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Original contribution

The FOXO1-miR27 tandem regulates myometrial invasion in endometrioid endometrial adenocarcinoma☆,☆☆

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Summary Micro-RNA (miRNA) signatures influence the prognosis of cancer, but little is known about their role in myometrial invasion in endometrioid endometrial adenocarcinoma (EEC). We studied miRNA expression signatures in noninvasive and invasive EEC focusing on the alteration of miR-27 and its main target, FOXO1 as well as their relationship with the clinicopathological parameters and other genetic alterations such as *PIK3CA* mutations. In 25 tumors and 5 normal endometria, unsupervised hierarchical clustering analysis showed that normal endometria and noninvasive EEC were grouped together and separately from invasive and advanced stage tumors. Of the 20 miRNAs differentially expressed in noninvasive (stage IA) and myoinvasive adenocarcinomas (stage IB and IC), miR27 was overexpressed in invasive adenocarcinomas, and its expression increased linearly according to stage. Results were validated by quantitative real-time reverse transcription polymerase chain reaction in an independent series of 44 EEC. By in situ hybridization, miR-27 expression was limited to the stroma. Using quantitative real-time reverse transcription polymerase chain reaction, the expression of proapoptotic transcription factor FOXO1 was down-regulated in invasive compared with noninvasive tumors. Furthermore, we found that the expression of active caspase 3 was higher in noninvasive than invasive EEC. When stratified by *PIK3CA* mutations, all invasive tumors down-regulated FOXO1, but only nonmutated adenocarcinomas showed miR-27 overexpression. In conclusion, we propose that the miR27-FOXO1 tandem inhibits apoptosis and represents an alternative pathway for tumor cell survival in *PIK3CA*-nonmutated EEC.
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1. Introduction

Small noncoding RNAs of 20 to 22 nucleotides or microRNAs (miRNAs) have been found to have a pivotal role in oncogenesis by posttranscriptionally inhibiting the expression of many different genes. So far, over 1500 human miRNAs have been identified. Similarly to protein-encoding

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genes, miRNAs have a critical role in the regulation of oncogenes and tumor suppressor genes [1].

The expression of miRNAs in certain neoplastic tissues differs from that of their normal counterparts, and consequently, the expression of the genes controlled by them is also different [2]. To date, several miRNA signatures have been described in various types of cancer. These signatures provided some specific miRNAs that are up-regulated or down-regulated in certain tumors and have prognostic significance [3]. miRNAs have also been related to the regulation of the neoplastic microenvironment, and several groups have demonstrated their expression and secretion both by neoplastic cells and the microenvironment, such as macrophages and fibroblasts [4-6]. miR-27 has been reported as an oncomiR in gastric, endometrial, and esophageal carcinomas [7-9], mainly as a cell cycle regulator. Two isoforms of miR-27 have been described, namely, miR-27a and miR-27b. The isoforms differ only in 1 nucleotide in their sequence and have similar effects on their target genes [10].

Although the pathogenesis of endometrioid endometrial adenocarcinoma (EEC) has been investigated widely, little is known about the molecular mechanisms responsible for myometrial invasion and metastasis.

The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway is frequently activated in human epithelial cancers. Activated AKT regulates the expression of various downstream target genes including *FOXO* family genes, which in turn regulate key cellular processes such as apoptosis and cell cycle. Activation of the PI3K/AKT signaling pathway through *PTEN* and/or *PIK3CA* (the p110a catalytic subunit of PI3K) mutations occurs frequently in endometrial adenocarcinomas [11]. *PIK3CA* mutations, predominantly in exons 9 and 20 are found in up to 39% of cases and coexist frequently with *PTEN* mutations and are associated with adverse prognostic factors such as high-grade and myometrial invasion [11].

Recently, miRNA deregulation has been studied in early (stage IA) and advanced stage EEC (stages III and IV) to elucidate their role in its oncogenic pathways [12].

We investigated the miRNA profiles of noninvasive and invasive EEC focusing on the alteration of a specific miRNA, miR-27, its main target, FOXO1, and their relationship with *PIK3CA* mutation.

2. Materials and methods

2.1. Case selection

A series of 69 patients with EEC and available fresh frozen and formalin-fixed, paraffin-embedded tissue were enrolled for the study, including 25 cases in the initial series for miRNA expression profiles and 44 in the validation series. The patients underwent hysterectomy at Hospital de la Santa Creu i Sant Pau from 1997 to 2011. Tumors were staged according to the 1988 staging system of the International Federation of Gynecology and Obstetrics. All diagnoses were reviewed by 2 pathologists, and the grade and percentage of epithelium and stroma were evaluated. Clinical and pathologic information were recorded from the hospital charts and pathology reports. As control, we used 5 samples of normal endometrium of age-paired women obtained from nonneoplastic hysterectomy specimens. Written informed consent and approval by the hospital ethics committee were obtained.

2.2. Tissue microarray, immunohistochemistry, and laser capture microdissection

Two representative 0.6-mm tissue cores were arrayed using a tissue microarray (TMA) workstation (Beecher Instruments, Sun Prairie, WI). A hematoxylin and eosin–stained section was made to confirm the presence of the original areas selected from each tumor. Subsequently, serial-sectioned slides were obtained.

Immunohistochemical staining of the TMA was performed with antibodies against FOXO1 (1:25; Cell Signaling Technology, Danvers, MA) and Cleaved Caspase 3 (1:1000; Cell Signaling Technology). Cases were evaluated by 2 pathologists. Regarding FOXO1, a case was considered negative when no nuclear immunoreaction was observed, whereas a positive case showed nuclear immunostaining. As for Cleaved Caspase 3, any amount of cytoplasmic staining was considered as positive. The intensity (0-3) and the percentage of stained cells were used to produce a score.

Five representative cases were microdissected using the Laser Microdissection System LMD7000 (Leica Microsystems, Wetzlar, Germany). Ten micrometer-thick sections obtained from frozen tissue were fixed on PEN membrane frame slides (Leica Microsystems) and stained with hematoxylin and eosin. Tissue fractions of normal, tumor stroma, and tumor epithelial cancer cells were isolated into separate microcentrifuge tubes.

2.3. RNA isolation and real-time reverse transcription polymerase chain reaction

Total RNA for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was extracted from fresh frozen tissue using Trizol reagent (Invitrogen, Carlsbad, CA) and the RNeasy Microkit (Qiagen, Valencia, CA) as specified by the manufacturer's instructions and used for miRNA gene expression profiling and FOXO1 messenger RNA (mRNA) and miR27 expression quantification. One microgram of total RNA was used for complementary DNA synthesis according to the protocol provided with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA); and 10 ng of total RNA was used to convert miRNA into complementary DNA according to the protocol provided with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The product was amplified and quantified using

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