Abstract

Well-differentiated endometrioid carcinoma (EC) is a low-grade cancer with relatively indolent behavior. However, even with well-differentiated histology, it sometimes tends to invade extensively and shows metastatic potential, suggesting that this is a group of cancers with heterogeneous behavior. On the other hand, due to its tendency for younger onset, the treatment strategy for EC frequently considers fertility preservation, highlighting the need for a more accurate evaluation of myometrial invasion through biopsy and imaging diagnostics. We previously reported the involvement of the CXCR4–CXCL12 and CXCL14 axes in EC invasion. Accordingly, we here investigated whether CXCR4 expression could reflect invasive potential and explored its interaction with cancer-associated fibroblasts (CAF) that produce chemokines in the tumor microenvironment. Immunohistochemical expression of CXCR4 was assessed in 71 cases of EC (14 of EC confined to the endometrium, 57 of myoinvasive EC), 6 cases of endometrial intraepithelial neoplasia (EIN), and 42 cases of non-carcinomatous conditions. CXCR4 expression was significantly higher in myoinvasive EC than in non-cancerous conditions, EIN, and endometrium-confined EC. By univariate and multivariate analysis, CXCR4 expression significantly reflected myometrial invasion. CXCR4 expression in the biopsied and resected specimens correlated weakly positively. Invasion and wound-healing assays were performed culturing an EC cell line in CAF-conditioned medium. The invasion and wound healing potentials were dependent on CXCR4 and CAF. Our study demonstrated that CXCR4 expression is an independent factor in myometrial invasion and can support diagnostic evaluation prior to treatment in the biopsy sample.

Keywords: Cancer associated fibroblast, CXCR4, endometrioid carcinoma, myometrial invasion, uterine conservative therapy

Introduction

Endometrial cancers (ECs) are categorized into three grades according to the International Federation of Gynecology and Obstetrics (FIGO) Grading system. Grades 1 and 2 are considered low-grade cancers, whereas Grade 3 is a high-grade cancer. Low-grade ECs exhibit an indolent biological behavior; however, once interactions with the surrounding stroma, such as in microcystic, elongated, and fragmented (MELF) pattern invasion, are observed, these tumors exhibit aggressive behavior and have a high metastatic potential (1).

Our previous study reported the involvement of the CXCR4–CXCL12 and CXCR4–CXCL14 axes in the enhanced invasiveness observed in the MELF pattern. CXCR4 is a unique receptor, exclusively interacting with the endogenous ligands CXCL12 and CXCL14 (2,3). When CXCL12 binds to CXCR4, various downstream signaling pathways are initiated, leading to diverse responses, including increased intracellular calcium, gene transcription, chemotaxis, cell survival, and proliferation (4). Cancer-associated fibroblasts (CAFs) release CXCL12 and CXCL14 under various conditions, promoting pro-malignancy activities in cancer cells and in the tumor microenvironment. Through the CXCL12–CXCR4 axis, CAF activity contributes to increased tumor development and metastasis (5-7). Consequently, elevated CXCR4 expression has been identified as a poor prognostic biomarker in several cancers (8-10).

This study directly compared the expression of CXCR4 in myoinvasive ECs, ECs confined to the endometrium, and non-tumorous endometrium. Considering their role in the tumor microenvironment, we also examined whether an interaction between CXCR4 and cancer-associated fibroblasts is present in ECs.

Materials and methods

Study population and slide review

A retrospective search was conducted on 119 cases from 2002 to 2016, based on the patient files of the Department of Pathology, Kurume University School of Medicine. The study was approved by the Institutional Review Board of Kurume University (No. 21202). Informed consent was obtained for all patients enrolled in the study.

Seventy-one patients were diagnosed with FIGO grade 1 or 2 low grade EC (14 cases of EC confined to the endometrium and 57 of myoinvasive EC), 6 cases of endometrial intraepithelial neoplasia (EIN), and 42 with non-cancerous conditions, including 14 cases of endometrial hyperplasia without atypia, 7 cases of atrophic endometrium, 6 cases of adenomyosis, and 15 cases of proliferative-phase endometrium (PE). The morphological classification was independently confirmed by two pathologists (S.S. and H.Y.).

Antibodies, cell lines, and cell culture

The following antibodies and an antagonists were purchased: rabbit monoclonal CXCR4 (clone UMB2; Abcam, Cambridge, UK), and CXCR4 antagonist AMD3465 hexahydrobromide (ab120809, Abcam). The human normal fibroblast cell line SFTY, established from human dermis, was purchased from the JCRB Cell Bank (Osaka, Japan). The human EC cell line KCC-1b and the CAF cell line SMZ were established in our institute. KCC-1b has been established from a primary EC (11). SMZ was established from the peri-cancer stroma of human EC. Using flow cytometry, SMZ was confirmed to express CAF markers, of which 76.85% were CD90-

positive and 99% were fibroblast-activating protein (FAP)-positive (data not shown). KCC-1b and SMZ were cultured in Dulbecco's modified Eagle's medium (DMEM)(Nissui, Tokyo, Japan) with 10% non-heat-inactivated fetal bovine serum, while SF-TY was cultured in DMEM with 20% fetal bovine serum.

Immunohistochemical analysis

Serial sections (4-μm-thick) were obtained from selected paraffin-embedded blocks and mounted onto coated glass slides using a Dako Real kit/HRP (DAB) (K5007; Dako Cytomation, Glostrup, Denmark). Briefly, the slides were deparaffinized in xylene and dehydrated using a graded series of alcohol solutions. Antigen retrieval was performed in citrate buffer (pH 6.0) for 30 min at 95°C in a pressure cooker. Endogenous peroxidase activity was blocked by incubating the sections in 10% H_2O_2 for 10 min at room temperature. Nonspecific binding sites were blocked using a protein block (X0909; Dako Cytomation) for 30 min. Sections were then incubated overnight with rabbit monoclonal anti-CXCR4 antibody (1:750) in a humidified chamber. The slides were washed in Tris-buffered saline with Tween 20 (TBST) and were incubated with horseradish peroxidase rabbit/mouse for 60 min at room temperature. The slides were again washed in TBST. DAB was applied and removed by rinsing with distilled water. The slides were counterstained with hematoxylin. Scoring was based on the amount and intensity of the membranous and cytoplasmic CXCR4 staining: negative (<1% of the positive cells), sporadic (some isolated positive cells, but <5%), focal (small cell clusters, but <25% of the positive cells), or diffuse (25% of stained cells). The intensity was graded as light, moderate, or strong. Diffuse and strong staining was designated as "++," sporadic and focal staining of any intensity as "+," and negative as

58 " –." Stained tissue sections were scored and examined without knowledge of clinicopathological data or
59 patient outcome (H-score).
60
61 ***In vitro* invasion assay**
62 Two million cells, plated in the top chamber of a Transwell device with a Matrigel-coated membrane (24-well
63 insert; pore size, 8 mm; BD Biosciences, Franklin Lakes, NJ, USA) were cultured in a medium without serum
64 or growth factors, and a medium supplemented with serum and chemoattractant, such as CAF-conditioned
65 medium (SMZ-CM) and normal fibroblast cell line (SFTY-CM), was added to the lower chamber. The cells
66 were incubated for 24 h, and cells that did not invade the pores were removed using a cotton swab. The cells on
67 the lower surface of the membrane were stained using a Diff-Quick Staining Set (Sysmex Corporation, Kobe,
68 Japan) and counted. Three independent experiments were performed.
69
70 **Wound-healing assay**
71 Scratch wound-healing assays were performed in 24-well tissue culture plates (Corning Inc., Corning, NY,
72 USA). One million cells plated in a chamber were cultured in a medium without serum or growth factors, and a
73 medium supplemented with serum and chemoattractant, such as SMZ-CM and SFTY-CM, were then added into
74 the chamber. Twenty-four hours after the cells were seeded (by which time the cell confluence usually reached
75 90–100%), scratches were made using a 200-μL pipette tip. The wells were then washed twice with medium and
76 cultured for an additional 24 h and 48 h, followed by an assessment of the wound area. Three independent

experiments were performed.

Statistical analysis

Multiple comparison tests using non-repeated measures analysis of variance ($p < 0.01$) and the Student–Newmann–Keuls test were performed for non-cancerous lesions, such as atrophic endometrium, adenomyosis, proliferative phase endometrium, endometrial hyperplasia without atypia, and neoplastic lesions, such as EIN, EC confined to the endometrium, and myoinvasive EC. The cut-off ratio was assessed using receiver operating characteristic (ROC) curve analysis and computed as 95% confidence intervals (CIs) of sensitivities and specificities. The areas under the curve (AUC) and 95%CIs were derived from logistic regression models for prediction of CXCR4 H-score positivity. Sensitivities and specificities were determined for cut-off points defined by Youden's index, which maximized the sum of the sensitivities and specificities.

Univariate analysis and multivariate logistic regression were performed with CXCR4 ≥ 12.5 /< 12.5 as the objective variable for patients with confirmed cancer. For multivariate analysis, variables were selected using the backward method. Spearman's rank correlation coefficient was used to test the correlation of CXCR4 immunostaining expression between the biopsy and resected specimens.

Results

Clinicopathological review

Seventy-one cases classified as low-grade EC were defined as having a highly differentiated morphology

characterized by minimal solid components with complex fusion of tubular and papillary structures, forming a labyrinthine pattern. These patients had a median age of 60 years (range: 25–88 years). EC stage distribution is shown in Table 1: 14 cases had EC confined to the endometrium, 57 cases showed myometrial invasion, of which 14 cases had invasion of less than half of the myometrial thickness, and 43 cases had invasion exceeding half of the myometrial thickness. Among cases of myometrial invasion, 28.2% (20 cases) exhibited vascular invasion.The myometrial invasion patterns were classified based on the surrounding stromal reaction at the invasive front. The most commonly observed stromal reactions were as follows: pushing pattern, characterized by a lack of prominent stromal reaction and clear demarcation from the myometrium; inflammatory pattern, characterized by lymphocytic and plasma cell infiltration; myxoid pattern, which included mucinous and edematous changes in the stroma, with neutrophil infiltration due to destruction of glandular structures; and fibrous pattern, resembling adenoma malignum with single glandular infiltration and notable fibrosis or absence of stromal reaction at the invasive front (Figure 1a). These findings were observed in varying proportions, with the dominant patterns being the pushing pattern, in 11 cases (19%), inflammatory pattern, in 6 cases (10%), myxoid pattern, in 5 cases (8%), and various patterns in 35 cases (59%). Adnexal metastasis was identified in 4.2% (3 cases), with one case lacking myometrial invasion. Lymph node metastasis was observed in 12.7% (9 cases).

Immunohistochemical analysis

CXCR4 expression was observed in the cell membrane and cytoplasm. The distribution of CXCR4 varied in the biopsy and resected specimens, appearing as patchy, diffuse, and sporadic patterns (Figure 1a). CXCR4 showed significantly higher expression in the resected specimens of myoinvasive carcinoma than in specimens related to noncancerous conditions, including adenomyosis, atrophic endometrium, PE, and endometrial hyperplasia without atypia, as well as in cases of EIN and EC confined to the endometrium ($p < 0.01$, $p < 0.05$, $p < 0.01$, respectively) (Figure 1b). No correlation was observed between the myoinvasive morphological patterns of EC (myxoid, inflammatory, fibrous, pushing) and CXCR4 expression. To establish a specific cutoff value for the CXCR4 H-score, we determined the best cutoff for resected specimens using the Youden index, based on an ROC curve, which yielded a cutoff value of 12.5. This cutoff demonstrated a sensitivity of 67.9% (95% CI: 55.4–80.4) and a specificity of 80.3% (95% CI: 70.4–88.7). The AUC was 0.74 (95% CI: 0.65–0.83) (Figure 1c). Clinical and pathological characteristics of the 71 patients with EC divided by the cutoff value are presented in Table 2. Among these patients, 58 (81.7%) showed CXCR4 expression. Although CXCR4 expression showed no correlation with age, FIGO grade, pathological stage, lymphovascular invasion, cervical invasion, adnexal metastasis, or pelvic lymph node metastasis (median age 60 years, range 25–88 years, $p = 0.21$ for age; $p = 0.84$ for FIGO grade; $p = 0.49$ for pathological stage; $p = 0.07$ for lymphovascular invasion; $p = 0.69$ for cervical invasion; $p = 0.55$ for adnexal metastasis; $p = 0.15$ for pelvic lymph node metastasis), univariate and multivariate analysis revealed a significant correlation between CXCR4 expression and myometrial invasion (odds ratio 4.59, 95% CI 1.25–16.8, $p < 0.05$ and $p < 0.02$, respectively).

Furthermore, although weak, a correlation was observed between resected specimens and biopsy specimens (r = 0.252, 95% CI 0.012–0.452, p = 0.038) (Figure 2).

In vitro invasion assay and *in vitro* wound-healing assay

KCC-1b had been confirmed to express CXCR4 in a previous study (1). Cell invasion was enhanced by co-culturing these cells with SMZ-CM. Furthermore, the cell invasion ability was higher in SMZ-CM than in SFTY-CM co-cultures. When the CXCR4 antagonist AMD3465 was added to SMZ-CM, cell invasion ability was reduced (Figure 3a). The wound area measurement revealed marked wound healing ability under SMZ-CM culture conditions than under control and SFTY-CM conditions, which was suppressed by adding AMD3465 (Figure 3b).

Discussion

In routine clinical practice, some surgically resected EC specimens have shown extensive myometrial invasion and lymph node metastasis, despite the initial expectation of these being low-grade cancers with minimal myometrial invasion. This suggests that, morphologically, ECs can be a heterogeneous group with varying invasive capabilities and that the interaction between the tumor microenvironment and EC cannot be overlooked. Therefore, ancillary studies utilizing biomarkers can facilitate an understanding of EC aggressiveness and behavior. Our results revealed that the semi-quantitative expression level of CXCR4, determined by the H-score, was significantly higher in myoinvasive ECs than in non-tumorous endometrium, EIN, and EC confined to the endometrium, which suggests that CXCR4 expression is specific to myoinvasive cancer (Figure 1b). Using a cut-

off ratio of 12.5, univariate and multivariate analyses of clinicopathological factors confirmed that myometrial invasion is an independent factor associated with CXCR4 overexpression. Based on these findings, we speculated that CXCR4 plays an essential role in myometrial invasion.

To support these results further, we conducted in vitro investigations using invasion and wound-healing assays with a co-culture of CAF-conditioned medium (CAF-CM) and normal fibroblast cell-conditioned medium (SFTY-CM). Human EC cell lines co-cultured with CAF-CM exhibited enhanced invasiveness and repair ability as compared to those co-cultured with SFTY-CM. Moreover, adding a CXCR4 antagonist suppressed cell invasion and repair processes (Figure 3a,b). These results suggested that EC cells expressing CXCR4 acquired invasive capabilities through contact with CAFs during the myoinvasive process, and that this ability is dependent on CXCR4.

As a histologically observed stromal reaction at the myoinvasive front is thought to reflect the tumor microenvironment, we examined the differences in CXCR4 expression in terms of the morphological patterns of the stromal reaction (Figure 1a). However, we did not observe any significant differences in CXCR4 expression levels. Several possible reasons for this may exist. Firstly, it could be attributed to the small number of cases in each subgroup after classification based on invasion patterns. Secondly, ligands produced by CAFs, such as CXCL12 or CXCL14, may better reflect the microenvironment than CXCR4. Nonetheless, regardless of the stromal reaction pattern, CXCR4 could be a marker reflecting myometrial invasion. Therefore, observing the correlation between CXCR4 expression in biopsy and resected samples is crucial for using CXCR4 as a diagnostic marker. Although a significant positive correlation was observed (Figure 2), the weak correlation remains as a

study limitation. It is due to be attributed to the fact that biopsy samples were mainly obtained through curettage or aspiration, which can lead to significant sample degradation. While there is room for improvement in quantitative methods to account for variations during sampling, despite the limitation of a weak correlation, CXCR4 immunostaining seems to have the potential ability to assist in pathological diagnoses. For example, particularly in cases where evaluating myometrial invasion becomes challenging due to the presence of uterine fibroids or adenomyosis. Additionally, as EC onset occurs at a younger age, accurately evaluating myometrial invasion in biopsy samples becomes increasingly essential for making appropriate decisions regarding fertility preservation. In such cases, Immunostaining of CXCR4 to evaluate myometrial invasion in biopsy samples is expected to enhance assessment accuracy.

In conclusion, our study demonstrated that CXCR4 expression is an independent factor in the myometrial invasion of EC and can support the determination of myometrial invasion at the biopsy stage.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Chihiro Fukumitsu, Mayuka Akao, Sachiko Ogasawara, Kenta Murotani, Naotake Tsuda, and Sakiko Sanada. The first draft of the manuscript was written by Chihiro Fukumitsu and all authors commented on previous versions of the manuscript. The draft of the manuscript was finalized by Sakiko Sanada. The conception, and design of the research was done by Sakiko Sanada. All authors read and approved the final manuscript.

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222

Figure legends

FIG. 1. Representative Hematoxylin & Eosin staining, CXCR4 immunostaining of resection and biopsy EC samples, and CXCR4 semi-quantitative and cut off ratio analyses.

a) Representative hematoxylin and eosin, and immunohistochemical staining for CXCR4 of the corresponding resection and biopsy specimens showing the morphological patterns of the invasive front. CXCR4 expression was observed in the cell membrane and cytoplasm. The distribution of CXCR4 varied, appearing as patchy, diffuse, and sporadic patterns. Additionally, the figure shows the morphological characteristics of each myoinvasive front of endometrioid carcinoma (ECs). b) Semi-quantitative analysis of CXCR4 expression regarding each endometrial condition. Brute-force, multiple comparison tests were performed in non-cancerous endometrial conditions, such as atrophy, adenomyosis, proliferative phase endometrium, endometrial hyperplasia without atypia, neoplastic disease as endometrioid intraepithelial neoplasia (EIN), EC confined to the endometrium and myoinvasive EC. CXCR4 expression was significantly higher in myoinvasive EC than in non-cancerous conditions, EIN, and EC confined to the endometrium ($p < 0.01$, $p < 0.05$, and $p < 0.01$, respectively). c) To establish a specific cutoff value for the CXCR4 H-score, the best cutoff for resected specimens was identified using the Youden index, based on the ROC curve, which yielded a cutoff value of 12.5. This cutoff demonstrated a sensitivity of 67.9% (55.4–80.4) and a specificity of 80.3% (70.4–88.7). The areas under the curve for the H-score was 0.74 (95% CI 0.65–0.83).

FIG. 2. The correlation of CXCR4 expression between biopsy and resection specimens

Weak but significant positive correlation between biopsy and resection specimens was observed (r = 0.252 [95%CI

243 0.012–0.452], p = 0.038).

244

245 **FIG. 3.** Invasion and wound-healing of endometrial cells under conditioned medium culture conditions

246 a) Invasion assay under culture conditions using normal fibroblast-conditioned medium (SFTY-CM) and cancer-

247 associated fibroblast (CAF)-conditioned medium (SMZ-CM). Compared to SFTY-CM, cell invasion ability

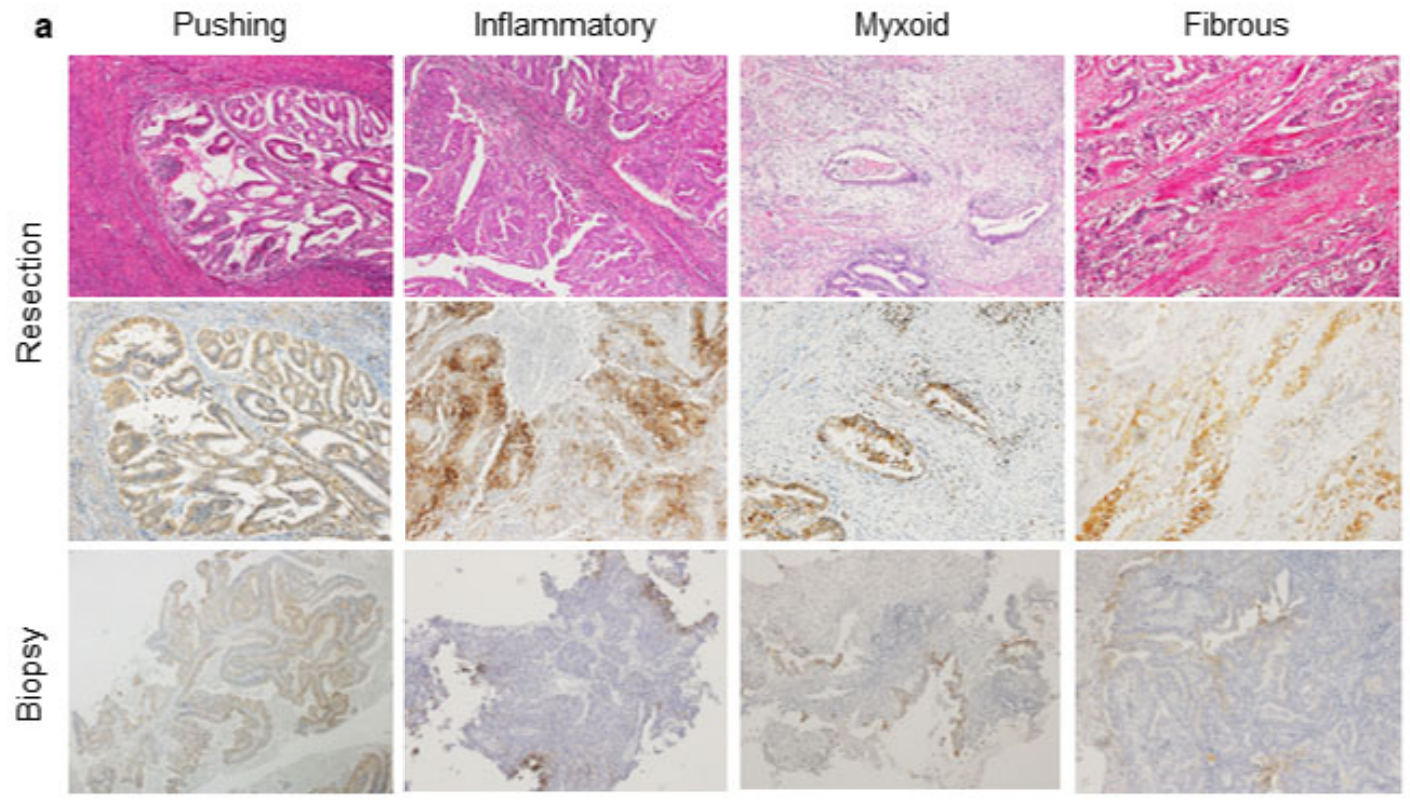
248 increased under the SMZ-CM culture condition. Both conditions resulted in decreased invasion ability when a

249 CXCR4 antagonist (AMD3465) was added. However, the decrease ratio was much greater with SMZ-CM. b) A

250 wound-healing assay was performed under the same conditions as used in the invasion assay. Wound area

251 measurement revealed remarkable wound healing ability under SMZ-CM culture conditions than under control

252 and SFTY-CM conditions. This ability was suppressed by adding AMD3465.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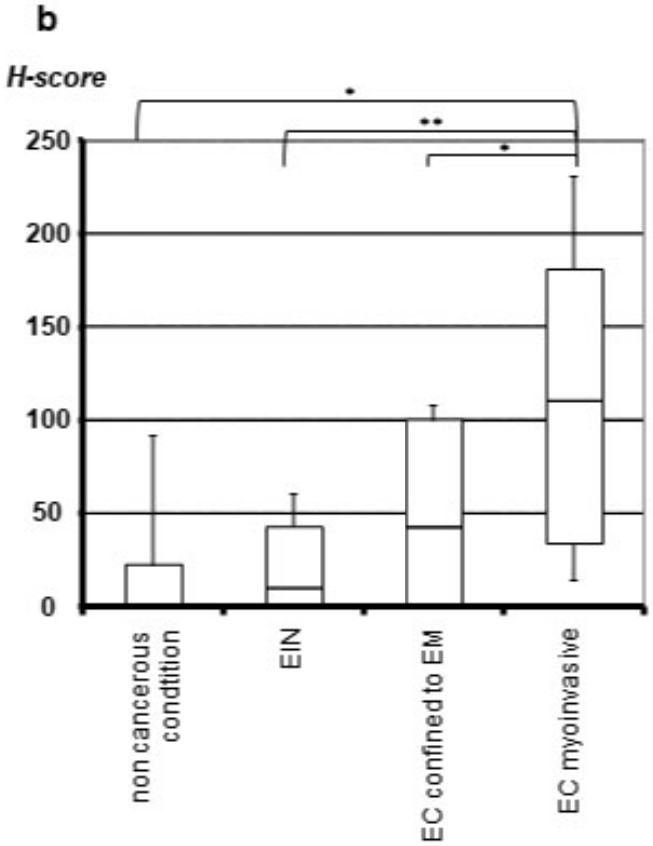
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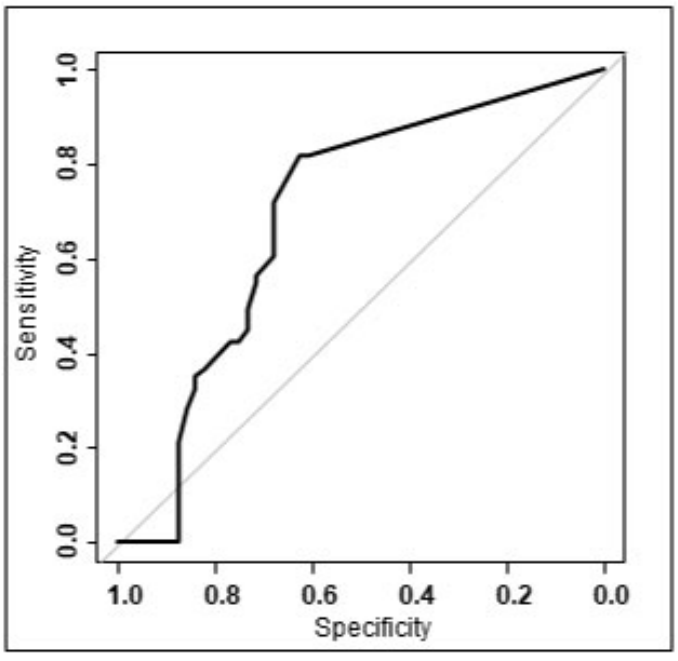
EC myoinvasive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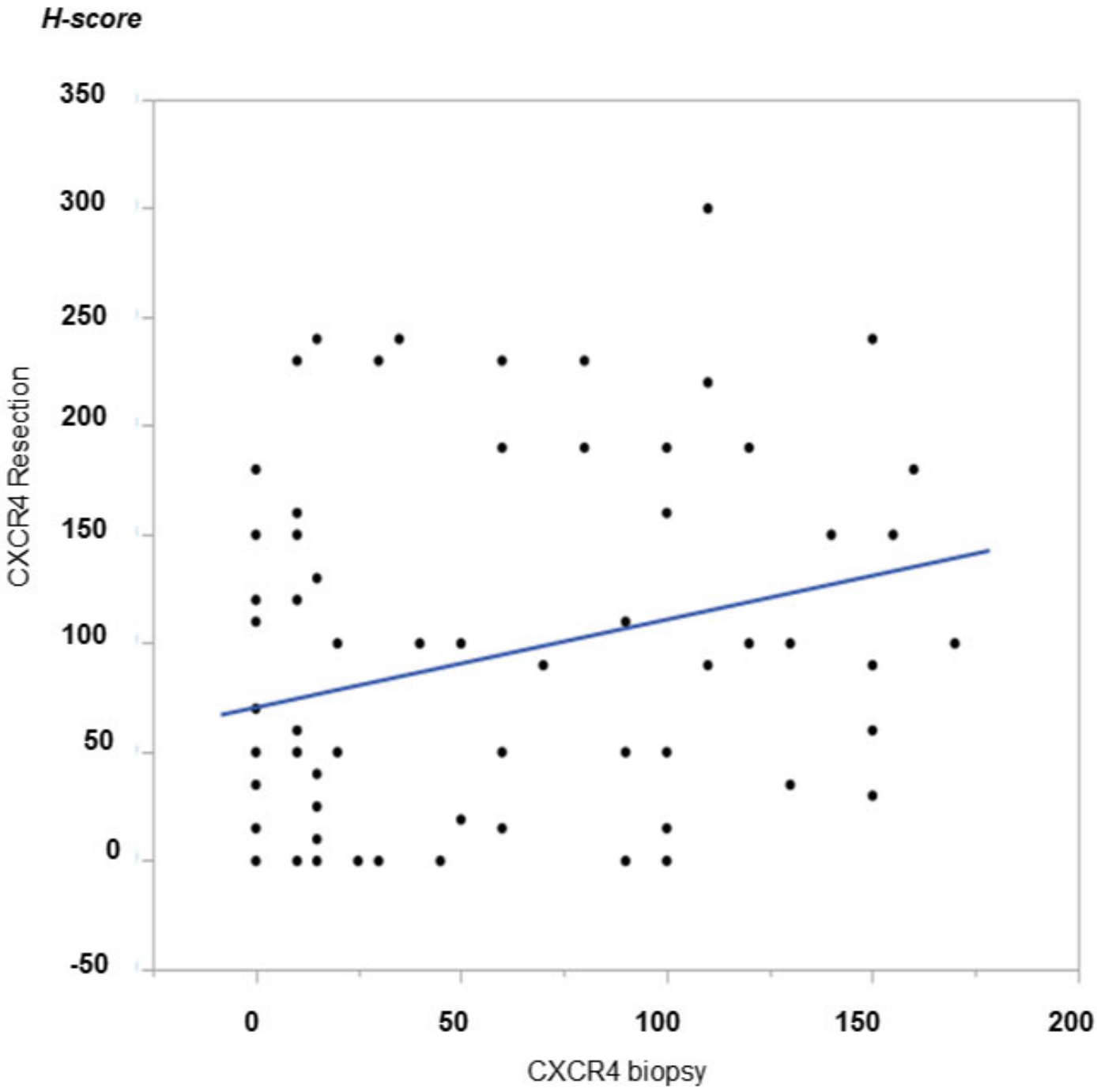
H-score
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CXCR4 Resection
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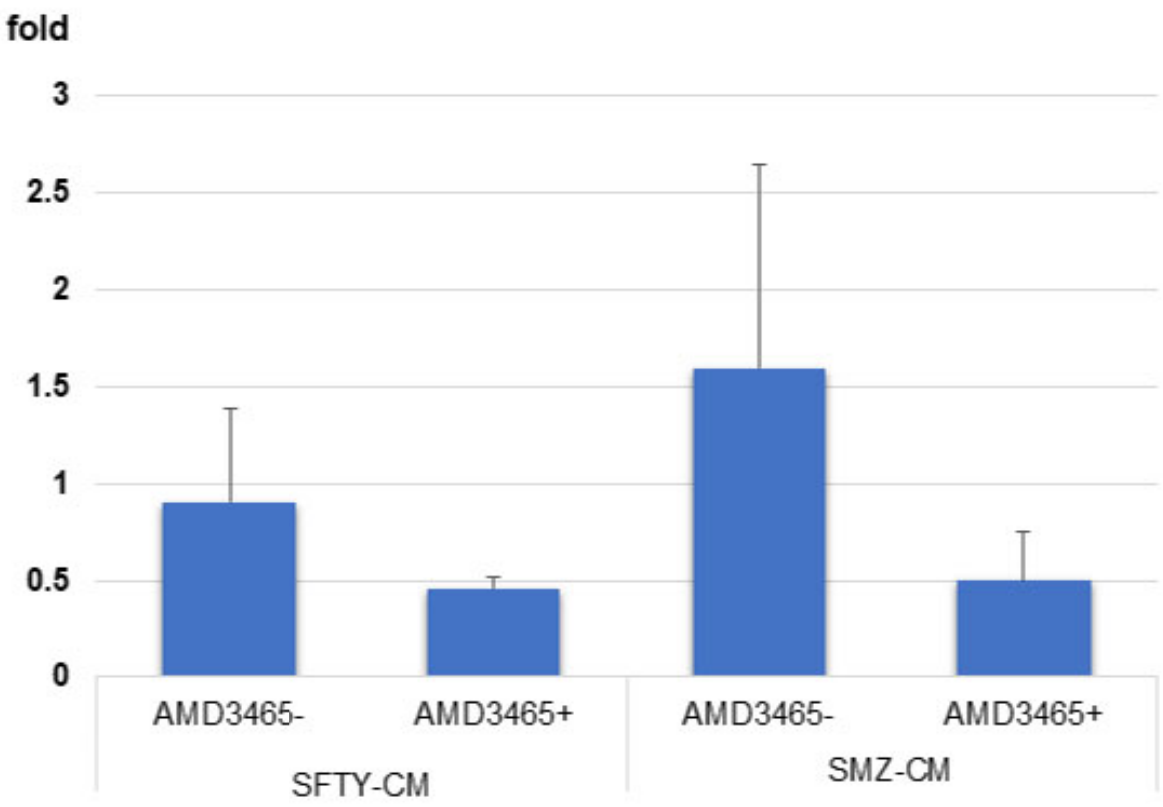
a


fold
3
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b

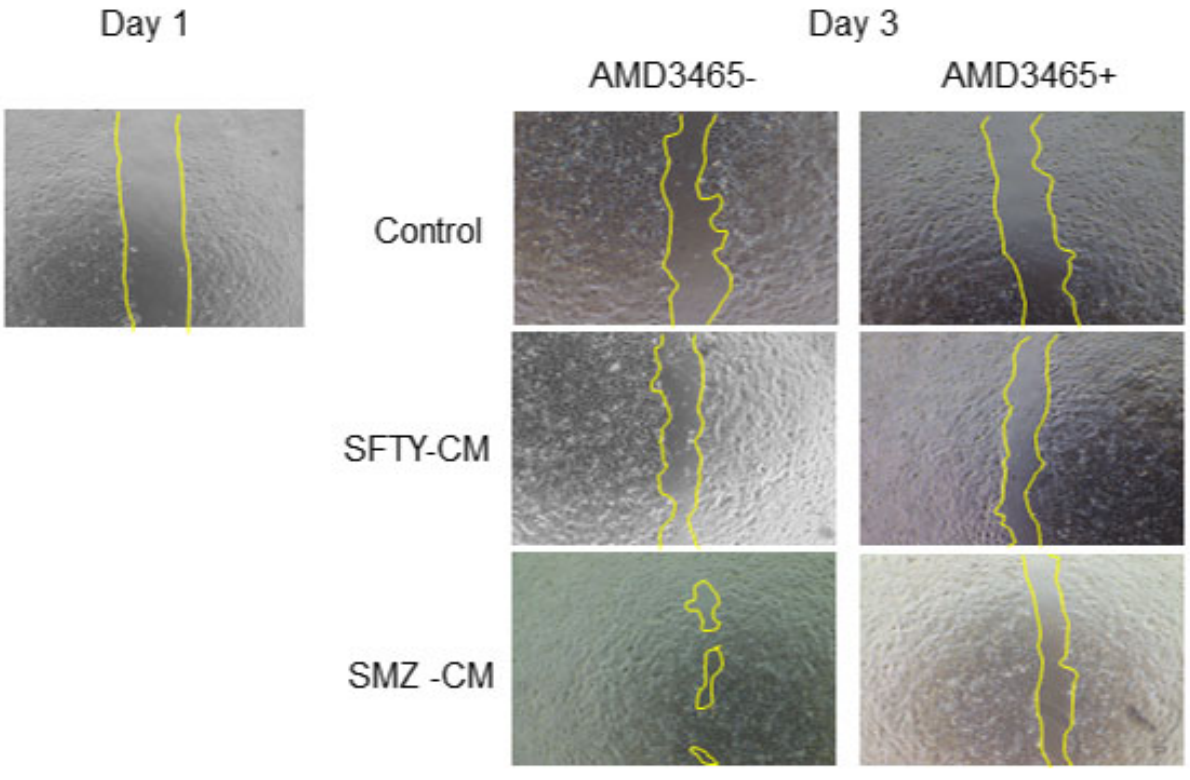
Day 1
Day 3
AMD3465-
AMD3465+
Control
SFTY-CM
SMZ -CM

TABLE 1. Clinicopathological characteristics of 71 endometrioid carcinoma patients

	n (%)
Age, median (range)	60 (25-88)
FIGO grade	
G1	62 (87.3)
G2	9 (12.7)
Pathological stage	
pT1a	20 (28.2)
pT1b	35 (49.3)
pT2	7 (9.9)
pT3a	8 (11.3)
pT3b	1 (1.4)
Myometrial invasion	
confined to endometrium	14 (19.7)
identified	57 (80.3)
Lymphovascular invasion	
identified	20 (28.2)
not identified	51 (71.8)
Cervical invasion	
identified	8 (12.7)
not identified	63 (88.7)
Adnexal metastasis	
identified	3 (4.2)
not identified	68 (95.8)
Lymph node metastasis	
Positive	9 (12.7)
Negative	56 (78.9)
unknown or not performed	6 (8.5)

TABLE 2. Semi-quantitative analysis of expression of CXCR4 at the cut off ratio 12.5

	CXCR4		P value	
	Negative	Positive		
	No. of patients N=14 (%)	No. of patients N=57 (%)	univariate	multivariate
	Median 56	Median 60		
Age	(range 37-76)	(range 25-88)	0.21	NS
FIGO grade			0.84	NS
G1	12 (85.7)	50 (87.7)		
G2	2 (14.3)	7 (12.3)		
Pathological stage			0.49	NS
pT1a	5 (35.7)	15 (26.3)		
pT1b	7 (50.0)	28(49.1)		
pT2	1 (7.1)	6 (10.5)		
pT3a	1 (7.1)	7 (12.3)		
pT3b	0 (0)	1 (1.8)		
Myometrial invasion			0.02	0.02
not identified	6 (42.9)	8 (14.0)		
identified	8 (57.1)	49 (86.0)		
Lymphovascular invasion			0.07	NS
not identified	14 (100)	37 (64.9)		
identified	0 (0)	20 (35.1)		
Cervical invasion			0.69	NS
not identified	12 (85.7)	51 (89.5)		
identified	2 (14.3)	6 (10.5)		
Adnexal metastasis			0.55	NS
not identified	14 (100)	55 (96.5)		
identified	0 (0)	2 (3.5)		
Lymph node metastasis			0.15	NS
Negative	14 (100)	42 (73.7)		
Positive	0 (0)	9 (15.8)		
unknown or not performed	0 (0)	6 (10.5)		

NS : not significant

Although no significant association of CXCR4 expression with age, FIGO grade, pathological stage, lymphovascular invasion, cervical invasion, adnexal metastasis, or pelvic lymph node metastasis was observed, univariate and multivariate analysis revealed a significant correlation between CXCR4 expression and myometrial invasion (odds ratio 4.59, 95%CI 1.25–16.8, $p < 0.05$ and $p < 0.02$, respectively).