Table 3 Queries and consensus-building using the Delphi method

No.	Query	Result of vote
	Definition	
1	Lymphatic vessel invasion is defined as invasion of tumour cells into lymphatic vessels	Agreement (100%)
2	Blood vessel invasion is defined as invasion of tumour cells into blood vessels	Agreement (100%)
	Assessment of BLI	
1	Scanning at 4× magnification should be used to identify suspected lesion. Then further assessment should be made at higher magnification	Agreement (87.5%)
2	BLI should not be determined in lesions presenting desmoplastic reaction	No agreement (50.0%)
3	BLI should not be determined in lesions with spicula in the space between tumour nest and stroma	Agreement (87.5%)
4	Lymphatic fluid, macrophage or blood cells in the space between tumour nests and stroma should be determined as BLI	No agreement (50.0%)
5	A tumour cluster with space around the cluster, but with vague periphery should not be determined as BLI	No agreement (25.0%)
6	Assessment of BLI should be performed in the invasive front of tumour	No agreement (50.0%)
7	Assessment of BLI should be performed with largest section containing the deepest invasive area of tumour	No agreement (50.0%)
	Staining for assessment of BLI	
1	Histochemical staining for assessment of blood vessel invasion should be performed with a whole section from the largest slice containing the deepest invasive area of the tumour	No agreement (50.0%)
2	Histochemical staining for assessment of blood vessel invasion should be performed with a section representative of the tumour	No agreement (25.0%)
3	Immunohistochemical staining for assessment of blood vessel invasion should be performed with a whole section from the largest slice containing the deepest invasive area of the tumour	No agreement (50.0%)
4	Histochemical staining for assessment of blood vessel invasion should be performed with a section representative of the tumour	No agreement (25.0%)
	Assessment of blood vessel invasion	
1	Elastica-stained internal elastic membrane without space around the tumour cluster should be determined as blood vessel invasion	Agreement (87.5%)
2	Elastica-stained internal elastic membrane without vascular endothelium around the tumour cluster should be determined as blood vessel invasion	Agreement (87.5%)
3	In elastica staining, thick arteries surrounded by the tumour cluster should be identified. If elastica-stained internal elastic membrane covering more than half of the circumference surrounding the tumour cluster is observed, the lesion should be diagnosed as blood vessel invasion	Agreement (87.5%)
4	Even without the presence of arteries, if elastica-stained internal elastic membrane covering more than half of the circumference surrounding the tumour cluster is observed, the lesion should be determined as blood vessel invasion	Agreement (87.5%)
5	Elastica-stained internal elastic membrane covering more than half of the circumference surrounding the tumour cluster without other vascular structure (ie, smooth muscle) is determined as blood vessel invasion	Agreement (87.5%)
6	Elastica-stained internal elastic membrane covering more than half of the circumference surrounding the tumour cluster without endothelial cells in H&E staining should not be determined as blood vessel invasion	No agreement (12.5%)
7	Tumour invasion into the elastica-stained vessel should be determined as blood vessel invasion	Agreement (100%)
8	Elastica-stained internal elastic membrane not covering the circumference surrounding the tumour cluster should not be determined as blood vessel invasion	No agreement (12.5%)
	Assessment of lymphatic vessel invasion	
1	An indistinguishable lesion should not be determined as lymphatic vessel invasion	Agreement (100%)
2	A tumour extending along Auerbach's neural plexus should not be determined as lymphatic vessel invasion	No agreement (75.0%)
3	A lumen with mucin should not be determined as a lymphatic vessel in case of mucinous carcinoma	No agreement (37.5%)
4	Lymphatic vessel invasion should not be determined in a lesion with small cancer cell cluster and desmoplastic reaction	No agreement (50.0%)
5	Small vessels indistinguishable between lymphatic and blood vessels should be determined as lymphatic vessel, if a thick vascular wall cannot be identified	No agreement (50.0%)
6	D2-40 positive vessel should be interpreted as lymphatic vessel regardless of the presence of endothelium	No agreement (37.5%)
7	D2-40 positive vessel without endothelium should not be interpreted as lymphatic vessel	No agreement (12.5%)
8	D2-40 negative vessel suspected as a lymphatic vessel in H&E staining should be interpreted as lymphatic vessel	No agreement (37.5%)
9	D2-40 positive cells covering all of the circumference surrounding the tumour cluster is the requirement for lymphatic vessel invasion	No agreement (0%)
10	D2-40 positive cells covering more than half of the circumference surrounding the tumour cluster is the requirement for lymphatic vessel invasion	No agreement (62.5%)
11	D2-40 positive endothelial cells covering all of the circumference surrounding the tumour cluster is the requirement for lymphatic vessel invasion	No agreement (0%)
12	D2-40 positive endothelial cells covering more than half of the circumference surrounding the tumour cluster is the requirement for lymphatic vessel invasion	Agreement (87.5%)
13	Tumour invasion into the D2-40- stained vessel is determined as lymphatic vessel invasion	Agreement (100%)

BLI, blood and lymphatic vessel invasion.

more consistent diagnosis. Pathological assessment is generally inexpensive and is available at many hospitals. So introducing a standardised pathological diagnosis can be a major contribution to current medical practice. So far, poor agreement has been reported on BLI diagnosis in many organs and conducting a multicentre study using BLI may be difficult. There are some criteria suggested by pathologists on vascular invasion and some of them were incorporated into our criterion.[23 24] While findings of H&E-stained slides should be further reviewed to search for findings with high concordance, developing objective criteria based only on H&E staining may be difficult. BLI and many pathological diagnoses are based on multiple associated histological findings. Therefore, investigation of the concordance in the histological findings associated with the diagnosis of BLI in our

Table 4 Current practice of pathological assessment at the department of pathology in different medical institutions in 2003

Institution	A	B	C	D	E	F	G	H
Number of cases	233	98	62	127	203	232	54	441
Number of colon cancer cases (%)	169 (72.5)	50 (51.0)	46 (74.2)	91 (71.7)	116 (57.1)	137 (59.1)	29 (53.7)	254 (57.6)
Number of rectal cancer cases (%)	64 (27.5)	48 (49.0)	16 (25.8)	36 (28.3	87 (42.9)	95 (40.9)	25 (46.3)	187 (42.4)
Male patients (%)	145 (62.2)	59 (60.2)	35 (56.5	68 (53.5	133 (65.5)	130 (56.0)	29 (53.7	262 (59.4)
Female patients (%)	88 (37.8)	39 (39.8)	27 (43.5)	59 (46.5)	70 (34.5	102 (44.0	25 (46.3)	179 (40.6)
Average age	65.0	64.2	67.4	68.0	63.4	64.3	64.4	63
Average number of blocks	20.1	34.4	4.8	6.4	8.8	18.0	6.3	8.0
Use of histochemical stainings	Some cases	All cases	None	Some cases	All cases	All cases	None	All cases
Range of histochemical stainings	Representative one block	All blocks	None	Representative one block	Blocks from largest slice of tumour	Representative one block	None	Representative one block
Use of immunohistochemical stainings	Some cases	None	None	Some cases	Some cases	Some cases	None	None
Antibody used in immunohistochemical stainings	D2-40	None	None	D2-40	D2-40, SMA	D2-40	None	None

framework was an important step. And the problem of diagnostic threshold or retraction artefacts remains unsolved and may be one of the reasons for poor concordance.[9] Elastica- staining and D2-40 staining have been reported to be useful in detecting BLI but may not always be associated with the detection of patients with higher risk.[14 25] Review of histochemical and immunohistochemical findings in this study suggested key findings with high concordance which may improve the quality of pathological diagnosis. Although the use of these stains alone may be insufficient, it can be used in combination with diagnostic criterion. D2-40 immunostaining was seen in lymphatic endothelium and in fibroblasts. D2-40-positive endothelial cells (but not D2-40 positive cells) was another important finding with high concordance.[26] Although histological variation was reported in blood vessel invasion, elastica-stained internal elastic membrane is the most important feature having consistent results.[27] Careful attention should be paid, however, to the lesion such as myenteric plexus ganglia invasion mimicking blood vessel invasion so as not to misdiagnose as blood vessel invasion in H&E staining. Use of some additional markers may be beneficial to reduce misdiagnosis of BLI without D2-40 expression or internal elastic membrane in the future.

Most pathological criteria to date have been developed through an informal approach by group experts in various scientific committees. A more standardised approach needs to be taken to develop pathological criteria to be used in all kinds of clinical or pathological settings. By analysing the current practice of pathologists and using the Delphi method, we have developed a framework for a new pathological diagnostic criterion. Although it still may not be sufficient to be used in multicentre trials, significant improvement over current practice is expected. We invite other pathologists' comment for its further revision and refinement. Routine elastica staining is low in cost and can be easily adopted by pathologists at different sites.[28] The applications of the present study's criterion to the daily diagnosis of BLI will likely result in the accumulation of more consistent results regarding blood vessel invasion. Although routine use of our criteria of lymphatic vessel invasion may not be feasible for advanced CRC where multiple immunostaining is required for the assessment, it may be useful for endoscopically resected pT1 CRC which is small in size and only few blocks are required.[29] The present report gives a set of criteria hopefully allowing a more consistent identification and reporting of BLI in CRC, and gives a template for the development of such diagnostic criteria that can be used in other areas of diagnostic pathology. Based on the framework that we propose, a more objective and consensus-based pathological diagnostic system can be created in many areas of diagnostic pathology.

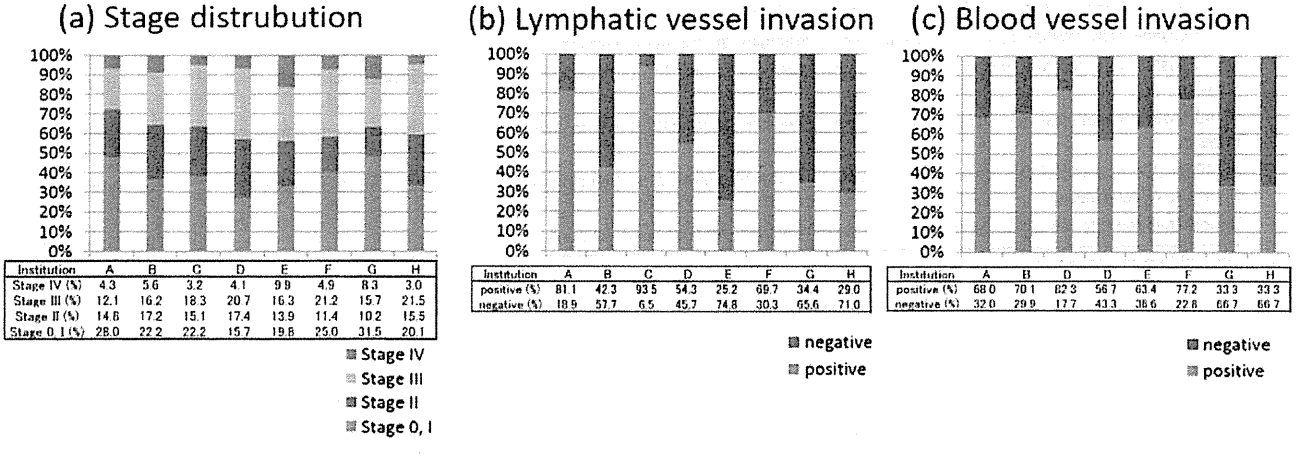

Figure 3 (A) Stage distribution of 1450 surgically resected colorectal cancer (CRC) cases among eight hospitals. (B) Distribution of positive cases of lymphatic vessel invasion among 1450 surgically resected CRC cases from eight hospitals. (C) Distribution of positive cases of blood vessel invasion among 1450 surgically resected CRC cases from eight hospitals.

Box 1 Established diagnostic criterion of blood and lymphatic vessel invasion (BLI) in Japanese society for cancer of the colon and rectum (JSSCR)

Definition
Lymphatic vessel invasion is defined as invasion of tumour cells into lymphatic vessels.
Blood vessel invasion is defined as invasion of tumour cells into blood vessels.

Assessment
Scanning at 4× magnification should be used to identify the suspected lesion. Then, further assessment should be made at higher magnification.
BLI should not be determined in lesions presenting spicula between the tumour nest and stroma.

Assessment of lymphatic vessel invasion
D2-40 positive vessel invasion should be determined as lymphatic vessel invasion, and the presence of D2-40 positive endothelial cells covering more than half of the circumference surrounding the tumour cluster should be the basis for diagnosis.
An indistinguishable lesion should not be determined as lymphatic vessel invasion.

Blood vessel invasion
In elastica staining, thick arteries surrounded by the tumour cluster should be identified. If elastica-stained internal elastic membrane covering more than half of the circumference surrounding the tumour cluster is present, the lesion should be diagnosed as blood vessel invasion.
Elastica positive vessel invasion should be determined as blood vessel invasion, and the presence of elastic-stained internal elastic membrane covering more than half of the circumference surrounding the tumour cluster should be the basis for diagnosis.
When the presence of elastica-stained internal elastic membrane covering more than half of the circumference surrounding the tumour cluster is identified, the lesion should be diagnosed as blood vessel invasion, even in the absence of accompanying artery, vascular structure or space.

Take home messages

- Current practice of pathology assessment was varied among institutions in Japan.
- Concordance was low for the pathological diagnosis of blood and lymphatic vessel invasion (BLI) in colorectal cancer, and was not any better when histochemical/immunohistochemical staining was provided.
- All histological findings associated with BLI from H&E staining were poor in agreement. However, observation of elastica-stained internal elastic membrane covering more than half of the circumference surrounding the tumour cluster as well as the presence of D2-40-stained endothelial cells covering more than half of the circumference surrounding the tumour cluster showed high concordance.
- Based on this observation, we developed a framework for pathological diagnostic criterion, using the Delphi method, which may serve as the basis for creating a standardised procedure for pathological diagnosis.

Author affiliations
[1]Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa,Chiba, Japan
[2]Department of Pathology, National Defence Medical College, Saitama, Japan
[3]Department of Diagnostic Pathology, Kurume University Hospital, Kurume, Fukuoka, Japan
[4]Department of Pathology, Kurume University School of Medicine, Kurume, Fukuoka, Japan
[5]Department of Pathology, Kyorin University Graduate School of Medicine, Tokyo, Japan
[6]Department of Pathology, Tokyo Metropolitan Komagome Hospital, Tokyo, Japan
[7]Division of Organ Pathology, Department Microbiology and Pathology, Faculty of Medicine Tottori University, Tottori, Japan
[8]Pathology Division, National Cancer Center Hospital, Tokyo, Japan
[9]Department of Pathology, Graduate School of Medicine and Dental Sciences, Niigata University, Niigata, Japan
[10]Clinical Trial Section, Research Center for Innovative Oncology, National Cancer Center Hospital East, Chiba, Japan

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PLOS ONE

Human Subperitoneal Fibroblast and Cancer Cell Interaction Creates Microenvironment That Enhances Tumor Progression and Metastasis

Motohiro Kojima[1☯], Youichi Higuchi[1☯], Mitsuru Yokota[2], Genichiro Ishii[1], Norio Saito[2], Kazuhiko Aoyagi[3], Hiroki Sasaki[3], Atsushi Ochiai[1]*

1 Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan, **2** Colorectal and Pelvic Surgery Division, National Cancer Center Hospital East, Kashiwa, Chiba, Japan, **3** Genetic Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan

Abstract

Backgrounds: Peritoneal invasion in colon cancer is an important prognostic factor. Peritoneal invasion can be objectively identified as periotoneal elastic laminal invasion (ELI) by using elastica stain, and the cancer microenvironment formed by the peritoneal invasion (CMPI) can also be observed. Cases with ELI more frequently show distant metastasis and recurrence. Therefore, CMPI may represent a particular milieu that facilitates tumor progression. Pathological and biological investigations into CMPI may shed light on this possibly distinctive cancer microenvironment.

Methods: We analyzed area-specific tissue microarrays to determine the pathological features of CMPI, and propagated subperitoneal fibroblasts (SPFs) and submucosal fibroblasts (SMFs) from human colonic tissue. Biological characteristics and results of gene expression profile analyses were compared to better understand the peritoneal invasion of colon cancer and how this may form a special microenvironment through the interaction with SPFs. Mouse xenograft tumors, derived by co-injection of cancer cells with either SPFs or SMFs, were established to evaluate their active role on tumor progression and metastasis.

Results: We found that fibrosis with alpha smooth muscle actin (α-SMA) expression was a significant pathological feature of CMPI. The differences in proliferation and gene expression profile analyses suggested SPFs and SMFs were distinct populations, and that SPFs were characterized by a higher expressions of extracellular matrix (ECM)-associated genes. Furthermore, compared with SMFs, SPFs showed more variable alteration in gene expressions after cancer-cell-conditioned medium stimulation. Gene ontology analysis revealed that SPFs-specific upregulated genes were enriched by actin-binding or contractile-associated genes including α-SMA encoding ACTA2. Mouse xenograft tumors derived by co-injection of cancer cells with SPFs showed enhancement of tumor growth, metastasis, and capacity for tumor formation compared to those derived from co-injection with cancer cells and SMFs.

Conclusions: CMPI is a special microenvironment, and interaction of SPFs and cancer cells within CMPI promote tumor growth and metastasis.

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* E-mail: aochiai@east.ncc.go.jp

☯ These authors contributed equally to this work.

Introduction

Although tumor size is a major prognostic factor in many cancers, prognosis in gastrointestinal cancer is stratified not by tumor size but by tumor spread [1]. Peritoneal invasion in colorectal cancer has been reported to be a strong prognostic factor, but this term was not well defined, and detection and diagnosis methods have been questioned [2–4]. Recent pathological reports have demonstrated that elastica stain, which highlights the peritoneal elastic lamina near the periotoneal surface, is useful for objective detection of peritoneal invasion. We and others have determined that peritoneal invasion defined as tumor invasion beyond the peritoneal elastic lamina (elastic laminal invasion: ELI) is a strong prognostic factor that can influence future pT criteria in the Union for International Cancer Control (UICC) TNM classification [5–7]. The peritoneum is a very thin membrane, within 500 μm thick, and the peritoneal elastic lamina exists within this membrane. The frequency of synchronous metastasis and recurrence is increased by 2 to 4 times when a tumor invades this narrow space [5]. These results may suggest that tumor progression and metastasis are facilitated by a cancer microenvironment formed by peritoneal invasion (CMPI). The extent of

CMPI can be identified by using elastica stain, and pathological features of CMPI can also be determined.

A tissue microarray facilitates the evaluation of protein expression for a large number of tissue blocks from a single specimen, and area-specific tissue microarrays have been reported to be useful for studying specific tumor areas in large cohorts [8]. After determination of CMPI by using elastica stain, a tissue core can be obtained from this area and a comparison with the features of other tumor areas can also be performed. This process may allow for an assessment of the important biological phenomena occurring in this cancer microenvironment.

Recent advances in cancer research have established the concept of cancer microenvironment that promotes tumor initiation, invasion, and metastasis [9]. Although the cancer microenvironment is composed of many types of cells, the use of area-specific tissue microarrays may allow for a focus on the cell components that characterize CMPI. Furthermore, if these cell components can be cultivated from the histologically corresponding subperitoneal region, a biological study to elucidate this putative cancer-promoting microenvironment can be performed.

The aim of this study was to explain how the colorectal cancer prognosis is affected by peritoneal invasion. We constructed area-specific tissue microarray system to determine the characteristic cell components of CMPI. Next, we cultivated specific fibroblast subpopulations from the submucosal and subperitoneal layers of the human colonic wall. The biological characteristics and gene profiles of submucosal fibroblasts (SMFs) and subperitoneal fibroblasts (SPFs) with or without cancer-cell-conditioned medium (CCCM) stimulation were compared. Subsequently, we constructed xenograft tumors by co-injection of cancer cells with either SPFs or SMFs. Our study proposed a new candidate for a cancer-promoting microenvironment in colon cancer and elucidated SPFs as crucial players in the enhancement of tumor progression and metastasis.

Patients and Methods

Ethics Statement

This study was approved by the National Cancer Center Hospital East Institutional Review Board (No: 19-021). A written general consent to use biologic materials for research was obtained from each participant prior to tissue acquisition. Animal experiments were approved by the Animal Ethics Committee of the National Cancer Center Hospital East (K11-032).

Patient Characteristics and Detection of ELI

Four hundred consecutive patients with TNM classification (5th edition) pT3 and pT4a colon cancer [10], undergoing surgery between 1996 and 2003 at the National Cancer Center Hospital East, were enrolled. Using elastica stain, we identified 173 cases with ELI and further examined these using area-specific tissue microarrays [5,8]. Of the 173 cases with ELI, 107 were pT3 and 66 were pT4a.

Construction of Area-Specific Tissue Microarrays

To elucidate the pathological features of CMPI in colon cancer tissue, we defined the cancer microenvironment as follows: (a) CMPI has a tumor border with peritoneal invasion and (b) the cancer microenvironment formed by submucosal invasion (CMSI) has a submucosal invasive tumor border (Figure 1A) [5]. The 2-point tissue microarray was then established as previously described [8]. Each tumor area was marked with ink on the histological slide; a single tissue core of 2 mm in diameter was obtained from each cancer microenvironment and transferred to a recipient block using a Tissue Microarrayer (Azumaya, Tokyo, Japan). In 24 cases, insufficient cancer tissue was obtained from the CMPI. However, sufficient tissue was obtained in 149 cases; these were analyzed histologically and immunohistochemically (See Materials and Methods S1, and Table S1).

Antibodies, Regents, and Immunohistochemistry

The antibodies, reagents, and the immunohistochemical procedures used are described in Materials and Methods S1 and the Table S2.

Evaluation of Area-Specific Tissue Microarray Sections

High-resolution slide images were acquired from all tissue cores with hematoxylin-eosin (H.E) and immunohistochemistry staining, using NanoZoomer 2.0-HT slide scanner (Hamamatsu photonics, Hamamatsu, Japan). All cores were examined using viewer software (NDP view: Hamamatsu photonics, Hamamatsu, Japan). When the area of fibrosis exceeded 50% of a whole tissue core with a 2 mm diameter upon H.E staining, it was defined as positive for marked fibrosis. On immunohistochemical staining, hot spots with CD3-, CD31-, and CD68-positive cells or vessels were selected in the viewer software, then an image of x20 magnification (0.51 mm^2) was taken, and saved as a JPEG file. Positive cells or vessels were counted in each image using morphometric software (WinRoof, Mitani Corporation, Fukui, Japan). Moreover, the area with highest alpha smooth muscle actin (α-SMA) expression in fibroblasts was selected, then a x20 magnification (0.51 mm^2) image was taken, and saved as a JPEG file. The ratio of the α-SMA positive area in the image was calculated using morphometric software, as described previously [11]. The α-SMA expression in normal muscle tissue, as determined by comparison with a serial H.E slide, was not evaluated. H.E and immunohistochemical staining data of CMPI was compared with that of CMSI to elucidate the histological characteristics of CMPI.

Primary Cells and Cell Lines

Submucosal tissue was obtained from sigmoid colon tissue more than 5 cm distant from the tumor. Colonic tissue was dissected from the muscular layer on the luminal side, and lamina propria and mucosal layer tissues were obtained. Next, the lamina propria was scrubbed away to obtain submucosal tissue. Subperitoneal tissue was obtained from the sigmoid colon mesentery at more than 5 cm distant from the tumor by using operating tweezers and scissors. These tissues were washed with phosphate-buffered saline (PBS) and incubated in 5% trypsin for 20 minutes, 3 times. The supernatant was centrifuged, plated on a dish, and submucosal fibroblasts (SMFs) and subperitoneal fibroblasts (SPFs) were obtained and then grown and maintained in MF-medium (Toyobo, Tokyo, Japan) [12]. All experiments were performed on cells within 8 passages.

The human colorectal cancer cell lines DLD-1 and Caco-2 were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, Saint Louis, MO) containing 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich, Saint Louis, MO), and 10% fetal bovine serum (FBS; Gibco, Palo Alto, CA).

Cell Proliferation Assay, Immunocytochemical Staining, and Flow Cytometry Analysis

Cell proliferation assays, immunocytochemical staining, and flow cytometry analyses were performed as described in Materials and Methods S1.

Stimulation of Fibroblasts by Cancer Cell Medium

Initially, $1.7\times10^4/cm^2$ of fibroblasts and DLD-1 cells were grown separately in DMEM containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS for 48 hours, and then were starved for 24 hours. Next, the medium was removed from the fibroblasts, and the medium from the starved DLD-1 cells was added to the fibroblasts for 24 hours to establish fibroblasts with cancer-cell-conditioned medium (CCCM) stimulation. As control, fibroblasts were starved for 48 hours (yielding fibroblasts without CCCM). SPFs and SMFs either with or without CCCM were assessed by using immunocytochemistry or gene expression analysis. As for the evaluation of immunocytochemical α-SMA expression, the area with highest α-SMA expression was selected, then a x20 magnification (0.51 mm^2) image was taken, and saved as a JPEG file. The ratio of α-SMA positive area in the image was calculated using morphometric software (WinRoof, Mitani Corporation, Fukui, Japan).

Gene Expression Analysis using Microarray

Three sets of SPFs and SMFs, either with or without CCCM, obtained from 3 different patients, were used in this study. We used GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA). Target cDNA was generated from 100 ng of total RNA extracted from each sample using a 3′ IVT Express Kit (Affymetrix, Santa Clara, CA). The procedures for target hybridization, washing and staining for signal amplification were performed according to the supplier's protocols. The arrays were scanned with a Gene Chip Scanner 3000 (Affymetrix, Santa Clara, CA), and the intensity of each feature of the array was calculated by using GeneChip Operating Software, version 1.1.1 (Affymetrix, Santa Clara, CA). The average intensity was standardized to the target intensity, which was set equal to 1000, to reliably compare different arrays. The values were log transformed and median centered. The programs GeneSpring (Agilent Technologies, Santa Clara, CA) and Excel (Microsoft Corporation, Redmond, WA) were used to perform the numerical analyses for gene selection.

Xenograft Transplantation and Tumor Formation Assay

Either 1×10^6 human colorectal cancer cells Caco-2 or DLD-1 alone, or with either 1×10^6 SPFs or SMFs, were injected subcutaneously (s.c.) into the back of SCID mice (8–12 weeks of age; CLEA, Tokyo, Japan). Tumor volumes were calculated weekly as described previously [13]. Mice injected with Caco-2 alone or with either SPFs or SMFs were killed after 10 weeks, and those injected with DLD-1 alone or with either SPFs or SMFs were killed after 8 weeks, and tumor weights were evaluated. For distant metastatic analysis, lung and liver tissue was removed and fixed in 10% formalin, and for the analysis of lymph node metastasis, neck and inguinal adipose tissue was also removed and fixed; all tissues were histologically examined. We used 8 mice in each group.

To elucidate the capacity of fibroblasts to enhance tumor formation, serial dilutions of Caco-2 or DLD-1 cancer cells were similarly co-injected with either 1×10^6 SMFs or SPFs. Tumor formation was evaluated 4 weeks after the injection. We used 4 mice for each group.

Statistical Analysis

X^2 test and Student's t test were used in the tissue microarray analysis, cell proliferation assay, xenograft transplantation, and tumor formation assay. A $P<0.05$ was defined as statistically significant. In the microarray analysis, gene expression data were analyzed using GeneSpring GX12 (Agilent Technologies, Santa Clara, CA). Row data were summarized by using MAS5 and normalized by log transformation and median centering for numerical analyses for gene selection. For principal component analysis (PCA), we used probe sets that were reliably measured and varied by 3-fold above the global median in at least 2 samples (approximately 10%); analyses were performed using GeneSpring GX12. The differentially expressed probe sets used in supervised hierarchical clustering were selected based on $P<0.05$ and fold change (FC) >2.0. P values were calculated using one way ANOVA with Benjamini and Hochberg FDR multiple testing correction. Hierarchical clustering with weight-average linkage clustering was performed using Cluster and Treeview programs (Michael Eisen, Stanford University, genome-www.stanford.edu). The functional annotation clustering of Gene Ontology Enrichment analysis was performed using DAVID software, with the classification stringency set to "High", and the significant clusters were selected based on an enrichment score >2.0 and a $P<0.01$ (Fisher's exact test after Benjamini and Hochberg FDR multiple testing correction) [14,15].

Results

Histological Features of ELI

Not only tumor cells, but also varieties of stromal cells constitute a distinct cancer microenvironment, and some promote tumor metastasis [9]. First, we elucidated the significant histological features of CMPI to shed light on phenomena occurring in this milieu by using area-specific tissue microarrays. The clinicopathological features of the 149 cases used are shown in Table S1. On H.E staining, we found extensive fibrosis (over 50%) more frequently in CMPI than was seen in CMSI (Figure 1B). The ratio of α-SMA positive area in CMPI was also higher than that seen in CMSI (Figure 1C). The proportions of T lymphocytes, macrophages, or microvessels evaluated using CD3, CD68, or CD31, respectively, were not significantly different between CMPI and CMSI (Figure 1D–F). Both in CMPI and CMSI, plump spindle-shaped fibroblasts were major source of α-SMA expression, and the ratio was successfully analyzed by using morphometric software (Figure 2A–D). Considering our previous results, which indicated that peritoneal invasion defined by ELI was closely associated with distant metastasis, we hypothesized that fibroblasts in the subperitoneal layer could be implicated not only in prominent fibrosis and activation, but also in the tumor's progression and metastasis. We then decided to isolate fibroblasts from the subperitoneal layer that histologically corresponded to peritoneal invasion. Fibroblasts from the submucosal layer were used as controls.

Isolation and Characterization of Cultured Human SPFs and SMFs

At first, we evaluated the morphological and biological characteristics of SPFs and SMFs in a normal state. Both cultured human SPFs and SMFs showed similar spindle-shaped morphologic characteristics (Figure S1A–B). SPFs and SMFs from 3 patients could be cultured over 10 passages, except for 1 SPF case (data not shown). Immunocytochemistry and flow cytometry revealed the obtained SPFs and SMFs were consistent with fibroblasts (Figure S1C–D). We found weak α-SMA expression in a few SPFs and SMFs. The doubling time for SPFs and SMFs was 79.9 hours and 36.3 hours, respectively, and the growth of SMFs was faster than that of SPFs ($P<0.05$), which suggested a biological difference between SPFs and SMFs (Figure S1E).

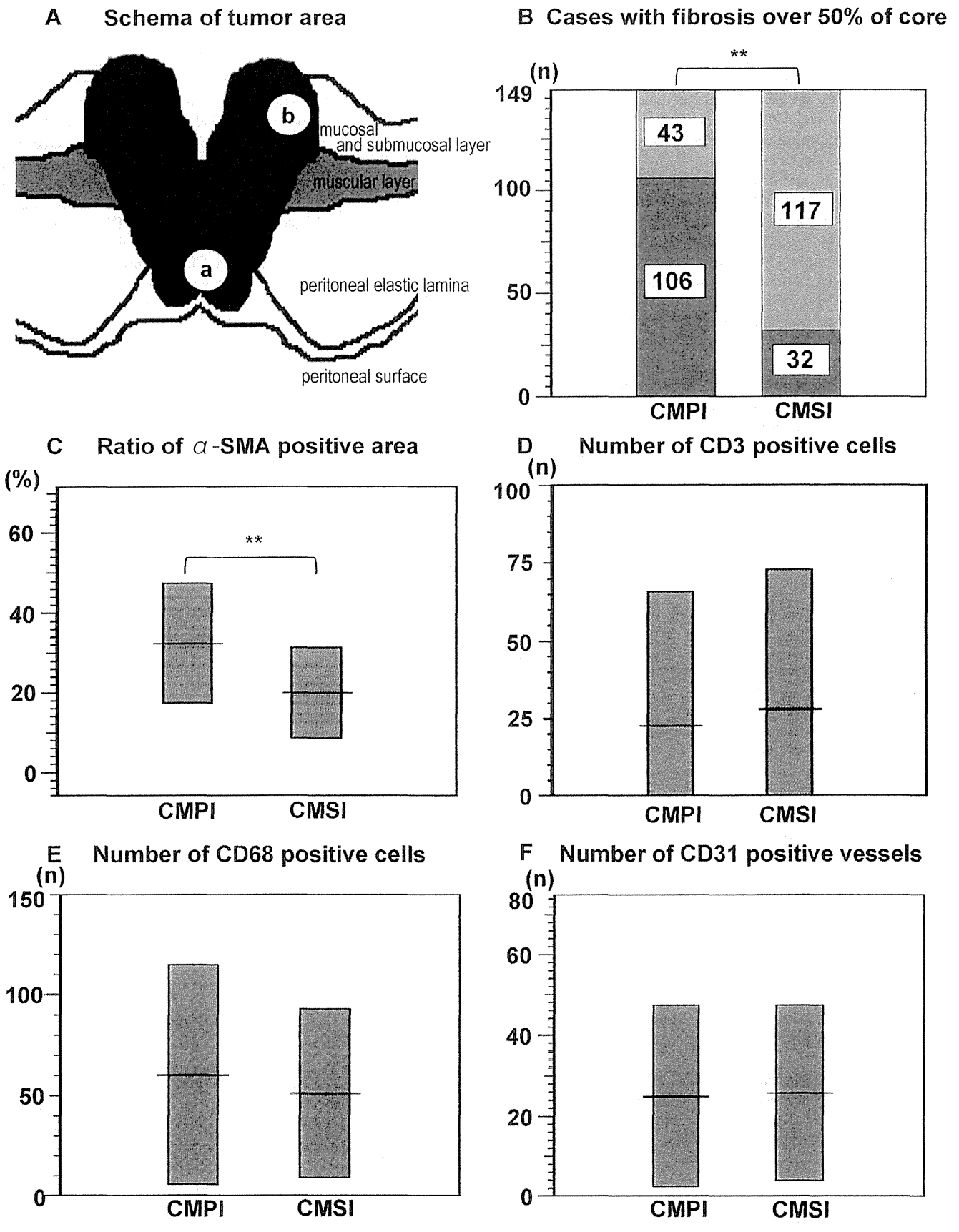

Figure 1. Pathological features of tumor microenvironment explored by using area-specific tissue microarray. (A) Schema of the cancer microenvironment formed by peritoneal invasion (CMPI) and the cancer microenvironment formed by submucosal invasion (CMSI) defined as

(a) invasive front with peritoneal invasion and (b) submucosal invasive front, respectively. (B) The distribution of fibrosis in human colon cancer tissue. Dark gray bars show the number of the cases with fibrosis over 50% of the core from each tumor area, and light gray bars show the number of the cases without extensive fibrosis. Core samples with CMPI showed a higher frequency of marked fibrosis than did core samples with CMSI ($P<0.01$). (C) Distribution of α-SMA expression in human colon cancer tissue. CMPI showed higher α-SMA expressions than those seen in CMSI ($P<0.01$). (D) Distribution of CD3-positive cells in human colon cancer tissue. Numbers of CD3-positive cells were not significantly different between CMPI and CMSI. (E) Distribution of CD68-positive cells in human colon cancer tissue. Numbers of CD68-positive cells were not significantly different between CMPI and CMSI. (F) Distribution of CD31-positive vessels in human colon cancer tissue. Numbers of the CD31-positive vessels were not significantly different between CMPI and CMSI. Results in (B) are presented by case numbers, and those in (C–F) are presented as the mean ± SD of 149 cases (**$P<0.01$).

doi:10.1371/journal.pone.0088018.g001

Gene Expression Profiling Comparison between SPFs and SMFs

To assess the phenotypical differences between SPFs and SMFs, the gene expression profiles of fibroblasts with or without CCCM stimulation were compared. PCA revealed 4 distinct clusters, depending on their origin and CCCM stimulation, which overcame the individual variation (Figure 3A and B). Supervised cluster analysis also revealed 4 distinct clusters (Figure 3C). This indicated the phenotypical difference in fibroblasts within the colonic wall. And this difference depended on the histoanatomical location. Furthermore, the reaction to CCCM stimulation was also different.

Next, we compared gene expression profiles in these fibroblasts with and without CCCM stimulation, separately (Figure 3D–E). Data from SPFs without CCCM stimulation were enriched by the gene ontology (GO) terms "extracellular matrix" and "proteinaceous extracellular matrix", which formed annotation cluster 1. Major expracellular matrix (ECM) components of collagens (COL1A1, COL4A1, COL4A2, COL5A1, and COL16A1), laminin, or fibronectin were included in this cluster. Moreover, gene expression related to components that bind to the ECM was also upregulated in SPFs and formed annotation cluster 2. Annotation cluster 3 was enriched for GO terms associated with "granules" or "vesicles" (Figure 3D and Table S3). This result suggested the gene expression profile associated with basic function in fibroblasts is different between SPFs and SMFs within the colonic wall. The top 20 genes highly expressed in SPFs also included several ECM components. Genes associated with fibrogenesis or the myofibroblastic differentiation of FLI1 and NOX4 were also found in the top 20 genes (Table S4). Among other highly expressed genes in SPFs without CCCM stimulation, we found POSN, SPARC, or COL4A1, which are known to be highly expressed in the cancer stroma; and many of these are prognostic factors [11,16,17].

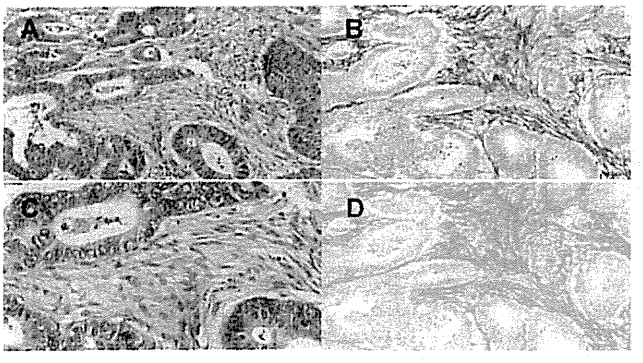

Figure 2. Histological features of fibrosis in the cancer microenvironment formed by peritoneal invasion (CMPI). (A) Histological features of stromal component of CMPI. Both in CMPI and the cancer microenvironment formed by submucosal invasion (CMSI), plump spindle-shaped fibroblasts were major sources of the stroma. (B) Marked α-SMA expression was found in fibroblasts. (C) Higher magnification more clearly revealed plump spindle-shaped fibroblasts. (D) Using morphometric software, we successfully detected and analyzed α-SMA expression.

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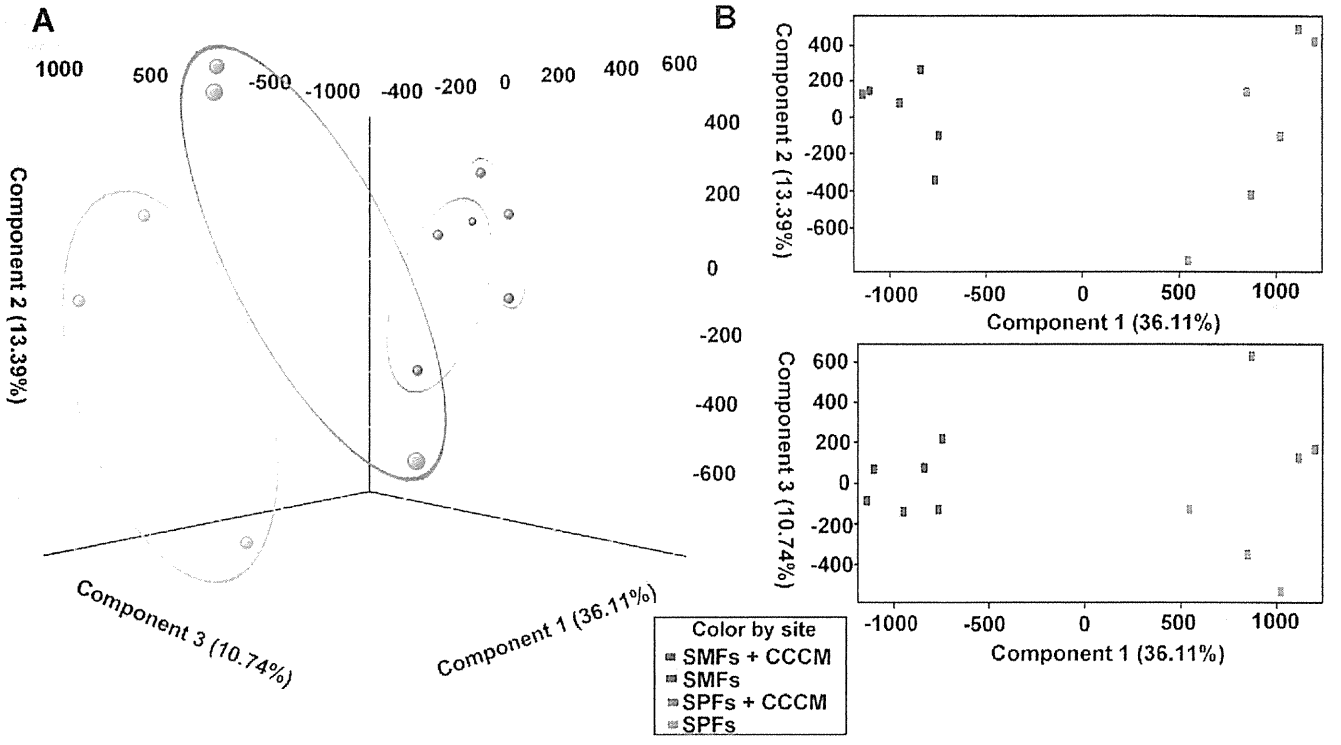
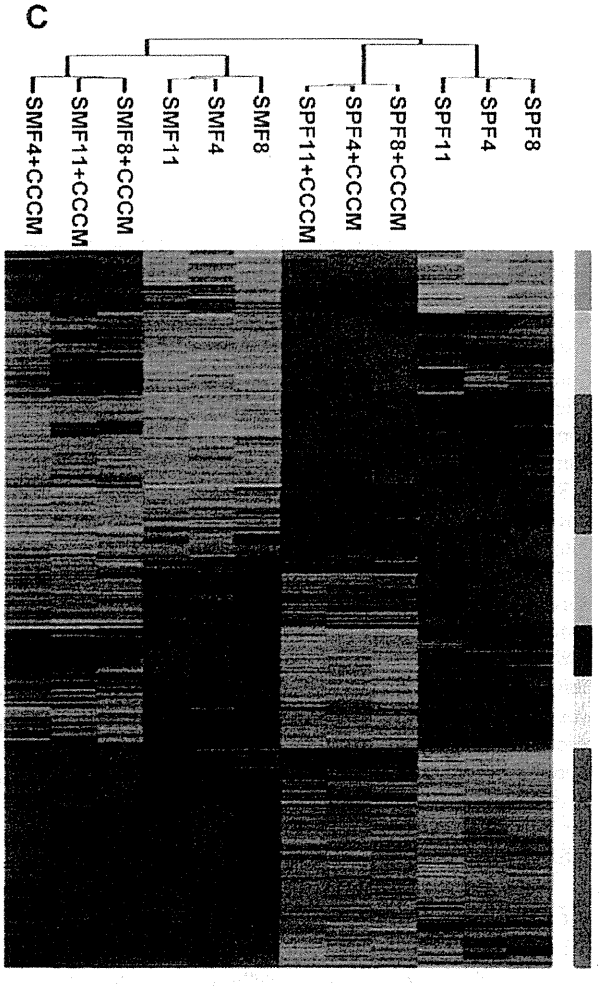
Most of the genes with increased expressions in SPFs without CCCM stimulation were also retained in the presence of CCCM stimulation; however, there were some differences (Figure 3D–E, Table S5–6). In GO analysis of SPFs with CCCM stimulation, the order of annotation clusters were changed compared to that seen in SPFs without CCCM stimulation. Among the top 20 genes, 13 genes were conserved and 7 genes were replaced. These results suggest a difference in the reaction to CCCM stimulation between SPFs and SMFs. The existence of SPFs-specific genes that are upregulated after CCCM stimulation was estimated.

We then analyzed these genes to establish the biological characteristics of SPFs after exposure to CCCM. A Venn diagram revealed 193 upregulated genes in SPFs and 59 in SMFs after CCCM stimulation. Of these, 51 were commonly upregulated both in SPFs and SMFs, 142 were SPFs specific, and only 8 were SMFs specific (Figure 4A). We then also focused on downregulated genes, and discovered 215 in SPFs and 146 in SMFs. Of these, 138 were commonly downregulated both in SPFs and SMFs, 77 were SPFs specific, and only 8 were SMFs specific (Figure 4B). These results suggested that SPFs showed more variable alteration in gene expression after CCCM stimulation. GO term analysis of SPF-specific genes downregulated after CCCM stimulation did not revealed any annotation cluster over 3.0 of the enrichment score (data not shown). In contrast, GO term analysis of SPFs-specific genes upregulated after CCCM stimulation revealed that terms such as "actin binding", "cytoskeletal binding protein", "contractile fiber", "LIM domain", "contractile fiber part", "sarcomere", and "myofibril" formed annotation cluster 1–3 (Figure 4C). Most of these genes were known to be related to cell contraction. Among the top 20 genes were many cytoskeletal or contractility associated genes. Surprisingly, ACTA2 that encodes α-SMA was upregulated specifically in SPFs after CCCM stimulation (Figure 4D). This result was confirmed by immunocytochemistry (Figure 4E–F). Morphometric analysis in immunocytochemical expression revealed that α-SMA expression was upregulated specifically and significantly in SPFs after CCCM stimulation in protein level (Figure S2, $P<0.05$). Variable upregulation and downregulation of genes after CCCM stimulation was a significant feature in SPFs. ACTA2 encoding α-SMA was included in this SPFs-specific upregulated gene set. Cancer associated fibroblasts (CAFs) include α-SMA-positive activated myofibroblasts. Together with the result of area-specific tissue microarrays, marked α-SMA expression in CMPI is depended on the sensitive character of SPFs which may associated with the difference in cancer microenvironment.

SPFs Enhance Tumor Growth, Metastasis and Tumor Formation Ability more Strongly than do SMFs

To elucidate the functional differences of fibroblasts in the colonic wall, we injected human colorectal cancer cell lines Caco-2





D

Upregulated gene clusters in SPFs compared with SMFs

Annotation Cluster 1	Enrichment Score: 9.31	
Gene Ontology ID	Gene Ontology Term	*P* value
GO:0031012	extracellular matrix	<.01
GO:0005578	proteinaceous extracellular matrix	<.01
SP_PIR_KEYWORDS	extracellular matrix	<.01
Annotation Cluster 2	Enrichment Score: 3.92	
Gene Ontology ID	Gene Ontology Term	*P* value
GO:0030247	polysaccharide binding	<.01
GO:0001871	pattern binding	<.01
GO:0005539	glycosaminoglycan binding	0.02
GO:0030246	carbohydrate binding	0.05
Annotation Cluster 3	Enrichment Score: 3.92	
Gene Ontology ID	Gene Ontology Term	*P* value
GO:0031093	platelet alpha granule lumen	<.01
GO:0060205	cytoplasmic membrane-bounded vesicle lumen	<.01
GO:0031983	vesicle lumen	<.01
GO:0031091	platelet alpha granule	<.01
GO:0030141	secretory granule	0.09
GO:0044433	cytoplasmic vesicle part	0.29

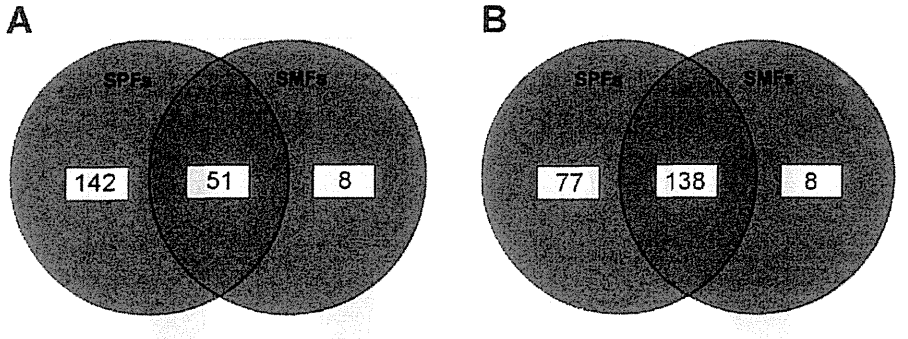
E

Upregulated gene clusters in SPFs with CCCM stimulation compared with SMFs with CCCM stimulation

Annotation Cluster 1	Enrichment Score: 10.12	
Gene Ontology ID	Gene Ontology Term	*P* value
SP_PIR_KEYWORDS	extracellular matrix	<.01
GO:0031012	extracellular matrix	<.01
GO:0005578	proteinaceous extracellular matrix	<.01
Annotation Cluster 2	Enrichment Score: 4.92	
Gene Ontology ID	Gene Ontology Term	*P* value
GO:0016477	cell migration	<.01
GO:0048870	cell motility	<.01
GO:0051674	localization of cell	<.01
Annotation Cluster 3	Enrichment Score: 4.66	
Gene Ontology ID	Gene Ontology Term	*P* value
GO:0001871	pattern binding	<.01
GO:0030247	polysaccharide binding	<.01
GO:0005539	glycosaminoglycan binding	<.01
GO:0030246	carbohydrate binding	0.11

Figure 3. Gene expression profiles in subperitoneal fibroblasts (SPFs) and submucosal fibroblasts (SMFs) with and without cancer-cell-conditioned medium (CCCM) stimulation. (A) Red is the microarray profile in SMFs with CCCM stimulation, blue is SMFs without CCCM stimulation, green is SPFs with CCCM stimulation, and silver is SPFs without CCCM stimulation. Three-dimensional representation of principal component analysis (PCA) component 1, 2, and 3. (B) Two dimensional representation of PCA components 1 and 2 (upper), and PCA components 1 and 3 (lower). Fibroblasts formed independent clusters, depending on histoanatomical site and the presence of CCCM stimulation. (C) Supervised cluster analysis in fibroblasts also revealed distinct clusters depending on histoanatomical site and the presence of CCCM stimulation. (D) Gene ontology analysis of upregulated genes in SPFs compared with SMFs. (E) Gene ontology analysis of genes upregulated in SPFs with CCCM stimulation, compared with SMFs with CCCM stimulation. Most of the genes with increased expressions in SPFs were retained after CCCM stimulation; however, there were some differences, and the order of annotation clusters were changed after CCCM stimulation.
doi:10.1371/journal.pone.0088018.g003

or DLD-1 s.c., alone, or along with either SPFs or SMFs, into SCID mice. At 7 weeks after the injection, all mice demonstrated tumor formation. The growth of tumors arising from cancer cells injected along with SPFs grew faster than that arising from the injection of cancer cells alone or co-injection with SMFs (Figure 5A). The final weights of tumors arising from cancer cells co-injected with SPFs were also larger than those arising from the injection of cancer cells alone or from co-injection with SMFs



C SPFs specific upregulated gene cluster after CCCM stimulation

Annotation Cluster 1	Enrichment Score: 4.73	
Gene Ontology ID	Gene Ontology Term	*P* value
GO:0003779	actin binding	< .01
SP_PIR_KEYWORDS	actin-binding	< .01
GO:0008092	cytoskeletal protein binding	0.01
Annotation Cluster 2	Enrichment Score: 3.97	
Gene Ontology ID	Gene Ontology Term	*P* value
SP_PIR_KEYWORDS	LIM domain	< .01
INTERPRO	IPR001781:Zinc finger, LIM-type	< .01
SMART	SM00132:LIM	< .01
Annotation Cluster 3	Enrichment Score: 3.84	
Gene Ontology ID	Gene Ontology Term	*P* value
GO:0044449	contractile fiber part	< .01
GO:0043292	contractile fiber	< .01
GO:0030017	sarcomere	< .01
GO:0030016	myofibril	< .01

D Top 20 in SPFs specifically upregulated genes after CCCM stimulation

Probe Set ID	*P* value	FC	Gene Symbol
206029_at	< .01	13.41	ANKRD1
206825_at	< .01	10.84	OXTR
205795_at	< .01	8.67	NRXN3
210511_s_at	< .01	8.09	INHBA
204671_s_at	< .01	7.66	ANKRD6
212473_s_at	< .01	7.57	MICAL2
202628_s_at	< .01	6.96	SERPINE1
229669_at	< .01	6.75	LOC100507263
201195_s_at	< .01	6.61	SLC7A5
200974_at	< .01	6.52	ACTA2
205518_s_at	< .01	6.49	CMAH
217452_s_at	< .01	6.32	B3GALT2
205442_at	< .01	6.21	MFAP3L
1558828_s_at	< .01	6.2	LOC728264
223063_at	< .01	5.84	C1orf198
204589_at	< .01	5.59	NUAK1
226869_at	< .01	5.44	MEGF6
213067_at	< .01	5.43	MYH10
203432_at	< .01	5.14	TMPO
206117_at	< .01	5.09	TPM1

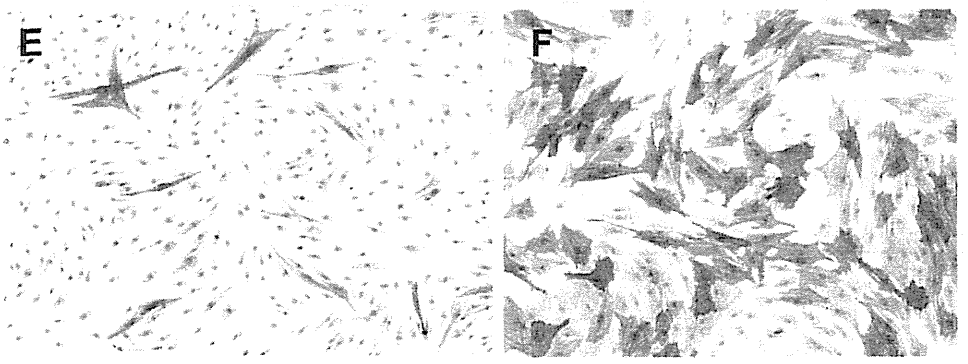
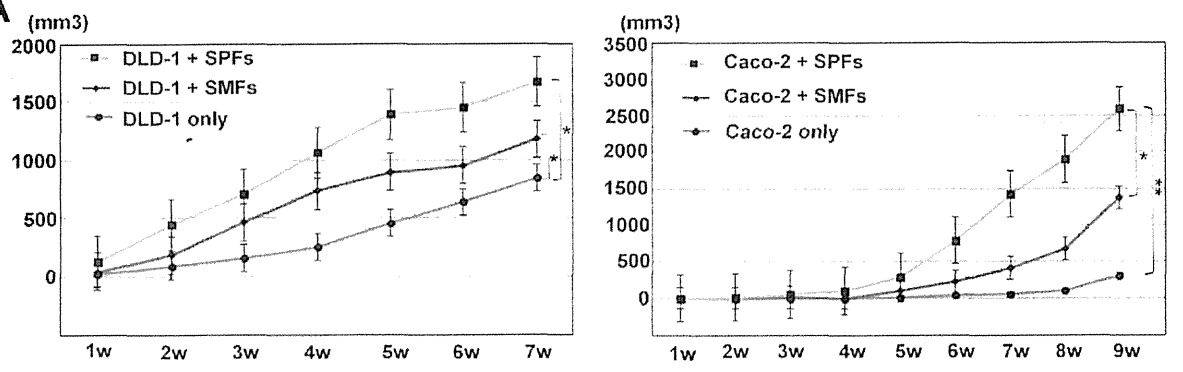
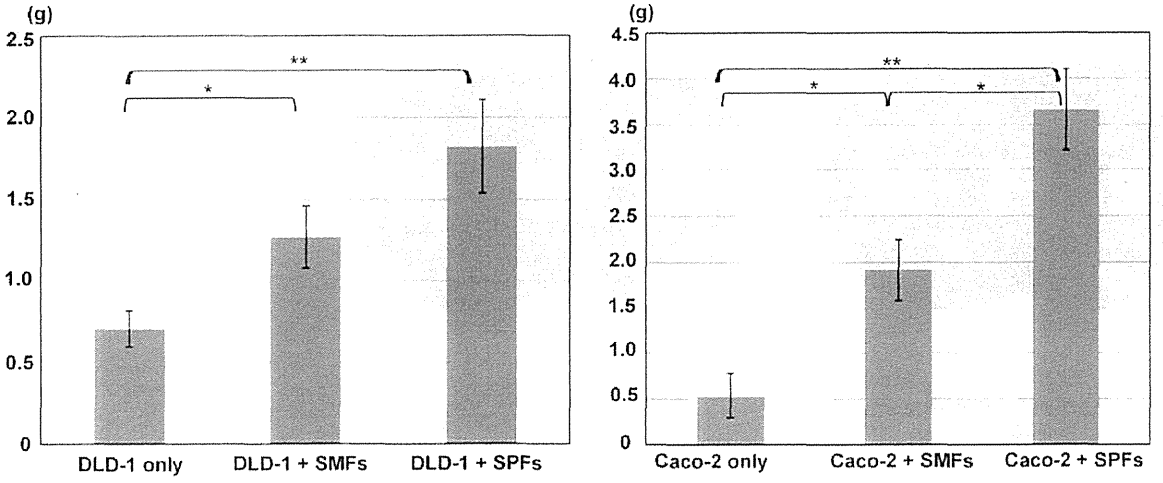


Figure 4. Gene modification in subperitoneal fibroblasts (SPFs) after cancer-cell-conditioned medium (CCCM) stimulation. (A) Genes upregulated by CCCM stimulation. (B) Genes downregulated by CCCM stimulation. (C) Top 3 annotation clusters in gene ontology analysis of SPFs-specific genes upregulated by CCCM stimulation. (D) Top 20 genes upregulated specifically in SPFs after CCCM stimulation. (E) Immunocytochemical α-SMA expression in SMFs after CCCM stimulation. (F) Immunocytochemical α-SMA expression in SPFs after CCCM stimulation. α-SMA expression was upregulated specifically in SPFs after CCCM stimulation (see also Figure S2).
doi:10.1371/journal.pone.0088018.g004

A



B



C

	Liver metastasis	Lung metastasis	Lymph node
Caco-2 + SPFs (10 weeks)	0/8	0/8	0/8
Caco-2 + SMFs (10 weeks)	0/8	0/8	0/8
DLD-1 + SPFs (8 weeks)	0/8	0/8	**4/8**
DLD-1 + SMFs (8 weeks)	0/8	0/8	**2/8**

D

Cells inoculated	Number of cancer cells	Tumor formation rate
Caco-2 only	1×10^6	0/4
Caco-2 + SPFs (1×10^6)	1×10^6	4/4
Caco-2 + SMFs (1×10^6)	1×10^6	4/4
Caco-2 only	5×10^5	0/4
Caco-2 + SPFs (1×10^6)	5×10^5	4/4
Caco-2 + SMFs (1×10^6)	5×10^5	4/4
Caco-2 only	1×10^5	0/4
Caco-2 + SPFs (1×10^6)	1×10^5	4/4
Caco-2 + SMFs (1×10^6)	1×10^5	3/4
Caco-2 only	5×10^4	0/4
Caco-2 + SPFs (1×10^6)	5×10^4	2/4
Caco-2 + SMFs (1×10^6)	5×10^4	0/4

Cells inoculated	Number of cancer cells	Tumor formation rate
DLD-1 only	1×10^6	3/4
DLD-1 + SPFs (1×10^6)	1×10^6	4/4
DLD-1 + SMFs (1×10^6)	1×10^6	4/4
DLD-1 only	5×10^5	1/4
DLD-1 + SPFs (1×10^6)	5×10^5	4/4
DLD-1 + SMFs (1×10^6)	5×10^5	4/4
DLD-1 only	1×10^5	0/4
DLD-1 + SPFs (1×10^6)	1×10^5	4/4
DLD-1 + SMFs (1×10^6)	1×10^5	1/4
DLD-1 only	5×10^4	0/4
DLD-1 + SPFs (1×10^6)	5×10^4	3/4
DLD-1 + SMFs (1×10^6)	5×10^4	1/4

Figure 5. Subperitoneal fibroblasts (SPFs) actively contribute to cancer progression. (A, left) Xenograft tumor growth in mice injected with DLD-1 human colorectal cancer cells alone (blue line, 840.7±112.6 mm^3 in 7 weeks), co-injected with DLD-1 cells and submucosal fibroblasts (SMFs; red line, 1178.0±177.6 mm^3 in 7 weeks), and co-injected with DLD-1 cells and subperitoneal fibroblasts (SPFs; green line, 1672.8±214.7 mm^3 in 7 weeks). The differences of tumor volume between DLD-1 cells alone and DLD-1 cells with SPFs, and between DLD-1 cells alone and DLD-1 cells with SMFs were statistically significant ($P<0.05$). (A, right) Xenograft tumor growth in mice injected with Caco-2 human colorectal cancer cells alone (blue line, 308.6±127.7 mm^3 in 9 weeks), co-injected with Caco-2 cells and SMFs (red line, 1363.1±284.3 mm^3 in 9 weeks), and co-injected with Caco-2 and SPFs (green line, 2595.1±349.5 mm^3 in 9 weeks). The differences of tumor volume between Caco-2 cells alone and Caco-2 cells with SPFs ($P<$

0.01), and between Caco-2 cells with SMFs and Caco-2 cells with SPFs ($P<0.05$) were statistically significant. Xenograft tumors derived from co-injection of cancer cells and SPFs grew faster than those derived from injection of cancer cells alone, or co-injection of cancer cells and SMFs. (B, left) Xenograft tumor weight in mice injected with DLD-1 cells alone was 0.71±0.11 g, co-injected with DLD-1 cells and SMFs was 1.27±0.19 g, and co-injected with DLD-1 cells and SPFs was 1.82±0.28 g in 8 weeks. The differences of tumor weight between DLD-1 cells alone and DLD-1 cells with SPFs ($P<0.01$), and between DLD-1 cells alone and DLD-1 cells with SMFs ($P<0.05$) were statistically significant. (B, right) Xenograft tumor weight in mice injected with Caco-2 cells alone was 0.53±0.24 g, co-injected with Caco-2 cells and SMFs was 1.91±0.34 g, and co-injected with Caco-2 cells and SPFs was 3.66±0.45 g in 10 weeks. The differences of tumor weight between DLD-1 cells alone and DLD-1 cells with SPFs ($P<0.01$), between DLD-1 cells alone and DLD-1 cells with SMFs ($P<0.05$), and between DLD-1 cells with SPFs and DLD-1 cells with SMFs ($P<0.05$) were statistically significant. Weights of xenograft tumors derived from co-injection of cancer cells with SPFs were higher than those of tumors derived from injection of cancer cells alone, or co-injection of cancer cells and SMFs (left: DLD-1, right: Caco-2). (C) Although the value did not reach statistical significance, xenograft tumors derived from co-injection of DLD-1 cells and SPFs showed twice the frequency of lymph node metastasis (n = 4) compared to those deriving from co-injection of DLD-1 cells and SMFs (n = 2). (D) Co-injection of cancer cells and SPFs result in enhanced tumor formation capacity. Results are presented as the mean ± SE of 8 mice (*$P<0.05$. **$P<0.01$).
doi:10.1371/journal.pone.0088018.g005

(Figure 5B). Although the difference was not statistically significant, tumors arising from the co-injection of DLD-1 cells with SPFs more frequently resulted in lymph node metastasis than did those formed from the co-injection of DLD-1 cells with SMFs (Figure 5C).

Next, the cancer cells being injected were serially diluted and tumor formation was evaluated at 4 weeks after the injection, as described previously [13]. Comparison with mice injected with cancer cells alone or co-injected with cancer cells and SMFs, enhanced tumor formation was found in mice co-injected with cancer cells and SPFs, and tumor formation was observed even when the injected cells were diluted to 5×10^4 (Caco-2) or 1×10^4 (DLD-1). These results suggested that SPFs enhanced tumor growth, metastasis, and tumor formation capacity, in comparison with the effect of SMFs; these findings may be related to the peritoneal invasion dependent clinical outcome in colon cancer.

Discussion

Fibroblasts are one of the most common types of stromal cells in connective tissue. Fibroblasts and loose connective tissue, which is one morphological type of connective tissue, are present throughout the body and contribute to the maintenance of the structural framework of most tissues, including the gastrointestinal tract [18]. Histologically, the gastrointestinal tract is composed of 5 layers that consist of the lamina propria, submucosa, muscular layer, subserosa, and serosa. Loose connective tissue and fibroblasts exist in every layer and have distinct physiological and pathological functions [19,20].

SPFs are known to produce peritoneal fluid and facilitate appropriate functioning of intra-abdominal organs [21]. Previous reports have shown that the marked contractile ability in SPFs was implicated in the colonic strictures in Crohn's disease [22,23]. In the field of peritoneal dialysis, SPFs have been reported to produce growth factors, cytokines, or chemokines in response to TGF-β stimulation and have been implicated in the failure of peritoneal dialysis [24]. However, the implication of SPFs in tumor progression is not known, and our study is the first to report the contribution of SPFs in tumor progression and metastasis that is dependent on peritoneal invasion in colon cancer.

Our findings seem to support the concept of microenvironmental regulation of cancer. The tumor microenvironment consists of distinct cell types, including fibroblasts, blood cells, vascular-originated cells, and more. They synergistically create a distinct microenvironment according to tumor progression, such as the core primary tumor microenvironment, the invasive tumor microenvironment, or the metastatic tumor microenvironment [9]. Area-specific tissue microarrays were very useful to expand this concept into the pathological phenomenon and biological study was then performed based on these results. Interestingly, the fibroblasts we obtained from the submucosal and subperitoneal tissues showed biological differences dependent on the histoanatomical site. In addition, our finding of marked phenotypical modification in SPFs suggests that fibroblasts from different histoanatomical sites show different reactions to cancer stimuli. We used Caco-2 with low tumorigenic and metastatic potential and DLD-1 with a higher tumorigenic and metastatic potential in this study. DLD-1 has been known to preferentially cause lymph node metastasis rather than lung or liver metastasis, and our data is in accordance with previous reports [25–28]. Together with the xenograft tumor results in which SPFs enhanced tumor growth and tumor formation in Caco-2 and DLD-1, and promote lymph node metastasis in DLD-1, we have clarified that this fibroblastic difference is implicated in colon cancer progression that is dependent on peritoneal invasion.

In general, fibroblasts within the tumor stroma, so-called CAFs, acquired a modified phenotype. CAFs are enriched in α-SMA positive active myofibroblasts and are known to play an active role in tumor progression [29,30]. Residual fibroblasts are one of the sources of CAFs, and residual fibroblasts exposed to cancer stimulation show phenotypical modification. Although the tumor-promoting ability of CAFs has been reported to be diverse and dependent on cancer origin, intra-tumoral diversity has not been clear [31]. Our data suggests the physiological diversity of fibroblasts within one organ produces the intra-tumoral diversity of CAFs. Therefore, gene profiles in fibroblasts with and without cancer CCCM stimulation may provide new insights into their diversity in colon cancer.

We are speculating that a fibroblast subpopulation with tumor-promoting capacity can be enriched in the subperitoneal layer. Their original phenotype may include a previously reported CAFs marker, and variable gene modification in response to cancer stimuli could be a characteristic feature of tumor-promoting fibroblasts. Recently, activated proteins in CAFs have been considered to be a target of therapy [32]. However, not all kinds of CAFs may promote tumor progression [31]. Our gene expression profile data in SPFs with and without CCCM stimulation may also be useful for future stromal-target therapy. SPFs with robust tumor promotion ability showed higher gene expression associated with an ECM component, and marked gene upregulation associated with cell contraction, including α-SMA, after CCCM stimulation. Recently both stromal-cell contractile ability and ECM stiffness have been reported to influence epithelial cell migration and invasion. Also, α-SMA is one of the representative markers of CAFs and myofibroblasts, and its expression is associated with biological contractile ability. Furthermore, α-SMA expression in tumor stroma was reported to be a prognostic factor in colorectal cancer. Therefore, our result suggests the importance of mechanotransduction theory in the study of the tumor microenvironment [33–35].

From the first categorization efforts reported by Lockhart-Mummery, primary colon cancer has been consistently stratified based on the extent of its spread into the bowel wall [36]. More recent pathological investigations have revealed that peritoneal invasion is a prognostic factor, and is a candidate for discriminating high-risk stage II colon cancer, and those patients who may receive benefit from post-operative therapy [3,37,38]. We and others reported the utility of elastica stain for the objective diagnosis of peritoneal invasion. We have proven that objective identification of peritoneal invasion is also useful for investigating biological phenomena specifically occurring in this tumor microenvironment [39]. Recently, Liang et al. proposed that pT3 tumors with ELI should be subdivided into further categories like pT3b [6]. Our findings support the subdivision of cases with ELI from those without ELI, and the diversity of the fibroblasts could be one factor associated with frequent metastases in cases with ELI.

In conclusion, fibrosis with α-SMA expression is a significant feature of the cancer microenvironment formed by peritoneal invasion in human colon cancer. The biological features and functions of fibroblasts in the subperitoneal tissue are different from those in submucosal tissue, and their phenotypical modification by cancer stimuli and contribution to tumor growth and metastasis are also different. Specifically, SPFs from the subperitoneal tissue showed characteristic biological features of a marked ECM component and contractile-associated gene expression, and functions that accelerate tumor formation and metastasis. Considering these comprehensive pathological and biological data, we propose that CMPI is a special microenvironment that promotes tumor growth and metastasis. In CMPI, SPFs and cancer cells interaction play an active and crucial role in tumor progression.

Supporting Information

Figure S1 Biological characteristics of subperitoneal fibroblasts and submucosal fibroblasts. (A) Immunocytochemical staining for vimentin in SPFs. (B) Immunocytochemical staining for vimentin in SMFs. (C) Flow cytometric analysis of SPFs and SMFs. (D) Immunocytochemical staining of SPFs and SMFs. Protein expression results were positive for vimentin and CD105, and negative for an epithelial marker (AE1/3), a neural marker (S-100), mesothelial markers (calretinin, CK8), endothelial markers (CD31, 34), and lymphocyte and monocyte markers (CD3, 14, 20, 45, 68), suggesting that the obtained cells were fibroblasts. We found weak α-SMA expression in a few SPFs and SMFs. (E) Growth curve of SPFs (blue) and SMFs (red). SPFs showed significantly longer doubling time than did SMFs ($P<0.01$). Results are presented as the mean ± SE of triplicate measurements ($^{**}P<0.01$).
(TIF)

Figure S2 Morphometric analysis of immunocytochemical α-SMA expression. α-SMA expression was upregulated specifically and significantly in SPFs after cancer-cell-conditioned medium (CCCM) stimulation. Results are presented as the mean ± SE of 3 experiments ($^{*}P<0.05$).
(TIF)

Table S1 Patient Characteristics Entered into Area-Specific Tissue Microarray.
(DOCX)

Table S2 Primary antibodies used in this study.
(DOCX)

Table S3 Upregulated gene clusters and composing genes in SPFs compared with SMFs.
(DOCX)

Table S4 Top 20 genes in SPFs compared with SMFs.
(DOCX)

Table S5 Upregulated gene clusters in SPFs with cancer-cell-conditioned medium (CCCM) stimulation compared with SMFs with CCCM stimulation.
(DOCX)

Table S6 Top 20 upregulated genes in SPFs with cancer cell-conditioned medium (CCCM) stimulation compared with SMFs with CCCM stimulation.
(DOCX)

Materials and Methods S1 Supplementary Materials and Methods.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MK YH GI AO. Performed the experiments: MK YH AO. Analyzed the data: MK YH KA HS. Contributed reagents/materials/analysis tools: MY NS. Wrote the paper: MK YH AO.

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Simona Gurzu, PhD
Orsolya Serester, PhD
Ioan Jung, PhD
Department of Pathology
University of Medicine and Pharmacy
Targu-Mures Romania

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Practical Utility and Objectivity: Does Evaluation of Peritoneal Elastic Laminal Invasion in Colorectal Cancer Overcome These Contrary Problems?

To the Editor:

The presence of peritoneal invasion separates pT3 and pT4a disease, and pT4a is known to be associated with a poor prognosis among patients with colorectal cancer (CRC).[1] In Japan, pathologists belonging to the Japanese Society for Cancer of the Colon and Rectum have worked together to review the current practice of pathologic assessments in different medical institutions.[2] In this review, in addition to the current status of many pathologic factors, tumor spread was also reviewed, and the distributions of pT1 to pT4 cases in 8 institutions were compared (Fig. 1). As a result, substantial differences in the frequency of pT3 and pT4 cases were found among the 8 institutions. Therefore, the previously indicated variability suggested by a review of previous reports on peritoneal invasion was confirmed for Japanese institutions.[3] As shown in a recently published, meticulous study by Liang et al,[1] elastic laminal invasion (ELI) in CRC can be used for the objective diagnosis of peritoneal invasion. These authors investigated ELI in 244 consecutively resected cases of pT3N0M0 CRC and found a strong association with the prognosis. An outstanding point in their study was the use of a single section to determine ELI, and they elucidated the availability of ELI in routine practice. Such results must be based on the proper selection of the section used for ELI evaluation. Liang and colleagues selected sections in which the tumor was closest to the peritoneal surface. Regarding this point, we would like to request a more precise description, which could be useful for concordant selections at multiple pathologic laboratories. Next, as they proposed, we agree with the use of ELI in discriminating pT3 cancers, on the basis of the results associated with the clinical outcome.[4,5] However, we still feel that some problems need to be answered. The most important problem is that immunohistochemistry or histochemistry may not always contribute to an improvement in concordance, as was confirmed in the D2-40 or elastic staining for the diagnosis of vascular invasion.[2] This problem must be assessed and solved in the future. Practical utility and objectivity must be considered together in future diagnostic pathology.

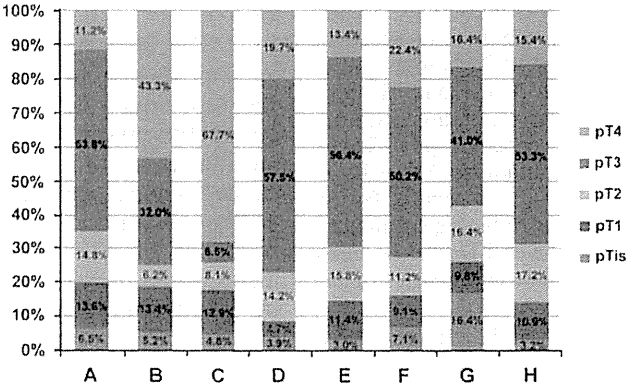

FIGURE 1. Distribution of pT1 to pT4 cases at 8 institutions in Japan. *X*-axis shows the institution, whereas the *Y*-axis shows the frequency of pT1 to pT4 cases. Substantial differences in the frequencies of pT3 and pT4 cases were observed among the 8 institutions.

Motohiro Kojima, MD, PhD*
Hideyuki Shimazaki, MD, PhD†
Keiichi Iwaya, MD, PhD†
Masayoshi Kage, MD, PhD‡
Jun Akiba, MD, PhD§
Yasuo Ohkura, MD, PhD‖
Shinichiro Horiguchi, MD, PhD¶
Kohei Shomori, MD, PhD#
Ryoji Kushima, MD, PhD**
Yoichi Ajioka, MD, PhD††
Shogo Nomura, MD, PhD‡‡
Atsushi Ochiai, MD, PhD*
*Pathology Division, Research Center for Innovative Oncology
‡‡Department of Biostatistics, National Cancer Center Hospital East, Chiba
†Department of Pathology, National Defense Medical College, Saitama
‡Department of Diagnostic Pathology, Kurume University Hospital
§Department of Pathology Kurume University School of Medicine, Kurume, Fukuoka

‖Department of Pathology, Kyorin University Graduate School of Medicine
¶Department of Pathology, Tokyo Metropolitan Komagome Hospital
**Pathology Division, National Cancer Center Hospital, Tokyo
#Department of Pathology, Sanin-Rosai Hospital, Tottori
††Department of Pathology, Graduate School of Medicine and Dental Sciences, Niigata University, Niigata

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Re: Practical Utility and Objectivity

Does Evaluation of Peritoneal Elastic Laminal Invasion in Colorectal Cancer Overcome These Contrary Problems?

In Reply:

The letter by Kojima and colleagues reveals marked variation in the reporting rate of pT3 and pT4 colorectal cancers at 8 Japanese institutions. Although factors such as cancer center specialization might partly explain the markedly increased incidence of pT4 disease at certain institutions, some of this variation is likely attributable to regional differences in pathologist interpretation of serosal surface involvement. The latter possibility highlights the need for more objective techniques to confirm or exclude serosal involvement by colorectal carcinoma. Assessment for peritoneal elastic lamina invasion, studied earlier by Kojima and colleagues and more recently in our study using a single section of tumor tissue is an approach that may help address this issue.[1,2] As Kojima and colleagues point out in their letter, with our method of applying an elastic stain to a single tumor section, the choice of the section is important. In response to their specific question requesting a more precise description of how to choose this section, we would offer the following. First, our selection criteria were defined in very simple terms—the section in which the tumor appears to be closest to the peritoneal surface, following review of all sections of the tumor. Admittedly, there is a degree of subjectivity in this assessment, and in some cases it is unclear whether the surface of the adipose tissue is serosal or not. However, from our experience, the choice of section closest to the surface is obvious in many cases. Pathologists should be aware that the closest serosal surface to the tumor may be an area of serosal retraction or invagination. Occasionally, there is fibrosis in the adipose tissue between the tumor and the closest peritoneal surface. Importantly, the section of tumor closest to the peritoneal surface is not necessarily the section with the greatest depth of invasion beyond the muscularis propria. Often the choice of section will come down to 2 sections for direct comparison, and, although we did not physically measure the distance to the surface of the adipose tissue, that is a practical option which some pathologists might consider. It is important to be aware that, with the single-section approach, the elastic lamina will not be identified in the majority of cases (59% in our study). However, failure to identify the elastic lamina on the most suspicious section does not seem to matter, as the cases with no elastic lamina identified have a similar prognosis to those cases in which the elastic lamina is identified but elastic lamina invasion is not present. Elastic lamina invasion appears promising as a more objective measure of peritoneal involvement. However, we fully agree with Kojima and colleagues that further study is needed to determine whether use of this technique can actually improve interobserver agreement in reporting of serosal involvement.

Wen-Yih Liang, MD*†‡
Thomas Arnason, MD, FRCPC‡
Gregory Y. Lauwers, MD‡
*Department of Pathology and Laboratory Medicine, Veterans General Hospital-Taipei, Taipei
†Department of Pathology, National Yang-Ming University School of Medicine, Taiwan, Republic of China
‡Department of Pathology Gastrointestinal Pathology Service, Massachusetts General Hospital and Harvard Medical School, Boston, MA

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特集　膵がん治療の新たな展開

膵がんに対する術後補助療法のエビデンスと今後の展望*

佐々木満仁**
上野秀樹**
林　秀幸**
柴　知史**
森実千種**
近藤俊輔**
奥坂拓志**

Key Words：pancreatic cancer, adjuvant therapy, chemotherapy, chemoradiotherapy, gemcitabine

はじめに

膵がんにおいて，根治を期待できる唯一の治療が切除術であるが，早期発見が困難ながん腫なため，診断時に約80％が切除不能進行例である．また切除例においても高率に再発をきたすため，術後の5年生存割合は10から20％程度[1)~3)]にとどまる．そのため，治療成績向上をめざし，術後補助療法として化学療法，化学放射線療法などの集学的な治療が試みられ，術後補助療法による生存割合の向上が報告されている．

本稿では，術後補助療法について今までに報告された研究，現在進行中の研究を中心にレビューし，今後の展望を考察する．

背　景

膵がんは近年増加傾向にあり，本邦の人口動態統計では，2011年に約2万9千人が膵がんで死亡している(男性5位，女性4位)[4)]．

膵腫瘍は組織学的には，外分泌腫瘍と内分泌腫瘍とに分けられ，さらに外分泌腫瘍は嚢胞腫瘍，膵管内腫瘍，異型性過形成および上皮内がん，浸潤性膵管がん，腺房細胞腫瘍に細分される．膵がんの約90％は浸潤性膵管がんであり，その中でも最も頻度が高い組織型は管状腺がんである．

膵がん患者によくみられる自覚症状には腹痛や背部痛，食欲不振，体重減少，黄疸などがあるものの，膵がんに特異的な症状ではないため，画像診断の進歩がみられた現在でも早期診断が困難である．診断の時点で切除不能な進行がんの状態が多いことや，切除可能な状態で発見され切除術を受けても80％前後が再発をきたすことから，膵がんの予後は不良である．

Stage別の予後としては，Stage I～IIでは生存期間中央値：16.4～36か月，5年生存割合：16.6～45.2％，stage IIIでは生存期間中央値：12.5か月，5年生存割合：7.3％，stage IVでは生存期間中央値：7.8か月，5年生存割合：3.5％と報告されている[5)6)]．

術後補助療法

前述したように，膵がんでは切除術を受けた場合でも高率に再発し，術後の5年生存割合は10から20％程度と不良である．そのため，治療成績向上をめざし，補助療法として術後に放射線療法や化学療法などの集学的治療が試みられてきた．

* Current status and future prospects of adjuvant therapy for resected pancreatic cancer.

** Mitsuhito SASAKI, M.D., Hideki UENO, M.D., Hideyuki HAYASHI, M.D., Tomofumi SHIBA, M.D., Chigusa MORIZANE, M.D., Shunsuke KONDO, M.D. & Takuji OKUSAKA, M.D.: 独立行政法人国立がん研究センター中央病院肝胆膵内科〔〒104-0045 東京都中央区築地5-1-1〕; Hepatobiliary and Pancreatic Oncology Division, National Cancer Center Hospital, Tokyo 104-0045, JAPAN

表1　膵がん術後補助化学放射線療法に関する第III相試験

試験名	報告年	術後補助療法	患者数	無再発生存期間中央値(月)	全生存期間中央値(月)	2年生存割合(%)	P値(log-rank)	備考
GITSG9173[7]	1985	5-FU併用放射線療法	21	11	20	43	0.035	
		経過観察のみ	22	9	11	18		
EORTC40891[8]	1999	5-FU併用放射線療法	104(60)	17.4	24.5(17.1)	51(37)	0.208	乳頭部領域がんを含む
		経過観察のみ	103(54)	16	19.0(12.6)	41(23)	(0.099)	( )は膵頭部がんのみ
ESPAC-1[1]	2004	5-FU併用放射線療法あり	145	10.7	15.9	29	0.053	
		5-FU併用放射線療法なし	144	15.2	17.9	41		

1．術後補助化学放射線療法

1985年に米国のGastrointestinal Tumor Study Group(GITSG)から，膵がん術後に5-FU併用放射線療法を受けた患者群のほうが経過観察のみの群よりも有意に生存期間が優れていたことを示す小規模な第III相試験が報告[7]された．以降，現在にいたるまで，米国では術後補助化学放射線療法が標準的な補助療法として認識され，実臨床で実施されている．しかし，その後欧州で行われた2つの第III相試験では，いずれも術後補助化学放射線療法の延命効果を証明することはできなかったため，国際的なコンセンサスは得られていない．以下に主な術後補助化学放射線療法(表1)を概説する．

GITSG9173試験は，術後補助療法の有効性を最初に報告したランダム化比較試験である．1974年から1982年までかけて49例を，術後5-FU併用放射線療法(5-FU＋RT)と手術単独群に分けて比較したもので，中間解析で生存期間に有意差を認めたため有効中止されている．この試験では5-FU＋RT後に2年間の維持化学療法(5-FU)を継続するプロトコールであったため，予後向上が術後補助化学放射線療法によるものか維持化学療法によるものか疑問が残る試験であったこと，また症例集積に長時間を要し，予定症例の半分以下の症例登録で中止されているなど試験自体に質の問題があったことから，解釈が分かれる試験であった．

これに対し，欧州においてEuropean Organization for Research and Treatment of Cancer(EORTC)1987年から1995年にかけて218例を対象に，維持化学療法を行わず5-FU＋RTによる術後補助化学放射線療法群と手術単独群を比較するEORTC40891試験が実施された[8]．結果としては，両群に生存期間の有意差は認められず(生存期間中央値：17.1か月 vs. 12.6か月，2年生存割合：37% vs. 23%，$P=0.099$)，術後補助化学放射線療法の意義は証明されなかった．一方，この試験では対象に乳頭部領域がんを含んでおり，膵がんのサブグループでは生存曲線が術後補助化学放射線療法群のほうが良好に推移していたことから，試験としてunderpoweredであったという意見もある．

さらに，欧州にて1994年から2000年までに289例を対象に，術後補助療法として化学療法，または化学放射線療法の有効性を検討するEuropean Study Group for Pancreatic Cancer-1(ESPAC-1)試験が実施された[1]．この試験では，対象患者を手術単独群，5-FUを用いた術後補助化学放射線療法群，5-FU＋ロイコボリン(LV)による術後補助化学療法群，術後補助化学放射線療法→化学療法群の4群に割り付け，2×2 factorial designによって，それぞれ化学放射線療法と化学療法の有無による予後の違いが検討された．その結果，化学放射線療法を含む治療を受けた群の生存期間は同治療を受けなかった群に対して劣っていた(生存期間中央値：15.9か月 vs. 17.9か月，$P=0.053$)が，化学療法を含む治療を受けた群は同治療を受けなかった群よりも有意に良好(生存期間中央値：20.1か月 vs. 15.5か月，$P=0.009$)であった．

これらの結果から，その後の術後補助療法の

表2　膵がん術後補助化学療法に関する第III相試験

試験名	報告年	術後補助療法	患者数	無再発生存期間中央値(月)	全生存期間中央値(月)	2年生存割合(%)	P値(log-rank)	備考
Bakkevoldら[9]	1993	ADR+MMC+5-FU	31	NA	23	43	0.02	
		経過観察のみ	30	NA	11	32		
Takadaら[10]	2002	5-FU+MMC	81	NA	12.8	24.2	NS	
		経過観察のみ	77	NA	12.4	29.6		
ESPAC-1[1]	2004	5-FU+LVあり	147	15.3	20.1	40	0.009	
		5-FU+LVなし	142	9.4	15.5	30		
JSAP-01[11]	2006	5-FU+CDDP	45	8.6	12.5	NA	0.94	
		経過観察のみ	44	10.2	15.8	NA		
CONKO-001[2][12]	2007	GEM	179	13.4	22.8	48.5	0.045	
		経過観察のみ	175	6.9	20.2	40.0		
JSAP-02[3]	2009	GEM	58	11.4	22.3	48.3	0.19	
		経過観察のみ	60	5.0	18.4	40.0		
RTOG9704[13]	2008	5-FU併用放射線療法+GEM	221 (187)	NA (11.4)	18.8 (20.5)	NA	0.15	(　)膵頭部がんのみ
		5-FU併用放射線療法+5-FU	230 (201)	NA (5.0)	16.9 (16.9)	NA	(0.09)	
ESPAC-3[14]	2010	GEM	537	14.3	23.6	49.1	0.39	
		5-FU+LV	551	14.1	23.0	48.1		
JASPAC-1[15][16]	2013	S-1	187	23.2	NA	70	<0.0001	
		GEM	191	11.2	25.9	53		

NA：not available

開発は，米国では化学放射線療法を，欧州では化学療法をベースに進められていくこととなった．

2．術後補助化学療法

術後補助化学療法に関しては，欧州や本邦で研究が進められており，近年いくつかの第III相試験が報告された(表2)．それらの中で，5-FUを用いた術後補助化学療法に関しては，症例数の少ない第III相試験では明らかな延命効果は証明されなかったものの[9)~11)]，欧州で行われたESPAC-1(前述)では5-FU+LVによる術後補助化学療法を受けた群は受けなかった群よりも生存期間が有意に長かったことが示された．ESPAC-1は膵がん補助療法の第III相試験としては大規模であり，試験デザインの複雑さなどの批判はあるものの，切除患者に対する補助化学療法の延命効果を初めて示した試験として評価されている．

一方，進行膵がんでは標準薬となっているゲムシタビンは，術後補助療法としての有用性を示す第III相試験が2000年後半に相次いで報告された．まず2007年にドイツから，Charite Onkologie-001(CONKO-001)試験が報告[2)]された．この試験では，1998年から2004年に膵がん切除患者368例が登録され，術後ゲムシタビンの投与は経過観察のみよりも無再発生存期間を有意に延長(無病生存期間中央値：13.4か月 vs. 6.9か月，$P<0.001$)することが確認された．2007年時点で生存期間に関しては有意差が証明されなかった(生存期間中央値：22.1か月 vs. 20.2か月，5年生存率：22.5% vs. 11.5%，$P=0.06$)が，ゲムシタビン群で優れた傾向にあったことが示された．その後2008年に長期フォローアップ後の解析結果が報告され，生存期間に関してもゲムシタビンが経過観察よりも有意に優れていたことが示された(生存期間中央値：22.8か月 vs. 20.2か月，5年生存割合：21.0% vs. 9.0%，$P=0.045$)[12)]．

また，本邦でJapanese Study Group of Adjuvant Therapy for Pancreatic Cancer-02(JSAP-02)試験により，術後補助療法におけるゲムシタビンの有効性が検討され，2009年にその結果が報告された[3)]．2002年から2005年までに119例が登録され，CONKO-001試験と同様にゲムシタビン群の無病生存期間は手術単独群よりも有意に良好で