

Stromal signatures in endometrioid endometrial carcinomas

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The pattern of myometrial invasion in endometrioid endometrial carcinomas varies considerably; ie, from widely scattered glands and cell nests, often associated with a fibromyxoid stromal reaction (desmoplasia) and/or a lymphocytic infiltrate, to invasive glands with little or no stromal response. Recently, two distinct stromal signatures derived from a macrophage response (colony-stimulating factor 1, CSF1) and a fibroblastic response (desmoid-type fibromatosis, DTF) were identified in breast carcinomas and correlated with clinicopathologic features including outcome. In this study, we explored whether these stromal signatures also apply to endometrioid carcinomas and how their expression patterns correlated with morphologic changes. We studied the stromal signatures both by immunohistochemistry and *in situ* hybridization in 98 primary endometrioid carcinomas with (87 cases) and without (11 cases) myometrial invasion as well as in the corresponding regional lymph nodes metastases of 9 myoinvasive tumors. Desmoplasia correlated positively with the DTF expression signature. Likewise, mononuclear infiltrates were found in the stroma of tumors expressing CSF1. Twenty-four out of eighty-seven (27%) myoinvasive endometrioid carcinomas were positive for the macrophage signature and thirteen out of eighty-seven (15%) expressed the fibroblast signature. Eleven additional cases were positive for both DTF and CSF1 signatures (11/87; 13%). However, over half of the cases (39/87; 45%) and the majority of the non-myoinvasive tumors (8/11; 73%) failed to express any of the two stromal signatures. The macrophage response (CSF1) was associated with higher tumor grade, lymphovascular invasion, and *PIK3CA* mutations ($P < 0.05$). There was a concordance in the expression of the CSF1 signature in the primary tumors and their corresponding lymph node metastases. This study is the first characterization of stromal signatures in endometrioid carcinomas. Our findings shed new light on the relationship between genetically different endometrioid carcinomas and various stromal responses. Preservation of the CSF1 macrophage stromal response in the metastases leads support to targeting the CSF1 pathway in endometrioid endometrial carcinomas.

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There is increasing evidence that changes in the surroundings of cancer cells can promote their proliferation.^{1–7} Invasive cancer cells actively recruit stromal cells and interact with them to create a

tumor microenvironment that stimulates tumor growth and probably metastasis. This pathologic scenario has not been investigated in endometrioid endometrial carcinoma.

The pattern of myometrial invasion in endometrioid endometrial carcinomas may vary from widely scattered glands and cell nests throughout the myometrium, often associated with stromal retraction that simulates vascular invasion, to more crowded glands with varying degrees of differentiation and little stromal reaction.⁸ In fact, the term ‘adenoma malignum pattern’ has been used to describe

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invasive glands that are well differentiated and surrounded by normal appearing myometrium. More often, however, myoinvasion is accompanied by a fibromyxoid stromal reaction, a lymphocytic infiltrate, or both.

Recently, two distinct stromal signatures derived from a macrophage response to colony-stimulating factor 1 (CSF1) and a fibroblastic response (desmoid-type fibromatosis, DTF) were identified in breast carcinoma and correlated with clinicopathological features including the outcome.^{9–11} The findings suggest that these stromal signatures may have a role in the induction and progression of carcinomas.

In addition, the phosphatidylinositol 3 kinase/AKT pathway is frequently activated in endometrioid endometrial carcinomas and a high percentage of *PIK3CA* mutations have recently been reported.^{12–14} Activated AKT regulates the expression of various downstream target genes including nuclear factor- κ B (NF- κ B). Active NF- κ B, which stimulates inflammation, is known to be involved in the suppression of apoptosis in endometrioid carcinoma.¹⁵ In fact, it has been proposed that activation of the NF- κ B/AKT pathway might link inflammation to tumor promotion and progression.^{16,17}

In this study, we individualized the fibroblastic and macrophage patterns of stromal response in a series of endometrioid endometrial carcinomas and attempted to relate the stromal signatures with the clinicopathological features of the tumors and their molecular genetic alterations (*PIK3CA* mutations). We also investigated whether or not the DTF fibroblastic and CSF1 macrophage response signatures, as assessed at the protein and RNA levels in primary endometrioid endometrial carcinomas, were maintained in their corresponding lymph node metastases.

Materials and methods

Tissue Samples

We analyzed 98 endometrioid carcinomas obtained from the Tumor Tissue Bank and the Surgical Pathology files of the Hospital de la Santa Creu i Sant Pau, Barcelona, Spain, and from Università Cattolica del Sacro Cuore, Rome, Italy. Material was obtained at primary diagnosis in all cases. Of the 98 carcinomas, 9 had developed pelvic and/or para-aortic lymph node metastases. Tumors were examined and classified using the histopathological criteria recommended by the 2003 World Health Organization Classification and the staging classification proposed in 2009 by the International Federation of Gynecology and Obstetrics (FIGO). Tumors were classified into four groups: stage I, tumor confined to uterine corpus; stage II, extension to uterine cervix; stage III, involvement of pelvic

organs; and stage IV, spread beyond the pelvis. Cases were anonymized and the study was approved by the Institutional Ethics Committee.

Tissue Microarray Design

We constructed four tissue microarrays (TAs118–121) with representative samples of myoinvasive carcinomas. Samples of noninvasive (intra-endometrial) carcinomas were also obtained. Tumor areas were selected on H&E sections and marked on the corresponding paraffin blocks. Samples for the TMA varied depending upon tumor invasiveness. In intraendometrial (non-myoinvasive) carcinomas, samples were taken from the endometrial compartment, whereas in myoinvasive tumors, the tissue cores were obtained from the interface of invasive epithelium and stroma. One representative 1-mm tissue core was obtained from each selected zone. Tissue cores were precisely arrayed in a paraffin block using a tissue microarray workstation (Beecher Instruments, Sun Prairie, WI, USA). An H&E-stained section was obtained to confirm the presence of the original areas selected from each tumor.

Immunohistochemistry and *In-Situ* Hybridization (ISH)

Slides were cut at 4 μ m from TAs118–121. Deparaffination and antigen retrieval were done in a PT Link module (DAKO, Glostrup, Denmark) and immunohistochemistry was done in a Dako Autostainer (DAKO). The primary antibodies used were FCGR3a (CD16; mouse monoclonal 2H7, 1:40, AbD Serotec, Kidlington, UK), CD163 (mouse monoclonal 10D6, 1:100, Novocastra, Leica-Microsystems, Wetzlar, Germany), FCGR2a (CD32; mouse monoclonal EP888Y, 1:200, Abcam, Cambridge, UK), SPARC (mouse monoclonal ON-1, 1:1000, Invitrogen, Carlsbad, CA, USA), and MMP11 (mouse monoclonal SL3-05, 1:50, Calbiochem, Merck Millipore, Darmstadt, Germany). The antigen retrieval solution for CD16, CD32, and SPARC was citrate (pH 6) and for CD163 and MMP11 was EDTA (pH 8). The immunohistochemical reactions were visualized using EnVision+ system (DAKO, Carpinteria, CA, USA) using diaminobenzidine. For CD163, an additional incubation was done with EnVision Linker mouse (DAKO). The immunohistochemical reactions were visualized using mouse versions of the EnVision+ system (DAKO) using diaminobenzidine. Although commercial CSF1 and CSF1 antibodies are available, we found the quality of immunoreactions to be suboptimal and performed CSF1 and CSF1R ISH on TMA sections according to a previously published protocol.¹⁸ The scoring criteria are shown in Table 1. Scores 1 and 2 were considered positive. A case was considered to have the CSF1 macrophage response signature if it showed coordinated expression (score 1 or 2) of

Table 1 Scoring criteria

	<i>FCGR3a/CD163/FCGR2a</i>	<i>SPARC/MMP11</i>	<i>CSF1/CSF1R asISH</i>
Score 0	≤20 cell/1 mm	<5% Of the stromal compartment is positive	No paranuclear dot-like stain or >1 paranuclear dot-like stain per cell in less than 5% of the cells
Score 1	>20 to ≤50 cell/1 mm	5–30% Of the stromal compartment is positive	1–4 Paranuclear dot-like stains per cell in at least 5% of the cells
Score 2	>50 cell/1 mm	>30% Of the stromal compartment is positive	>5 Paranuclear dot-like stains per cell in at least 5% of the cells

CSF1 and the three CSF1 response proteins (CD163, FCGR3a, and FCGR2a). A case was considered to have the DTF fibroblastic signature if it showed coordinated expression (score 1 or 2) of SPARC and MMP11.

Definition of Histologic Features (Desmoplasia and Inflammatory Infiltrate) in the Tumor Microenvironment

We evaluated the H&E-stained sections for the presence of desmoplasia and inflammatory infiltrate in both the cases studied by immunohistochemistry and ISH on TAs118-121. The evaluation of desmoplasia was based on three criteria: stromal edema, immature (gray) or mature (pink) collagen, and stromal cellularity. The presence of two of these three criteria counted as desmoplasia. A case was considered to show desmoplasia if these features were present in more than one-third of the tissue core. An inflammatory stroma was defined as the presence of a cluster of mononuclear cells infiltrating more than one-third of the tissue core.

PIK3CA Mutational Analysis

PIK3CA were assessed on tumor DNA by PCR amplification and subsequent sequencing analysis. Mutational analysis was performed using previously reported PCR conditions and primers for exons 9 and 20 of *PIK3CA*.¹⁹ The thermal cycling conditions included an initial 12 min at 94 °C, followed by 40 cycles of 45 s at 94 °C, 45 s at specific annealing primer temperature of 52–62 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C. The PCR conditions for exon 9 of *PIK3CA* were optimized to avoid mispriming with the *PIK3CA* pseudogene spanning exons 9–13 on chromosome 22.²⁰ The PCR products were purified using the exoSAP-IT (USB, Cleveland, OH, USA) and subjected to direct sequencing using ABI PRISM big Dye terminator v1.1 cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing fragments were detected by capillary electrophoresis using an automated ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Table 2 Clinicopathologic features of 98 endometrioid carcinomas

<i>Total number of patients</i>	98
<i>Age (years; n = 98)</i>	
Average	65.2 (37–92)
<i>Tumor size (cm; n = 98)</i>	
Average	4.4 (1–14)
<i>Histologic grade (n = 98)</i>	
1	30 (31%)
2	40 (41%)
3	28 (28%)
<i>FIGO stage (n = 98)</i>	
IA	32 (33%)
IB	33 (34%)
II	7 (7%)
IIIA	8 (8%)
IIIC	13 (13%)
IVA	5 (5%)
<i>Myometrial invasion (n = 87)</i>	
<50%	26 (30%)
>50%	61 (70%)
<i>Vascular invasion (n = 80)</i>	
Yes	28 (35%)
No	52 (65%)
<i>PIK3CA mutation (n = 98)</i>	
Exon 20	13 (13%)
Exon 9	16 (16%)

Statistical Analysis

Statistical analysis was performed using the statistical package SPSS/win 18.0 (SPSS, Chicago, IL, USA). Pearson's χ^2 test was used to compute *P*-values for association testing. A value of *P* ≤ 0.05 was considered statistically significant.

Results

Clinical and Pathologic Findings

The clinicopathological features of the 98 endometrioid carcinomas are shown in Table 2. Briefly, 30 tumors were FIGO grade 1 (31%), 40 grade 2 (41%), and 28 grade 3 (28%). Eleven carcinomas were

confined to the endometrium (stage IA) and the other 87 had myometrial invasion. A total of 32 (33%) tumors were classified as FIGO stage IA, 33 (34%) stage IB, 7 (7%) stage II, 8 (8%) stage IIIA, 13 (13%) stage IIIC, and 5 (5%) stage IVA.

The CSF1 and DTF Stromal Signatures are Present in a Subset of Myoinvasive Endometrioid Carcinomas

We first investigated the CSF1 macrophage signature analyzing the expression of *CSF1* and three CSF1 response markers (CD163, FCGR3a, and FCGR2a) by ISH and immunohistochemistry on four endometrioid endometrial carcinoma TMAs. The markers were scored based on their expression in the cells of the stromal compartment, which encompassed mononuclear inflammatory cells, including macrophages. By ISH, *CSF1* was expressed in tumor epithelium in 62 cases (62/87; 71%; Figure 1a). Tumor expression of *CSF1* by ISH was associated with stromal expression of CSF1 response proteins: 35 of 62 (56%) cases with tumor *CSF1* expression showed concordant stromal expression of the three CSF1 response proteins, whereas immunoreaction for the latter was found in only 1 of 25 (4%) cases without tumor *CSF1* expression ($P=0.0001$). These data show that the epithelial expression of *CSF1* significantly correlated with the stromal expression of CSF1 response proteins and further suggest that CSF1 may have an important role in promoting the CSF1 macrophage response signature. As shown in Figure 1b, ISH revealed that *CSF1R* was expressed both in the tumor cells and stromal cells in 64 myoinvasive endometrioid endometrial carcinomas

(64/85; 75%). Most *CSF1R*-positive cases also expressed *CSF1* (58/64; 90%). The DTF fibroblastic stromal signature was studied by the stromal expression of SPARC and MMP11 markers. These markers were exclusively expressed by a subset of spindle stromal cells (Figure 2).

Twenty-four out of eighty-seven (27%) myoinvasive endometrioid endometrial carcinomas exhibited a positive macrophage signature and only thirteen out of eighty-seven (15%) had a fibroblast signature. Another 11 cases showed both DTF and CSF1 signatures (11/87; 13%). However, over half of the cases (39/87; 45%) and most of the non-myoinvasive cases (8/11; 73%) failed to express any stromal signature (Figure 2).

Relationship between Stromal Response Signature and Conventional Histological Findings

We tried to correlate the different stromal signatures (DTF fibroblastic and CSF1 macrophage signatures) with the morphologic changes in the tumor stroma. We found a strong association between the CSF1 macrophage signature and the presence of inflammatory cells ($P<0.001$; Figure 3). Thirty-one out of forty-seven (65%) tumors exhibiting an inflammatory response expressed the CSF1 signature. In contrast, only 4 out of 38 (11%; 4/38) tumors lacking an inflammatory response had the CSF1 signature. The relationship between the DTF stromal signature and the presence of desmoplasia was also significant (Figure 3). Whereas half of the cases that histologically showed a desmoplastic response had a DTF stromal signature (17/35; 48%), only 4 of 38 (13%) of

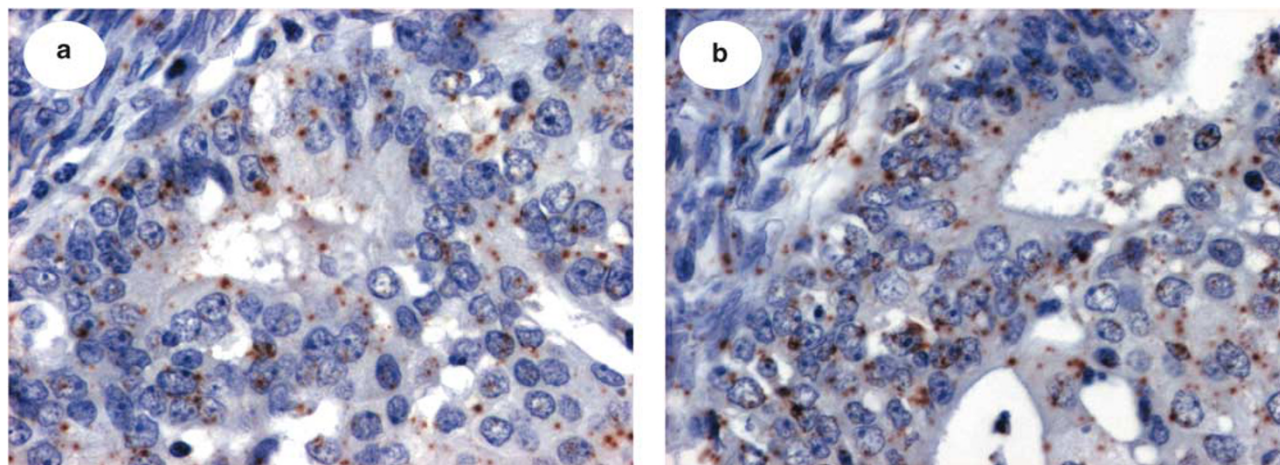
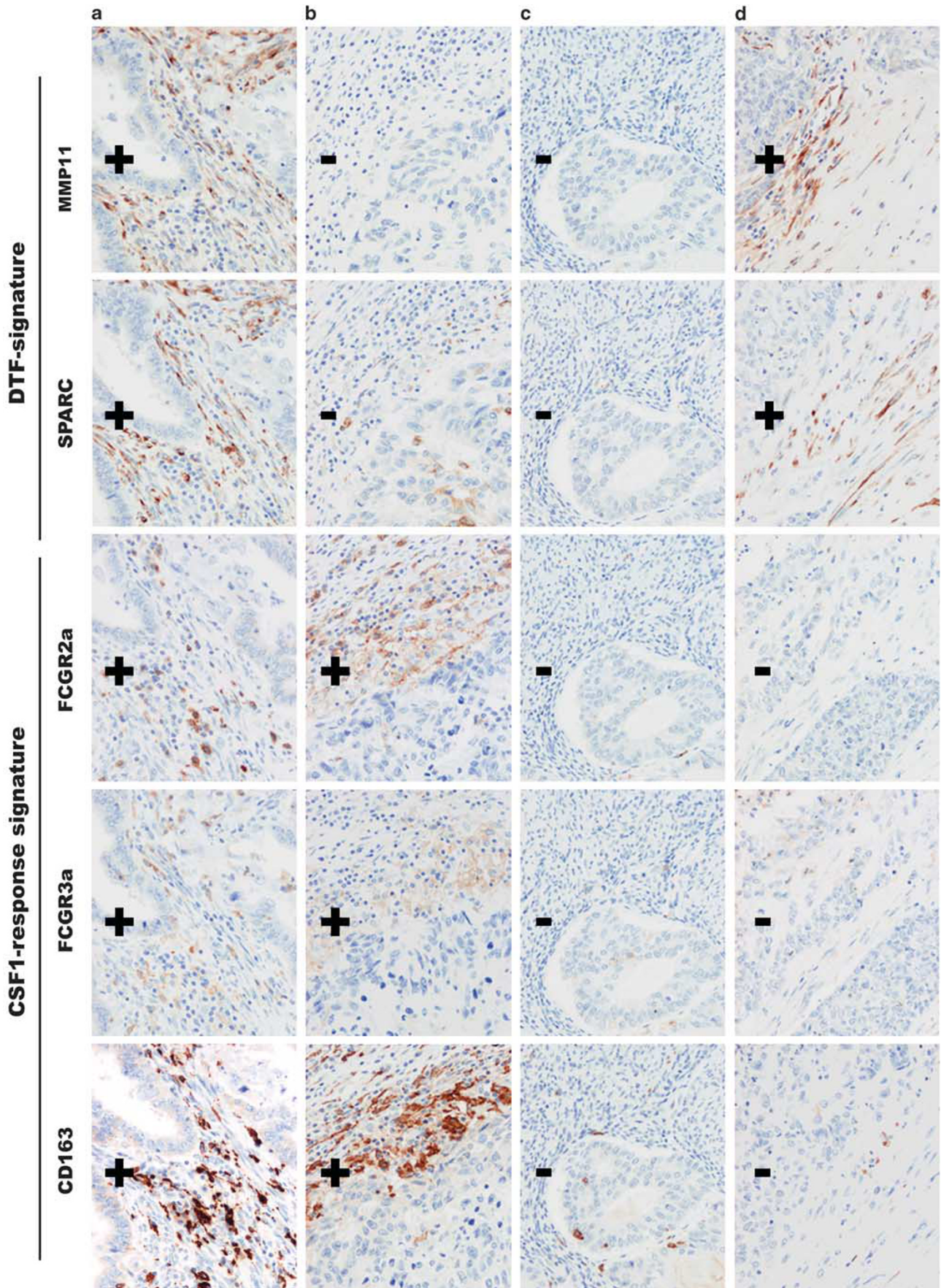


Figure 1 CSF1/CSF1R *in-situ* hybridization in endometrioid carcinomas. (a) The tumor cells show multiple brown dots (CSF1 mRNA). (b) The expression of CSF1R mRNA (brown dots) is seen both in the tumor cells and stroma.

Figure 2 Immunohistochemistry of three macrophage response markers (CD163, FCGR3A, FCGR2B) and two fibroblast markers (SPARC, MMP11) showing a representative case positive for both DTF fibroblastic and CSF1 macrophage signatures (a), a case positive only for CSF1 macrophage signature (b), a case without stromal response signature (c), and a case positive only for DTF fibroblastic signature (d).



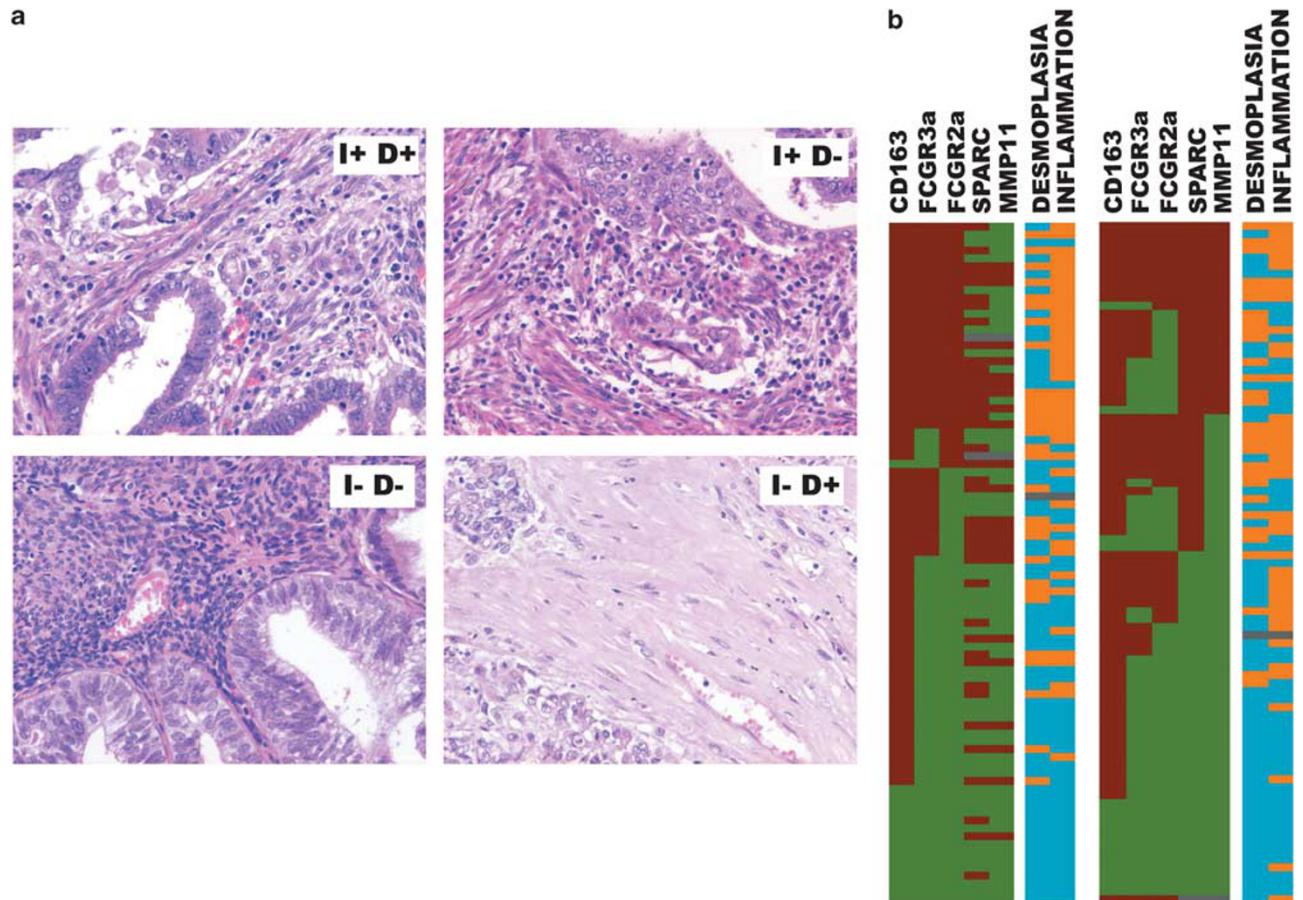


Figure 3 (a) H&E images with different stromal patterns in endometrioid endometrial carcinomas (I = inflammation, D = desmoplasia). (b) The heat-map shows the 98 EEC cases arrayed along the y axis and the immunohistochemical (CD163, FCGR3a, FCGR2a, SPARC, and MMP11) and histologic (desmoplasia/inflammation) findings arrayed on the x axis. Red (for immunohistochemical expression) and orange (for the histologic response) indicate the immunohistochemical expression or the histologic stromal response, whereas green (for immunohistochemistry) and blue (for histologic response) indicate the absence of them. Both panels show the same findings except when the tumors were arranged according to the CSF1 immunohistochemistry (left panel) and the DTF immunohistochemistry (right panel).

tumors lacking desmoplasia expressed the DTF fibroblastic signature ($P < 0.001$; Figure 3). Of the 39 double negative cases for DTF and CSF1 stromal signatures, 24 (62%) failed to show significant inflammation or desmoplasia on H&E examination.

Relation of Stromal Signatures with Clinicopathological Features

We attempted to relate the different response signatures with the clinicopathologic features, including age, tumor size, lympho-vascular invasion, myometrial invasion, tumor grade, tumor stage, and *PIK3CA* mutation status (Table 3). Tumors that expressed the macrophage signature were associated with higher grade, vascular invasion, and *PIK3CA* mutations. In contrast, the CSF1-DTF-group was composed of low-grade adenocarcinomas, lacking lymphovascular invasion, and without *PIK3CA* mutations. There was no significant association between the fibroblastic signature and any of the clinicopathologic features.

Stromal Signatures in Primary Endometrioid Endometrial Carcinomas and their Metastases

We compared the CSF1 and DTF stromal response patterns in the primary endometrioid endometrial carcinomas and their corresponding lymph node metastases. We found that five out of six (83%) primary tumors expressing the CSF1 macrophage signature maintained this feature in their metastases. Similarly, the three primary tumors lacking the CSF1 signature failed to express the CSF1 signature in their corresponding metastases. No correlation was identified for the DTF fibroblastic signature.

Discussion

The stroma can have an active role in the induction or promotion of oncogenesis.^{1,5,7,21–24} For example, tumor cells can actively recruit stromal cells (inflammatory cells, endothelial cells, and fibroblasts) into the tumor microenvironment and this recruitment is essential for tumor survival and growth. In invasive breast carcinomas, previous

Table 3 Correlation of stromal signatures with clinicopathologic features in myoinvasive endometrioid carcinomas

	<i>CSF1</i> + <i>DTF</i> − (%)	<i>CSF1</i> − <i>DTF</i> + (%)	<i>CSF1</i> + <i>DTF</i> + (%)	<i>CSF1</i> − <i>DTF</i> − (%)	P
<i>Myometrial invasion</i>					NS
< 50%	6/24 (25)	5/13 (38)	5/11 (45)	10/39 (26)	
> 50%	18/24 (75)	8/13 (62)	6/11 (55)	29/39 (64)	
<i>Vascular invasion</i>					0.029
Yes	12/21 (57)	4/12 (33)	5/11 (45)	7/36 (19)	
No	9/21 (43)	8/12 (67)	6/11 (55)	29/36 (81)	
<i>Histologic grade</i>					0.001
1	1/24 (4)	5/13 (38)	0/11 (0)	14/39 (36)	
2	11/24 (46)	3/13 (24)	7/11 (67)	18/39 (46)	
3	12/24 (50)	5/13 (38)	4/11 (33)	7/39 (18)	
<i>FIGO stage</i>					0.074
IA	2/24 (8)	6/13 (46)	4/11 (36)	9/39 (23)	
IB	7/24 (29)	4/13 (31)	4/11 (36)	18/39 (46)	
II	2/24 (8)	0/13 (0)	1/11 (9)	4/39 (10)	
IIIA	3/24 (13)	0/13 (0)	1/11 (9)	4/39 (10)	
IIIC	7/24 (29)	2/13 (15)	1/11 (9)	3/39 (8)	
IVA	3/24 (13)	1/13 (8)	0/11 (0)	1/39 (3)	
<i>PIK3CA mutation</i> ^a					0.000
Yes	16/24 (67)	3/13 (23)	4/11 (37)	3/38 (8)	
No	8/24 (33)	10/13 (77)	7/11 (63)	35/38 (92)	

^aExon 9 and exon 20 mutations.

studies have characterized two different stromal signatures that correlated with the clinicopathologic features of the tumor such as hormone status, tumor grade, and clinical outcome.^{9,10,25} In the current study, we have investigated whether these stromal signatures are expressed not only in the primary myoinvasive endometrioid endometrial carcinomas, but also in their metastases. Our results showed that distinct subsets of endometrioid endometrial carcinomas have the fibroblastic (*DTF*) and macrophage (*CSF1*) response signatures, whereas another subset of endometrioid endometrial carcinomas failed to express significantly either of the two stromal signatures. In the positive cases, desmoplasia correlated clearly with the *DTF* expression signature and, likewise, tumors exhibiting a mononuclear infiltrate in the stroma expressed mainly the *CSF1* macrophage response. Also, there was a significant positive correlation between the *CSF1* macrophage signature in the primary tumors and their matched lymph node metastases.

PIK3CA mutations, which occur in 24–39% of endometrioid endometrial carcinomas, dysregulate the phosphatidylinositol 3 kinase/*AKT* signaling pathway.^{12–14} Mutations are predominantly located in the helical (exon 9) and kinase (exon 20) domains, but they can also occur in exons 1–7.²⁶ *PIK3CA* mutations have been associated with adverse prognosis such as high histologic grade and myometrial invasion. Among the *AKT* targets, *NF-κB* is of particular interest. *NF-κB* is an important mediator of inflammation-induced tumor growth and progression, as well as an important modulator

of tumor surveillance and rejection.¹⁷ *NF-κB* activation occurs frequently in endometrioid endometrial carcinomas and explains the development of resistance to apoptosis in these tumors.¹⁵ In our series, *PIK3CA*-mutated endometrioid endometrial carcinomas were associated with the *CSF1* stromal signature, in which *CSF1* is produced by the tumor cells. In fact, activation of *NF-κB* pathway promotes tumor development by inducing the expression of genes that encode cytokines such as *CSF1*.¹⁷ *CSF1* has an important role in attracting inflammatory cells, including macrophages, into the tumor microenvironment (paracrine effect). These inflammatory cells, in turn, produce cytokines that promote tumor growth, angiogenesis, extracellular matrix breakdown, invasion, and metastasis. In addition, *CSF1* may promote tumor growth through interaction with *CSF1R* on the tumor cells (autocrine effect).

Some endometrioid endometrial carcinomas have an unusual pattern of myometrial invasion that shows widespread permeation of the myometrium with little or no host response. The deceptively bland appearance of the glands in many of these cases is analogous to that of the minimal deviation adenocarcinoma (adenoma malignum) of the cervix. Although two studies^{27,28} suggested that this pattern may have an adverse prognostic significance, a recent study, including a larger number of cases, has not confirmed this.²⁹ In our series, a subset of endometrioid endometrial carcinomas failed to demonstrate significant positive expression of either *DTF* or *CSF1* stromal signatures. These

findings indicate that there is a subset of EECs that evoke little or no inflammatory or stromal response. Interestingly, this group of tumors consisted largely of low-grade endometrioid endometrial carcinomas that lacked lymphovascular invasion and *PIK3CA* mutations. Further studies are needed to find out whether cases of 'adenoma malignum' lack CSF1 and DTF signatures.

When considering novel therapeutic approaches, it is important to determinate the stability of the potential target. Recently, a concordance between the CSF1 macrophage response signature in primary mammary and colorectal carcinomas and their corresponding lymph node metastases has been reported.³⁰ In a previous study, we also encountered a positive correlation between CD163-macrophages in primary endometrioid endometrial carcinomas and their matched lymph node metastases.³¹ In the current study, we found that the expression of CSF1 by the tumor cells of primary endometrioid endometrial carcinomas is maintained in most metastatic tumors. These findings suggest that the CSF1 macrophage response signature is determined by the intrinsic biology of the tumor and is independent of the microenvironment the tumor resides in. The presence of a preserved CSF1 macrophage response signature in three different types of metastatic cancer, ie, mammary, colorectal, endometrioid endometrial carcinomas, suggests that target therapy may represent an effective strategy in these cases. In fact, an alternative, emerging therapeutic modality focuses on targeting various microenvironmental processes.^{2,32} As the tumor stromal cells are thought to be 'normal' and genetically less labile than the tumor cells proper, resistance to therapy is less likely. Therefore, the tumor stroma may represent an excellent target for directed therapy.

In summary, the conclusions of our study, which represents the first attempt to characterize stromal signatures in endometrioid endometrial carcinomas, are:

1. Besides tumor cells, tumor microenvironment is worth to be investigated.
2. Desmoplasia correlated positively with the DTF expression signature. Likewise, a mononuclear (macrophage) infiltrate correlated strongly with the CSF1 expression signature.
3. Stromal signatures have significant clinicopathological associations.
4. CSF1 pathway may represent a promising therapeutic target in a subset of endometrioid endometrial carcinomas.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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