High-Risk Endometrial Carcinoma Profiling Identifies TGF-β1 as a Key Factor in the Initiation of Tumor Invasion

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Abstract

Endometrial cancer is among the three most common cancers in females in industrialized countries. In the majority of cases, the tumor is confined to the uterus at the time of diagnosis and presents a good prognosis. However, after primary surgery, 15% to 20% of these tumors recur and have limited response to systemic therapy. We carried out gene expression profiling of high-risk recurrence endometrial cancers to identify new therapeutic approaches targeting the molecular pathways involved in the acquisition of an aggressive tumor phenotype. A microarray gene-expression analysis on a total of 51 human endometrial carcinomas revealed 77 genes specifically altered in high-risk recurrence tumors (P < 0.001). The bioinformatics analysis of gene–gene interactions and molecular relationships among these genes pointed to a prominent role for TGF-β1 signaling in the acquisition of an aggressive phenotype. We further showed that TGF-β1 has a principal role at the initiation of endometrial carcinoma invasion through the promotion of the epithelial to mesenchymal transition that leads to the acquisition of an invasive phenotype in HEC-1A and RL95-2 cells. Impairment of this initial step with SB-431542, a specific TGF-β1 inhibitor, precluded further persistent endometrial carcinoma invasion. In conclusion, we showed that the characterization of the molecular changes associated with the acquisition of an aggressive phenotype represents a realistic strategy for the rational identification and characterization of new potential therapeutic targets in an effort to improve the clinical management and the outcome of high-risk endometrial cancer patients.

Introduction

Endometrial carcinoma is the most frequently diagnosed malignancy of the female genital tract in industrialized countries. Its incidence is estimated at 15 to 20 in 100,000 women per year (1). endometrial carcinoma is usually divided into type I and type II categories based on clinical behavior and morphologic phenotype, with good correlation to the molecular findings (2). The appearance of early symptoms explains why most cases are diagnosed at an early stage when disease is still confined to the uterus and presents a high percentage of survival (3). Nevertheless, a subset of endometrial tumors exhibits an aggressive phenotype characterized by lymphovascular invasion, high histological grade, and myometrial invasion, leading to poor prognosis. Approximately 25% of the patients who have undergone surgical staging are found to have extraperitoneal disease (4). endometrial carcinoma spreads primarily to the pelvic and paraaortic lymph nodes, as well as to the adnexa and pelvic viscera, with distant metastases via the hematogenous route having a low incidence. The mechanisms involved in this aggressive transformation and dissemination are largely unknown (5).

From a clinical point of view, an increased risk of relapse (high-risk phenotype), defined as patients with tumors invading deep into the myometrium or the cervical stroma or with extraterine spread and patients with uterine papillary serous carcinoma or clear cell carcinoma, represents a therapeutic challenge. Surgical treatment remains the cornerstone of therapy and, in particular, high-risk patients would seem to benefit from complete pelvic and paraaortic lymph node staging with adjuvant treatments tailored to the results of lymphadenectomy (6). Nowadays, treatment in advanced disease consists of chemotherapy, radiotherapy, or a combination of both, with decisions based on the presence of risk factors.
In recent years, there has been a renewed interest in incorporating chemotherapy into treatment paradigms for women with endometrial carcinoma (7). For patients with advanced disease (stage III and IV) a combination of chemotherapy with cisplatin and doxorubicin has been found to be superior to radiotherapy (8). Newer agents such as paclitaxel have shown promising survival and response rates in endometrial cancer patients as a single agent or in combination with cisplatin/carboplatin and doxorubicin chemotherapy (9). It remains to be seen whether adjuvant chemotherapy in patients with high-risk disease in a lower stage will improve survival and possibly replace adjuvant radiotherapy in some groups of patients (10). Interestingly, various targeted therapies including mTOR, EGFR and VEGF inhibitors, appear promising for subgroups of patients (11). Nevertheless, and as in most other cancer diseases, the molecular pathways involved in endometrial carcinoma onset and progression are very complex, and involve high penetrance genes as well as intricate interactions of multiple low penetrance genes.

Despite the great effort made to establish the pathogenesis of endometrial carcinoma, the key molecular events responsible for tumor invasion and dissemination are still not clearly delineated (12). Gaining insight into the molecular alterations involved in endometrial carcinoma progression and invasion may provide us with opportunities for developing new therapeutic approaches to inhibit tumor spread and to improve the outcome in high-risk endometrial carcinoma patients. The aim of this work was to characterize at the molecular level a phenotype of high-risk recurrence in endometrial carcinoma, and to identify potential therapeutic targets focused on this specific group of patients. For this, we carried out a global gene expression comparison between low- and high-risk endometrial carcinomas by microarray technology. Bioinformatics pointed to a reduced group of 98 genes, only 77 were annotated genes and thus identified by the Ingenuity Pathway Analysis Software (IPA), used to provide a global view of the pathways involved in the high-risk recurrence in endometrial carcinoma.

**Cell-inverted invasion assay**

HEC-1A endometrial carcinoma cells were cultured as described (14), and RL95-2 endometrial adenosquamous carcinoma cells were grown in DMEM/F12 medium containing 10% FBS and 1% penicillin/streptomycin. The invasion assay was conducted as described (15). EGF (60 ng/mL; Sigma) or TGF-β1 (2.5 ng/mL; R&D Systems Inc.) were used as chemoattractants, and SB-431542 (20 μM/L; Sigma) was used as a specific TGF-β1 signaling inhibitor by blocking TβRI kinase activity (16). Cells were allowed to migrate and invade into growth factor-reduced Matrigel for 15 days, stained with 4 μM/L alcian-acetyoxymethyl ester (Invitrogen), and visualized by confocal microscopy (Leica TCS SP2 confocal microscope) using a 20× objective. Optical sections were scanned at 5 μm intervals moving up from the underside of the membrane into the Matrigel. The fluorescence from each optical section was quantified with LCS Lite software (Leica Microsystems).

**Quantitative real-time PCR**

Cells invading into the Matrigel were isolated, homogenized in 800 μL PBS and centrifuged at 600 × g for 5 minutes at 4°C. The cellular pellet was then subjected to TRIzol Reagent (Invitrogen) extraction and purification using the RNaseasy Kit (Qiagen). Cells in the lower membrane compartment and from 2D cultures were directly subjected to TRIzol extraction and RNA purification. cDNA synthesis was carried out using MuLV reverse transcriptase (Applied Biosystems) according to the manufacturer’s protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and vimentin expression levels were measured by using TaqMan Gene Expression Assays (Applied Biosystems), containing specific primers and a TaqMan MGB probe for each gene, on a 7,500 quantitative Real-time PCR Machine and SDS software (Applied Biosystems). Data are represented as the fold mean change (2ΔΔCt) in gene expression relative to the control of at least 3 different experiments. The relative amount of vimentin gene transcripts was normalized to GAPDH.

**Western blot analysis**

HEC-1A and RL95-2 cells were treated with EGF (60 ng/mL) or TGF-β1 (2.5 ng/mL) for 2 hours in the presence or absence of the SB-431542 inhibitor (20 μM/L). Western blot was conducted as described (13), with the following primary antibodies: anti-phosphorylated Smad2 (1:250, Cell Signaling Technology Inc.), anti-phosphorylated ERK1/2 (1:250, Sigma), anti-Smad2/3 protein.
(1:500, Cell Signaling Technology Inc.), anti-ERK1/2 (1:500, Santa Cruz Biotechnology Inc.) or anti-β-actin (1:500, Sigma). Membrane-bound proteins were visualized with horseradish peroxidase (HRP)–conjugated secondary antibodies (1:1000, Sigma) using the Immobilon Western Chemiluminescent HRP Substrate.

**Wound healing assay**

A monolayer of HEC-1A cells at 90% confluence wounded with a pipette tip were allowed to close the wound for 72 hours in serum-free medium with TGF-β1 (2.5 ng/mL) or EGF (60 ng/mL), in the presence or absence of the SB-431542 inhibitor (20 μmol/L).

**Statistical analysis**

Statistical analyses were conducted using SPSS v15.0. Two-sided nonparametric tests were used to determine the differences between experimental conditions. A P < 0.05 indicated statistical significance.

**Results**

**Molecular profiling of high risk of recurrence endometrial carcinomas**

To identify genes involved in the acquisition of a high-risk phenotype, we carried out a gene expression analysis on a total of 51 human endometrial carcinoma using Agilent technology (13). Stages IA and IB endometrioid carcinomas were grouped as low risk of recurrence carcinomas (n = 19), and compared with the group with a high risk of recurrence including stages IC, II, III, or IV endometrioid, as well as serous-papillar and clear cell carcinomas (n = 32). According to the new FIGO classification (17), stage IA carcinomas comprised the low-risk group, whereas stages IB, II, III, and IV carcinomas comprised the high-risk group (see Supplementary Table S1 for breakdown of histological subtypes). We integrated into this group both histology type I and type II endometrial carcinoma as no association has been found between histology and outcome in women with advanced/recurrent disease (18).

The comparison between expression profiles from the low risk and high risk of recurrence groups rendered a list of 77 annotated genes differentially expressed (P < 0.001; Supplementary Table S2). The bioinformatics analysis of gene–gene interactions and molecular relationships among the identified genes pointed to a reduced number of molecular pathways associated with the risk of recurrence in endometrial carcinoma: estradiol and testosterone, hypoxia, insulin, MAPK (mitogen-activated protein kinase), or FOS-mediated pathways (Fig. 1; top). Interestingly, TGF-β1 signaling was found as the main
pathway altered at the core of the molecular network related to the acquisition of a high-risk recurrence phenotype (Fig. 1; magnification at the top). Of note, TGF-β1 itself was not found to be altered in high-risk endometrial carcinomas but supports the global network associated with high-risk relapse. TGF-β1 is known to participate in tumor promotion and, particularly, in epithelial to mesenchymal transition (EMT; ref. 19), allowing cells to move away from their epithelial cell community and to integrate into the surrounding tissue, even at remote locations.

**TGF-β1 promotes EMT in endometrial cancer cells**

To validate our gene expression approach to high-risk endometrial carcinoma and the involvement of the TGF-β1 pathway in the promotion of recurrences, we first evaluated the effect of TGF-β1 exposure on the endometrial cancer cell line HEC-1A. This cell line has been described as TGF-β1 sensitive and its metastatic ability partially depends on the correct activation of this route (20). Moreover, our group showed that when injected in mice either subcutaneously or orthotopically, HEC-1A cells generate highly undifferentiated and aggressive tumors representative of high-risk endometrial carcinoma (12). Cells treated with TGF-β1 (2.5 ng/mL) for 24 hours acquired a mesenchymal phenotype characterized by the loss of cell–cell contacts and the promotion of migratory structures as lamellipodia (Fig. 1; bottom). The addition of the specific TGF-β1 inhibitor SB-431542 [20 μmol/L; (16)] restored the epithelial architecture and the formation of compact colonies characteristic of this cell line, suggesting a specific role for TGF-β1 in the EMT of HEC-1A cell line (Fig. 1; bottom). Likewise, at the molecular level, cells treated with TGF-β1 exhibited an important increase in the mesenchymal marker vimentin, which was completely abrogated when cells were incubated in the presence of SB-431542 (Fig. 1; bottom). We obtained similar results with the RL95-2 endometrial cancer cell line, originated from an adenosquamous carcinoma that also belongs to the category of high-risk endometrial carcinoma (Supplementary Fig. S1A and S1B).

**TGF-β1 promotes invasion in endometrial cancer cells**

We next sought to assess whether TGF-β1 was capable of inducing tumor invasion in a 3-dimensional (3D) culture assay. For this, we adapted the inverted version of the classical Boyden chamber invasion assay to evaluate the invasive capacity of endometrial tumor cells and the inhibition of this process by an experimental therapy targeting TGF-β1. Although physiological conditions imply a more complex cellular context, with this in vitro assay we aimed to mimic tumor invasion as a key mechanism in the process of dissemination and metastasis. The acquisition of invasive capacities differentiates a primary lesion restricted to the organ of origin from an aggressive systemic disease.

As shown in Fig. 2, HEC-1A cells were able to migrate through the membrane, mimicking basement membrane invasion, and to invade into the Matrigel as an extracellular matrix, when TGF-β1 was applied on top of the Matrigel as a chemoattractant. Sequential images obtained every 15 μm from the membrane indicated that HEC-1A cells were able to invade in response to TGF-β1, whereas no basal migration/invasion was observed under control conditions (Fig. 2A). Likewise, TGF-β1 chemotraction was precluded when SB-431542 inhibitor was added to the medium (Fig. 2A). Similar results were obtained with the RL95-2 cell line (Supplementary Fig. S1C). The extent of HEC-1A invasion promoted by TGF-β1 attraction was quantified by measuring the fluorescence intensity at each confocal section every 25 μm from the membrane (Fig. 2B).

We finally wanted to confirm whether in these 3D experimental conditions TGF-β1 also promoted the EMT observed in 2D culture conditions. The expression levels of vimentin analyzed in the cells located in the lower compartment of the invasion chamber increased when TGF-β1 was applied at the top of Matrigel, and were restored to control untreated levels when the SB-431542 inhibitor was added (Fig. 2D). All these data indicate that TGF-β1 promotes EMT in the HEC-1A and RL95-2 cell lines, resulting in the acquisition of cell migration/invasion capabilities in a 3D culture assay. Furthermore, from a therapeutic point of view, the blockade of TGF-β1 by the SB-431542 inhibitor showed efficacy against endometrial tumor dissemination.

**Inhibition of TGF-β1 impairs EGF-induced endometrial tumor cell invasion**

To further evaluate TGF-β1 as a suitable therapeutic target against endometrial carcinoma invasion and thus against the acquisition of a high risk of recurrence phenotype, we analyzed the impact of TGF-β1 inhibition under conditions of chemoattraction where TGF-β1 was not directly applied to the 3D inverted invasion assay. For this, we selected EGF as a potent chemoattractant among a number of chemokines (21). It has to be noted that, in the 3D culture conditions, small amounts of TGF-β1 are present both in the Matrigel and in the serum, but this level was not able to promote either evident HEC-1A migration or invasion (Fig. 1; data not shown). Only when EGF was added to the serum at the top of Matrigel as the chemoattractant were HEC-1A cells able to migrate through the basement membrane and to invade into the matrix (Fig. 3A and B).
Notably, when SB-431542 was incorporated to the culture conditions, EGF-promoted HEC-1A invasion was completely abrogated (Fig. 3A and B). Smad proteins are the most important mediators of TGF-β1 signaling, and SB-431542 blocks this signaling pathway by inhibiting the TβR1 kinase activity required for the Smad2/3 phosphorylation (16). Western blot showed that SB-431542 effectively repressed TGF-β1 pathway activation, as analyzed by Smad2 phosphorylation, without interfering with the EGF signaling pathway, as evidenced by ERK1/2 phosphorylation (Fig. 3C). As mentioned above, similar results were obtained with the RL95-2 endometrial cancer cell line (Supplementary Fig. S1C and S1D).

Moreover, EGF induced little to no invasion in the absence of serum (data not shown), all indicating that the presence of TGF-β1 in the serum was not sufficient but necessary for the promotion of HEC-1A cell invasion under EGF chemoattraction conditions.

**Inhibition of TGF-β1 impairs the initial steps of endometrial tumor cell invasion**

To confirm this last point, we first analyzed the ability of TGF-β1 and EGF to stimulate HEC-1A migration in a wound healing assay. TGF-β1 stimulated the chemotactic migration of HEC-1A, accelerating wound closure compared with control serum-deprived conditions (Fig. 4A).
In addition, targeting TGF-\(\beta\)1 activity with SB-431542 almost completely blocked both the basal and the TGF-\(\beta\)1–stimulated migration ability of HEC-1A cells (Fig. 4A). Interestingly, treatment with EGF did not significantly enhance the ability of HEC-1A cells to migrate with respect to the control, which was further precluded when we blocked TGF-\(\beta\)1 signaling (Fig. 4A).

In addition, when we analyzed in detail the model of HEC-1A cell invasion under TGF-\(\beta\)1 and EGF chemoattraction conditions, we observed that cells were able to migrate through the porous membrane and minimally invade the Matrigel when TGF-\(\beta\)1 was located at the top of Matrigel (Fig. 4B). By contrast, EGF attraction promoted the formation of spheroids of HEC-1A cells deeply invading the matrix in a cell-collective rather than individual migration/invasion manner (Fig. 4B). This evidence together with the wound-healing assay suggested that TGF-\(\beta\)1 could play a key role in the early steps of endometrial carcinoma invasion, associated with EMT and the acquisition of a migratory/invasive phenotype. This represents a limiting step as deep invasion promoted by EGF only occurs upon the acquisition of this phenotype.

To further confirm this hypothesis, we conducted a time course analysis of 3D HEC-1A cell invasion under EGF chemoattraction conditions, adding the TGF-\(\beta\)1 inhibitor to the medium 6 days later, thus allowing the small amounts of TGF-\(\beta\)1 present in the serum...
the time necessary for the induction of EMT. Cells were then allowed to invade for 10 more days and the extent of invasion was analyzed by confocal microscopy (see scheme in Fig. 4C). A, images of HEC-1A cells incubated with EGF (60 ng/mL) or TGF-β1 (2.5 ng/mL) in the absence or presence of SB-431542 (20 μmol/L) added to the medium at day 6 once TGF-β1–dependent EMT had occurred; and EGF (60 ng/mL) plus SB-431542 (20 μmol/L) at day 0 before TGF-β1–dependent EMT. D, quantification of HEC-1A cell colonies formed under the different chemotraction conditions in the time course invasion assay. Bars, SEM.

Discussion

The invasion of endometrial cancer cells through the myometrium and their migration to the nearby lymph nodes are key factors related to a poor prognosis (3). Despite the characterization of molecular events associated with the development of type I and type II endometrial carcinoma such as alterations in PTEN, β-catenin, KRAS, p53, or HER2/neu, the molecular pathology of myometrial infiltration that defines the initial steps of invasion in endometrial carcinoma is almost unknown (12, 22, 23). Thus, an important clinical impact would be associated with discovering genes or canonical pathways associated with advanced endometrial carcinomas, unmasking the principal common mechanisms of tumor aggressiveness, providing novel markers and developing rational therapies.

We first characterized the molecular phenotype of high risk of recurrence endometrial carcinomas. Focusing
on the core of the network that supports the molecular determinants of endometrial carcinoma recurrences (Fig. 1), we found molecules already associated with an aggressive phenotype like HIF1A (24) or NR3C1 (25). Hormones have also been associated with high risk of recurrence, especially related to postmenopausal women with prolonged life expectancy, changes in reproductive behavior, and the prevalence of overweight and obesity (26). Interestingly, the majority of the genes at the core of the molecular network related to high risk of recurrence in endometrial carcinoma refer to the process of trophoblast invasion. Genes such as FOS, MMP9, MAPK1, RHOA, and TGF-β1 have been described as main directors of the process of embryo implantation (27, 28). In fact, the molecular events characterizing trophoblast implantation as a physiological process of invasion into the uterus, strictly controlled both at the time and space levels, have been paralleled to the process of endometrial tumor invasion in an uncontrolled manner (29).

In particular, TGF-β1 was located in a central position at the molecular network, suggesting a critical role for this cytokine in the high invasive ability of endometrial cancer cells. TGF-β1 signals are largely known as tumor-promoters of cellular responses such as proliferation, survival, migration and invasion (30). TGF-β1 overexpression has been reported to be associated with metastatic phenotypes and poorer patient outcome (31). Regarding the uterus, the TGF-β1 pathway has been associated with decidualization, whereas its disruption in both endometrial hyperplasias and carcinomas resulted in the loss of growth inhibition, acting as a key factor in the early steps of endometrial carcinogenesis (32). On the other hand, TGF-β1 receptor type II and other components of the signaling pathway have been associated with myometrial infiltration, local spread and distant metastasis in endometrial carcinomas (33). Our group also linked the TGF-β1 pathway with the increased invasive ability promoted by ETV5 transcription factor during the initial steps of endometrial carcinoma dissemination (22). In this new study, we showed that TGF-β1 induces changes in the endometrial cancer cell lines HEC-1A and RL95-2 compatible with EMT both at the morphological and molecular levels. In addition, an increase in HEC-1A and RL95-2 invasion capacity was found when TGF-β1 was used as the chemoattractant, but was completely abolished in the presence of its specific inhibitor SB-431542, indicating that TGF-β1 plays a role in endometrial tumor cell invasiveness. The 3D cell invasion assay stimulated by EGF as the chemoattractant was used to further evaluate the real impact and dependence on TGF-β1 signaling in the acquisition of an aggressive tumor phenotype. Our results show that TGF-β1 is a limiting step in deep tumor invasion, as EMT promoted...
by TGF-β1 was not sufficient but was indispensable when EGF was used as the chemoattractant.

Moreover, EGF attraction promoted the generation of HEC-1A cell spheroids deeply invading the matrix, whereas under TGF-β1 stimulation conditions, cells spread at lower fields in the Matrigel rather than deeply invading the matrix. It has been described that the spheroid morphology of endometrial epithelial cells can develop single-lumen polarized glandular structures resembling those observed in endometrial tissue (34). These spheres seem to form a cooperating unit with a highly persistent and directional migration type into the 3D matrix. In vivo, clustered migration would minimize cell loss, favor high local MMP expression, and protect cancer cells from immunological assault (35). We hypothesize a stepwise process where TGF-β1 stimulates EMT in the endometrial tumor cells, enabling them to migrate through the porous membrane before the development of the EGF-dependent invasive glandular structures (Fig. 5). This cooperation has been recently reported, as TGF-β1 stimulation of EMT enabled an EGF-driven breast cancer model to abandon its inherent branching architecture and form large, undifferentiated masses that were hyperinvasive in response to EGF (36). Interestingly, when we applied both EGF and TGF-β1 as chemoattractants, we observed the same result as when we applied TGF-β1 alone, with no spheroid formation (data not shown). This suggests that once TGF-β1 has initiated tumor infiltration through the promotion of EMT, its contribution must be counteracted for further persistent invasion. This could account for the extensively discussed evasive character of EMT in carcinomas (37).

All these data suggest a role for TGF-β1 in early endometrial carcinoma invasion, associated with the EMT process and the acquisition of a migratory/invasive phenotype during myometrial infiltration. Translated to the appearance of secondary metastatic lesions, we hypothesized that TGF-β1 should promote the transit of epithelial-circulating tumor cells into an invasive phenotype during implantation into the metastatic niche, previously to the post-EMT–persistent invasion and micrometastasis formation. Thus, a therapeutic approach targeting TGF-β1–promoted migration and invasion of both primary and secondary tumors represents an attractive strategy for the prevention and treatment of local and distant recurrences in endometrial carcinoma. Interestingly, because TGF-β1 signals seem to play a critical function for EGF-promoted invasion, the combination of both TGF-β1 and EGFR inhibitors could be promising as a therapeutic strategy. To this regard, Erlotinib has shown an overall objective response rate of 12.5% in recurrent or metastatic endometrial cancer (38). In addition, the integration of oncology parameters associated with an aggressive phenotype (i.e., the TGF-β1 pathway) together with the histopathology parameters actually used in the clinic would help to better stratify increased risk of recurrence.

In conclusion, it is imperative to decipher the molecular events associated with the advanced stages of endometrial carcinoma, a disease that has seen a dramatic increase in cancer mortality in the recent decades. In the present study, (i) we characterized the molecular changes associated with the acquisition of an aggressive phenotype in human endometrial carcinoma; (ii) we identified and characterized TGF-β1 as a main director of endometrial cancer cell invasion; and (iii) we showed that our approach represents a realistic strategy for the rational identification and characterization of new potential therapeutic targets, in an effort to improve the clinical management and the outcome of high-risk endometrial cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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