Expression of cancer-associated fibroblast markers in advanced colorectal cancer

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Received May 18, 2017; Accepted December 11, 2017

DOI: 10.3892/ol.2018.8097

Abstract. Colorectal cancer is one of the most common causes of mortality from cancer worldwide. Previous studies have demonstrated that cancer-associated fibroblasts (CAFs) promote neoangiogenesis and tumor growth for various tumors. The present study analyzed CAF markers, including α-smooth muscle actin (α-SMA), collagen I, platelet-derived growth factor receptor-β (PDGFR-β), and D2-40 (antibody recognizing podoplanin), and vessel markers, including cluster of differentiation (CD)31 and CD34, for 121 advanced colorectal cancer cases using a digital image analyzing technique. The association between CAF markers and vessel markers with clinicopathological factors was investigated. Furthermore, the association between CAF markers with each other, and their association with vessel markers was analyzed. Mean/median expression area of stromal and vessel markers in tumors were collagen I, 26.787%; D2-40, 1.372%; PDGFR-β, 11.646%; α-SMA-positive and desmin-negative myofibroblasts (α-SMA subtraction), 15.372%; CD31, 3.635%; and CD34, 2.226%. The expression area of α-SMA subtraction was significantly correlated with collagen I (P<0.001, correlation rho=0.509). High levels of α-SMA subtraction (P=0.002), collagen I (P=0.040), and PDGFR-β (P=0.040) expressions tended to be associated with high venous invasion. D2-40 did not correlate with other CAF and vessel markers. These results indicated that individual CAFs may have different expression patterns, and different strength effects for venous invasion in advanced colorectal cancer stroma.

Introduction

Cancer tissues are composed of cancer cells and the surrounding stroma including fibroblasts, vascular endothelial cells, and extracellular matrix. Recent studies have focused on the cancer-associated fibroblasts (CAFs), a major cellular component of the cancer stroma, and have demonstrated that CAFs promote neoplastic angiogenesis and tumor growth in various tumors (1-6).

Collagen I, D2-40 (antibody recognizing podoplanin), Platelet-derived growth factor receptor-β (PDGFR-β), and α-smooth muscle actin (α-SMA) have been known as molecular/histopathological markers of CAFs (7). Podoplanin (D2-40) expression of CAFs from various cancers have been studied (8). The majority of recently reports identified podoplanin (D2-40) expression of CAFs as an unfavorable marker of prognosis, such as lung cancer (9), breast cancer (10), and esophageal adenocarcinoma (11), while podoplanin expression of CAFs was shown as a favorable prognosis indicator of colorectal cancer (12,13). Some previous studies have reported one of the above CAF markers, but there are no papers for analyzing the relationships among these CAFs using image technique in colorectal cancer. The relationships of CAF markers, such as collagen I, D2-40, PDGFR-β, and α-SMA in advanced colorectal cancer are still unknown. We speculate synergic effects of individual CAF markers are important, and may significantly contribute neoplastic angiogenesis.

In this study, we analyzed histopathological expression of CAF markers in the human advanced colorectal cancers, as well as vessel markers (CD31 and CD34), because CAFs are thought to promote neoplastic angiogenesis in the cancer stroma. In addition, we examined the relations among the CAF/vessel markers and clinicopathological factors.

Materials and methods

Tissue specimens. A total 121 tumor samples from patients who underwent curative surgical resection for advanced colorectal cancer at the Hirosaki University Hospital between January 2008 and December 2009 were included. Informed consent was obtained from each patient regarding the use of clinical records and pathological specimens. Cancer had invaded the subserosa layer of the colorectal wall, and the clinical stages were stage IIA, IIB, or III according to the TNM classification of the UICC (14). Lymph nodes were evaluated histologically. No patient received preoperative chemotherapy, and no patient had metastasis of other
Evaluation of immunohistchemical staining expression area.

We measured the percentage of immunostaining-positive lesions in the total cropped, binarized area for each immunostained slide. The binarized image shows immunostaining-positive and -negative lesions as black and white, respectively. For the examination of α-SMA-positive myofibroblasts, we made an α-SMA-demin subtraction image using the subtraction mode of the ImageJ software (Fig. 1) because α-SMA became positive for muscle tissues, such as muscularis mucosa and muscular layer, in addition to the myofibroblasts. The subtraction image shows the value of α-SMA-positive and desmin-negative myofibroblasts in the cancer stroma. We called this subtraction image as α-SMA subtraction. D2-40-, PDGFR-β-, and collagen I-positive lesions were made binarized and calculated expression area (the positive percentage of the cropped area) by using ImageJ software in the cancer stroma as CAF markers. CD31 and CD34 positive lesions were also made binarized and calculated as vessel markers by using ImageJ software.

Statistical analysis. The value of immunohistochemical expression area and intensity and pathological factor were compared by using Pearson's chi-square test or Fisher's exact test for categorical data. Normally distributed and homoscedastic data were analyzed by two-sample t-test, and non-normally distributed data were analyzed by the Wilcoxon rank sum test for continuous data. The median immunostaining expression area (α-SMA subtraction, CD31, and CD34) and each staining intensity score (collagen I, D2-40, and PDGFR-β) were compared by using Kruskal-Wallis test. P<0.05 was considered to indicate a statistically significant difference. Each of the mean/median immunostaining expression percentage in the cancer stroma was compared using Spearman's rank correlation coefficient. Correlation was defined as statistically significant if the rho value (r) was >0.4. All statistical evaluations were performed using R (http://www.r-project.org) and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan).

Results

Expression of CAF/vessel markers of colorectal cancer tissues. The clinicopathological characteristics of the 121 colorectal
Figure 1. Imaging analysis for α-smooth muscle actin (α-SMA) subtraction. (A) Immunohistochemical expression of desmin (magnification, x40); (B) binarization image of desmin (magnification, x40); (C) hematoxylin and eosin staining (magnification, x40); (D) immunohistochemical expression of α-SMA (magnification, x40); (E) binarization image of α-SMA (magnification, x40); and (F) image which obtained by subtracting desmin from α-SMA (α-SMA subtraction) (magnification, x40).

Figure 2. Intensity score for collagen I, D2-40, and platelet-derived growth factor receptor-β (PDGFR-β; magnification, x40). The expression intensity was evaluated by scoring in collagen I, D2-40, and PDGFR-β. Score 1, low intensity; score 2, moderate intensity; and score 3, high intensity.
Table I. Clinicopathological characteristics of 121 colorectal cancer cases.

| Clinicopathological features | Number of patients (%) |
|-----------------------------|-------------------------|
| Age, median (range)         | 67.4 (26-93)            |
| Sex                         |                         |
| Male                        | 66 (54.5)               |
| Female                      | 55 (45.5)               |
| Location                    |                         |
| Colon                       | 77 (63.6)               |
| Rectum                      | 44 (36.4)               |
| Histological type           |                         |
| Well-differentiated tubular | 11 (9.1)                |
| adenocarcinoma (tub1)       |                         |
| Moderately differentiated   | 99 (81.8)               |
| tubular adenocarcinoma (tub2)|                         |
| Papillary adenocarcinoma (pap)| 3 (2.5)                |
| Poorly differentiated       | 5 (4.1)                 |
| adenocarcinoma (por)        |                         |
| Mucinous adenocarcinoma (muc)| 3 (2.5)                 |
| Stage                       |                         |
| IIA                         | 64 (52.9)               |
| IIIB                        | 48 (39.7)               |
| IIIC                        | 9 (7.4)                 |
| Venous invasion             |                         |
| Low (v0, v1)                | 89 (73.6)               |
| High (v2, v3)               | 32 (26.4)               |
| Lymphatic invasion          |                         |
| Low (ly0, ly1)              | 80 (66.2)               |
| High (ly2, ly3)             | 41 (33.8)               |
| Lymph node metastasis       |                         |
| Negative                    | 64 (52.9)               |
| Positive                    | 57 (47.1)               |

There was no relation between lymphatic invasion and expression of CAF/vessel markers except for CD34. Much expression area of CD34 was correlated with high lymphatic invasion (P=0.048) (Table IV). There was no relation between lymph node metastasis and expression of CAF/vessel markers except for α-SMA subtraction (Table V). Weak expression area of α-SMA subtraction was significantly correlated with positive lymph node metastasis (P=0.025). Strong expression area of α-SMA subtraction was significantly correlated with negative lymph node metastasis (P=0.025).

**Correlation among CAF/vessel markers.** We evaluated the correlation among the expression of CAF/vessel markers. Spearman’s correlation rho, and the scatter plots are shown in Table VI, and Fig. 3, respectively. There was significant correlation between α-SMA subtraction and collagen I (P<0.001, correlation rho=0.509) in expression area. The significant difference was not found between other CAF and vascular marker nor other CAF markers each other in expression area. There was not significant difference between collagen I and D2-40 (P=0.119), collagen I and PDGFR-β (P=0.665), PDGFR-β and D2-40 (P=0.940) in intensity score. There was not significant difference between expression area of CAF markers and three each CAF markers intensity score; collagen I (P=0.072), D2-40 (P=0.297), and PDGFR-β (P=0.386). There was not difference between CD31 expression area and three CAF markers of intensity score; collagen I (P=0.232), D2-40 (P=0.205), and PDGFR-β (P=0.657). There was not difference between CD3 expression area and three CAF markers of intensity score; collagen I (P=0.133), D2-40 (P=0.090), and PDGFR-β (P=0.641).

**Discussion**

Previous reports were accomplished about each CAF (13,16-19), but did not provide the information about the comparison of each CAF. We captured immunohistochemical staining images, and adjusted phases in each case by using Adobe Photoshop software (Adobe® Photoshop® CC 2014®; USA) for image registration, and compared the different antibodies in the same field. In the present study, we analyzed histopathological expression of CAF markers (collagen I, D2-40, PDGFR-β and α-SMA subtraction) in 121 cases of the surgically resected advanced colorectal cancers, using digital image analyses. High levels of α-SMA subtraction (P=0.002), collagen I (P=0.040), and PDGFR-β (P=0.040) expression areas tended to be associated with high venous invasion. α-SMA positive and desmin negative myofibroblasts in the advanced colorectal cancer is associated with malignant potential in previous study (16,17). Serum levels of Collagen I degradation telopeptide are correlated with staging and poor disease-free survival of colorectal patients (19). PDGFR-β expression in colorectal cancer stroma is associated with metastatic potential (18). Our data supported these previous reports. α-SMA subtraction and venous invasion had strongest positive correlation in the three markers (α-SMA subtraction, collagen I, and PDGFR-β), in spite of a median expression of the α-SMA subtraction not being so high (α-SMA subtraction 15.372%, collagen I 26.787%, and PDGFR-β 11.646%).

Expression of CAF/vessel markers, and venous invasion/lymphatic invasion/lymph node metastasis. Relationships between the expression of CAF/vessel markers, and venous invasion/lymphatic invasion/lymph node metastasis are summarized in Tables III, IV and V, respectively. Extensive expression area of α-SMA subtraction (P=0.002), collagen I (P=0.040) and PDGFR-β (P=0.040) were significantly correlated with high-grade venous invasion (Table III).
We evaluated the correlation among the expression of CAF/vessel markers. Collagen I, α-SMA subtraction, and PDGFR-β correlated with venous invasion. There was significant correlation between α-SMA subtraction and collagen I.
expression (P<0.001, correlation rho=0.509). PDGFR-β was not associated with collagen I nor α-SMA subtraction image, though high PDGFR-β expression was correlated with venous invasion. These data suggested that α-SMA subtraction, collagen I, and PDGFR-β might have differential strength effects for venous invasion and different expression patterns in advanced colorectal cancer stroma. Immunohistochemically, collagen I, α-SMA subtraction, and PDGFR-β widely

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### Table IV. Expression of CAF/vessel markers and lymphatic invasion of colorectal cancer.

| Lymphatic invasion | Low (ly0, ly1) n=80 (66.1%) | High (ly2, ly3) n=41 (33.9%) | P-value |
|--------------------|----------------------------|----------------------------|---------|
| **Expression area**|                            |                            |         |
| Collagen I         | 26.333                     | 27.673                     | 0.990   |
| D2-40              | 2.026                      | 2.950                      | 0.156   |
| PDGFR-β            | 12.789                     | 15.515                     | 0.188   |
| α-SMA subtraction  | 15.287                     | 17.465                     | 0.119   |
| CD31               | 4.113                      | 4.443                      | 0.665   |
| CD34               | 2.301                      | 2.838                      | 0.048†  |
| **Expression intensity** |                        |                            |         |
| Collagen I         |                            |                            | 0.604   |
| High               | 43 (35.5)                  | 20 (16.5)                  |         |
| Low                | 37 (30.6)                  | 21 (17.4)                  |         |
| D2-40              |                            |                            | 0.225   |
| High               | 28 (23.1)                  | 19 (15.7)                  |         |
| Low                | 52 (43.0)                  | 22 (18.2)                  |         |
| PDGFR-β            |                            |                            | 0.715   |
| High               | 32 (26.4)                  | 15 (12.4)                  |         |
| Low                | 48 (39.7)                  | 26 (21.5)                  |         |

†P<0.05, statistical significance CAF, cancer-associated fibroblast; PDGFR-β, platelet-derived growth factor receptor-β; α-SMA, α-smooth muscle actin.

### Table V. Expression of CAF/vessel markers and lymph node metastasis of colorectal cancer.

| Lymph node metastasis | Negative n=64 (52.9%) | Positive n=57 (47.1%) | P-value |
|-----------------------|-----------------------|-----------------------|---------|
| **Expression area**   |                       |                       |         |
| Collagen I            | 26.798                | 26.775                | 0.990   |
| D2-40                 | 2.228                 | 2.463                 | 0.698   |
| PDGFR-β               | 13.943                | 13.454                | 0.882   |
| α-SMA subtraction     | 17.242                | 14.659                | 0.025†  |
| CD31                  | 3.627                 | 4.098                 | 0.704   |
| CD34                  | 2.350                 | 2.632                 | 0.352   |
| **Expression intensity** |                       |                       | 0.168   |
| Collagen I            |                       |                       |         |
| High                  | 33 (27.3)             | 30 (24.8)             |         |
| Low                   | 31 (25.6)             | 27 (22.3)             |         |
| D2-40                 |                       |                       | 0.277   |
| High                  | 23 (19.0)             | 24 (19.8)             |         |
| Low                   | 41 (33.9)             | 33 (27.3)             |         |
| PDGFR-β               |                       |                       | 0.506   |
| High                  | 24 (19.8)             | 23 (19.0)             |         |
| Low                   | 40 (33.1)             | 34 (28.1)             |         |

†P<0.05, statistical significance CAF, cancer-associated fibroblast; PDGFR-β, platelet-derived growth factor receptor-β; α-SMA, α-smooth muscle actin.
expressed in the whole colorectal cancer stroma. On the other hand, the expression of D2-40 was generally localized in the cancer stroma, and was more frequently detected in the superficial parts of the cancer tissue. The expression patterns (location, intensity) of collagen I were similar to that of \(\alpha\)-SMA subtraction image, but PDGFR-\(\beta\) and D2-40 showed different expression patterns in advanced colorectal cancer stroma. The different expression pattern may influence venous invasion. It is necessary to further study the relationships between CAFs in colorectal cancer. CAF markers must become potential targets of future colorectal cancer treatment. Combination therapy of PDGFR tyrosine kinase inhibitor and anticancer drug was more effective than the anticancer drug alone (20,21). In the future, cancer treatment will be taylor made treatment, and its options will be expanded. Knowing the detailed characters of CAFs leads to taylor made treatment. In our study, it is possible that \(\alpha\)-SMA subtraction contributes most to venous invasion compare with collagen I, PDGFR-\(\beta\).

The significant differences were seen in the expression of collagen I, PDGFR-\(\beta\) and \(\alpha\)-SMA subtraction between low and high venous invasion. Expression area analysis was quantifiable by using digital image analyses. On the other hand, it was difficult to analyze the intensity of collagen I, \(\alpha\)-SMA subtraction, because the expression of \(\alpha\)-SMA subtraction and collagen I was highly expressed in most cases.

The intensity of the expression of PDGFR-\(\beta\) is likely to be unstable due to its small fluctuation in intensity. Therefore, there was no significant difference in the expression intensity. Interestingly, the expression of \(\alpha\)-SMA subtraction was low in lymph node metastasis cases in this study. We analyzed the CAF and vessel markers focused on the D2-40 expressed lesions, therefore D2-40 might have some influence for CAF and vessel markers expression. D2-40 (Podoplanin) is a 38-kDa mucin-type transmembrane glycoprotein with extensive O-glycosylation and high sialic acid content, and it has been implicated in tumor progression (22). Podoplanin promotes relocalization of ezrin to filopodia-like structure and platelet aggregation, so that podoplanin may be involved in cancer migration, invasion, and malignant progression (23-25). The majority of recently reports identified D2-40 expression of CAFs as an unfavorable marker of prognosis (9-11), but some reports described D2-40 in CAFs as a favorable marker for colorectal cancer (12,13). Choi et al analyzed early and advanced colorectal cancer (12). Yamanashi et al analyzed 120 advanced colorectal cancer cases (13). The detailed mechanism has not been understood why the expression of D2-40 becomes a favorable marker for colorectal cancer. Despite high venous invasion of \(\alpha\)-SMA subtraction expression, D2-40 expressed area might influence the potential of lymph node metastasis in this study. Collagen

![Figure 3. The Scatter plot for expression of platelet-derived growth factor receptor-\(\beta\) (PDGFR-\(\beta\)) and collagen I.](image)

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Table VI. Spearman's rank correlation rho in CAFs/vessel markers.

| CAF/vessel markers | Collagen I | D2-40 | PDGFR-\(\beta\) | \(\alpha\)-SMA subtraction | CD31 | CD34 |
|--------------------|-----------|-------|----------------|--------------------------|------|------|
| Collagen I         | 0.150     | 0.260 | 0.509          | 0.088                    | 0.133|      |
| D2-40              | 0.145     | 0.257 | 0.318          | 0.127                    | 0.227| 0.237|
| PDGFR-\(\beta\)    |           | 0.127 | 0.060          | 0.145                    |      |      |
| \(\alpha\)-SMA subtraction | 0.088 | 0.133 | 0.145 | 0.318 | 0.319 |
| CD31               |           |      | 0.319          |                          |      |      |
| CD34               |           |      |                |                          |      |      |

\(\rho>0.4\). CAF, cancer-associated fibroblast; PDGFR-\(\beta\), platelet-derived growth factor receptor-\(\beta\); \(\alpha\)-SMA, \(\alpha\)-smooth muscle actin.
I and PDGFR-β are recognized to have malignant potential in CAFs of colorectal cancer (18,19). These two markers do not have a relationship with lymph node metastases, but have a relationship with vein involvement in our study. There is possibility that α-SMA subtraction, collagen I, and PDGFR-β are associated with venous invasion rather than lymphatic invasion. Both vessel markers (CD31/CD34) were not statistically associated with CAF markers/other histological factors, except for the relationship between CD34 and lymphatic invasion.

Our results indicated that the patterns of expression for α-SMA subtraction, collagen I, D2-40, and PDGFR-β vary in CAFs of advanced colorectal cancer. Collagen I, α-SMA subtraction, and PDGFR-β were widely distributed in the colorectal cancer stroma, while D2-40 was limited. The expression of α-SMA subtraction, collagen I, and PDGFR-β were associated with high venous invasion. However, the relationship between CAF markers might be complicated to understand. There have been any previous studies for the relationships between CAF markers. We must further study CAF markers by analyzing with variable viewpoints (i.e., expression pattern, relationship between CAFs and strength for clinicopathological factors).

Acknowledgements
This study was supported by Grants-in-Aid for Science from the Ministry of Education, Culture, Sports, Science and Technology in Japan and a grant for Hirosaki University Institutional Research.

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