranes were blocked in Tris Buffered Saline with Tween 20 (TBST; 10 mM Tris–HCl [pH 8.0], 150 mM NaCl and 0.1% Tween 20) containing 5% non-fat dry milk (Nestlé, Vevey, Switzerland) for 1 h at room temperature. Membranes were then incubated with a rabbit monoclonal antibody against frizzled-6 (Abcam; cat. # 128916) overnight in 4 °C, washed with TBST and incubated with secondary antibody (Peroxidase-conjugated AffinPure goat anti-mouse, 1:20000 dilution, Jackson Immuno-Research Laboratories, Inc., West Grove, PA). After washing with TBST, bands were visualized by enhanced chemiluminescence (ECL; Pierce, Rockford IL), according to the manufacturer's instructions. Membranes were stripped, blocked and then re-incubated with anti-GAPDH antibody (Sigma). The developed X-ray films were scanned for quantification and analysis was performed on a Macintosh computer using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/). Loading was corrected by calculating the ratio between frizzled-6 and GAPDH values and was expressed as arbitrary units (AU).

2.5. Frizzled-6 silencing using siRNA

SK-LMS-1 uterine LMS cells were obtained from ATCC (Manassas, VA) and cultured according to ATCC instructions. Cells were used for silencing studies at sub-confluent conditions. Gene silencing-Frizzled-6 siRNAs (3 sequences) were purchased from Sigma-Aldrich (St. Louis MO). Silencing efficiency was estimated for each of the sequences and for the combination of all three.

Cellular invasion was measured using Boyden chamber assay with Matrigel-coated PVPF filters with 8 μm pores as previously described [15]. Zymography was performed as previously detailed [15]. Scratch assay ("wound healing" assay) was performed as follows: 24 h prior to the experiment, cells transfected with si-FRZ6 construct were seeded to create a confluent monolayer. Cell monolayers were scraped with a pipette tip to create approximately similarly sized scratches. Cells were incubated for 24 h, and images acquired at 0 and 24 h. Scratch closure was analyzed by comparing images obtained at these time points.