Pathological significance of fibroblast activation protein and its association with angiogenesis in colorectal carcinoma

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Background/aim
Fibroblast activation protein (FAP) as one of the complex tumor environment is expressed in activated fibroblasts and associated with poor prognosis in cancer. FAP expression in colon cancer lacks sufficient evidence to serve a significant role in angiogenesis. This study aimed to clarify the association of FAP expression with angiogenesis in the prognosis of colorectal carcinoma (CRC).

Materials and methods
A total of 50 biopsies of CRC were evaluated by immunohistochemistry for investigating FAP expression and microvascular density (MVD) using CD34 protein. In terms of FAP-positive cells and FAP staining intensity, tumors were classified as high and low expression. With respect to tumor vascularity, cases were classified into hypovascular tumors and hypervascular ones. Both of FAP expression and MVD were correlated with histological tumor grade, stage, and lymph node metastases and also with each other.

Results
FAP expression was significantly higher in malignant cases than normal nontumor tissue samples. The percentage of FAP-positive cells was significantly correlated with grade, T-stages, and lymph node metastases, while FAP intensity was significantly associated with high tumor stage only. Hypervascularity was significantly correlated with high T-stages and lymph nodes metastasis. A significant correlation was found between FAP expression percentage and MVD.

Conclusion
This study indicates that FAP is overexpressed in primary CRC and is associated with poor prognosis. The authors suggested that FAP may be used as a prognostic marker and could be reliable for predicting the angiogenic activity of CRC. Further studies are recommended applying FAP as a diagnostic marker for CRC and for evaluating its promising role as an excellent target for antitumor therapy.

Keywords:
CD34, colorectal carcinoma, FAP, immunohistochemistry, MVD

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Introduction

Colorectal cancer is one of the most frequent cancers worldwide with one of the highest mortality rate. The incidence of colorectal carcinoma (CRC) was 6.1% in 2018 [1]. Liver and lung are the commonest sites of metastasis and the major cause of death in metastatic CRC [2]. The 5-year relative survival rate for localized tumor is 90%, while patients with distant metastasis at diagnosis survival rate are only 13% [3].

Cancer not only forms an assembly of malignant cells, but they form a complex environment of a variety of stromal cells, including fibroblasts, vascular cells, and inflammatory cells. Especially cancer-associated fibroblasts were shown to have a key role in many tumors involving their growth, metastasis, and progression [4].

In this respect, an interesting target is the fibroblast activation protein (FAP), which is expressed in activated fibroblasts, but not in quiescent ones, and was shown to be associated with poor prognosis in various malignant tumors, including CRC [5]. Generally, activation of fibroblasts leads to changes in their morphology to appear with more stellate-shaped rather than spindle-shaped forms. Moreover, the activated fibroblasts are able to migrate, proliferate, and produce the extracellular matrix [6].

FAP is a type ll membrane-bound glycoprotein belonging to the dipeptidyl peptidase 4 family, which is expressed by carcinoma-associated fibroblasts and serves important roles in tumor occurrence and prognosis [7]. Given the scientific evidence, the functions of FAP have been considered to be associated with its proteolytic activity. This activity is involved in tissue remodeling, helping the neoplastic cells to invade the surrounding tissue, penetrate the wall of blood vessels, and spread forming distant metastasis [8].

On the other hand, angiogenesis is a common phenomenon in most cancers and is essential for tumor development and for its regional and metastatic growth [9]. When tumor mass is supported only by the host blood vessels, it remains in a limited size with slow progression. On the contrary, neoangiogenesis is supported by the tumor cells through secretion of proangiogenic agents, which stimulate more rapid growth and tumor progression [10]. It had also been shown that tumor aggressiveness was determined by the degree of its angiogenic activity and could be assessed by measuring the microvascular density (MVD) [11].

Only few data on angiogenesis and its association with clinicopathological features and prognostic outcomes have been reported. This study aimed to investigate FAP expression using immunohistochemistry and explore its association with angiogenesis and the clinicopathological characteristics in patients of primary colorectal adenocarcinoma.

Materials and methods

Sampling

Formalin-fixed, paraffin-embedded tissue samples from 50 patients of CRC were included in this study. The specimens were selected randomly from the Department of Pathology, Faculty of Medicine, Cairo University. The pathology report and hematoxylin- and eosin-stained slides were revised, and tumor grading was performed according to World Health Organization (WHO) classification. The presence of lymph node metastasis and T-stage was reviewed. Ten fields from each slide were investigated for fibroblast infiltration and MVD using immunohistochemistry.

Ethical approval

The study was approved by the Ethical Committee of the National Research Centre with approval number 7413042021.

Immunohistochemical study

FAP and CD34 expression was examined in all tissues using streptavidin–biotin technique. Two slides from each case were deparaffinized, hydrated, and incubated in 3% hydrogen peroxide for 30 min to block the internal peroxidase activity. Antigen retrieval was done by microwave pretreatment for 10 min in 0.01 M citrate buffer. For each case, one slide was incubated at 4°C overnight with monoclonal antibodies against FAP (Medico Pharma trade) with a dilution 1 : 200. The second slide was incubated with monoclonal antibodies to CD34 at a dilution 1 : 500 (Dako Corporation, Copenhagen, Denmark). These steps were followed by 30 min of incubation with biotinylated horse antimouse antibody at room temperature, avidin–biotin peroxidase complex for 50 min at room temperature, and finally diaminobenzidine for 3–5 min. The slides were counterstained with hematoxylin, dehydrated, and mounted.

FAP immunoreactivity was evaluated semiquantitatively as the percentage of positive staining in stromal cells, as well as the maximal staining intensity (0=none, 1=weak, 2=intermediate, and 3=strong) by two experienced pathologists who were blinded to clinical parameters and the clinical outcomes of the patients. Semiquantitative analysis of stromal staining was assessed as 0, 1+, 2+, and 3+. Score 0 was defined as the complete absence or weak FAP immunostaining in less than 1% of the tumor stroma, score 1+ was focal positivity in 1–10% of stromal cells, score 2+ was positive FAP immunostaining in 11–50% of stromal cells, and score 3+ was positive FAP immunostaining in more than 50% of stromal cells. In terms of the percentage of FAP-positive cells, samples containing less than 10% of positive cells were classified as low, whereas samples containing at least 10% of positive cells were classified as high. In terms of FAP staining intensity, samples with intensities 0 or 1 were reconsidered low, whereas samples with intensities 2 or 3 were considered high by Henry [12].

MVD was carried out on the immunohistochemical-stained slides using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England) at the Department of Pathology, National Research Center. We place the slide to be examined on the stage of the microscope, and focus it at power magnification (×100) for counting blood vessel density. The light source is set to the required level. Successful adjustment of illumination is checked for on the video monitor. Any single brown-stained cell that indicates an endothelial cell stained with CD34 was counted as a single vessel. Branching structures were counted as a single vessel, unless there was a break in the continuity of the structure. Individual vessel counts were performed at ×100 magnification in 10 fields of the highest neovascularization areas. The median MVD was calculated for each case. The median MVD was used as the cutoff value for the MVD. Cases with mean number equal or less than this median were considered as hypovascular tumors and cases with mean number more than this median were considered as hypervascular tumors [13,14].

Both of FAP expression and vascular density were correlated with histologic grades, T-staging, and lymph nodes status. Also, they were correlated with each other.

Statistical analysis

χ^2 test was applied to examine the correlation between fibroblast infiltration, vascular densities and histologic grade, lymph node metastases, and tumor tissue invasion (T-staging). *P* value less than 0.01 was considered significant.

Results

Immunostaining for FAP and CD34 was evaluated in 50 cases of colon carcinoma. According to WHO, 6 cases were GI, 30 cases of GII, and 14 cases of GIII. On reviewing T-staging of the cases, 5 cases were T1, 12 cases T2, 14 cases T3, and 19 cases were T4. Of all cases of colon carcinoma, 40 cases showed lymph nodes metastasis (N1 and N2) and 10 cases were lymph node-negative (N0).

Expression of FAP in colorectal carcinoma

The expression of FAP at the protein level in stromal cells was analyzed in 50 cases of CRC and 10 nontumoral adjacent tissues using immunohistochemistry (IHC). A significant high percentage of FAP-positive stromal cells was observed in tumors as compared with nontumoral tissues ($P<0.01$). High percentage (at least 10%) of FAP-positive cells was found in 32 cases (64%) of CRCs, whereas high FAP intensity was observed in 29 cases (58%). FAP staining was detectable in only 2 cases (20%) out of the 10 nontumoral tissue samples, both of them showed low FAP percentage and weak staining-intensity positive cells (score 1).

Pathological parameters were compared in tumors with FAP expression (both percentage and intensity). We found that the percentage of FAP-positive cells was significantly more frequently found in CRCs with high grade, advanced stages, and lymph node metastases ($P<0.01$ in each). High FAP intensity was significantly associated with tumors showing high tumor stage ($P<0.01$). Tumors with lymph node metastases showed higher FAP staining intensity than nonmetastatic carcinoma but the correlation was nonsignificant. On the other hand, FAP staining intensity was non-significantly correlated with tumor grade (Table 1, Fig. 1).

Microvessel density

In this study, the MVD median was 150 and was used as the cutoff value for the MVD. MVD was significantly higher ($P<0.01$) in malignant cases than nontumor tissue, where the mean microvessel counts were 135 and 55 in both groups, respectively. Of the 50 colonic carcinomas, 31 cases (62%) were hypervascular and the remaining 19 cases (38%) were hypovascular.

Histologic grade was not significantly different ($P>0.01$) between hypovascular and hypervascular tumors. On the contrary, hypervascularity was

Table 1 Correlation between FAP expression and clinicopathological parameters in colorectal carcinoma

Clinicopathological parameters	No	FAP frequency high low *P* value (32) (18)	FAP intensity high low *P* value (29) (21)
Grades			
I	6	2 (33.3%) 4 (66.7%)	3 (50%) 3(50%)
II	30	18 (60%) 12 (40%) <0.01	17 (56.7%) 13 (43.3%) >0.01
III	14	12 (85.7%) 2 (14.3%)	9 (64.3%) 5 (35.7%)
T-staging			
T1	5	1 (20%) 4 (80%)	0 (0%) 5 (100%)
T2	12	5 (41.7%) 7 (58.3%) <0.01	3 (25%) 9 (75%) <0.01
T3	14	10 (71.4%) 4 (28.6%)	10 (71.4%) 4 (28.6%)
T4	19	16 (84.2%) 3 (15.7%)	16 (84.2%) 3 (15.8%)
Lymph nodes			
N0	10	4 (40%) 6 (60%)	5 (40%) 6 (60%)
N1	26	16 (61.5%) 10 (38.5%) <0.01	14 (53.8%) 12 (46.2%) <0.01
N2	14	12 (85.7%) 2 (14.3%)	10 (71.4%) 4 (28.6%)

FAP, fibroblast activation protein.

significantly ($P<0.01$) correlated with high T-stages and lymph node metastasis (Table 2, Fig. 2).

Correlation between FAP expression and microvessel density

A significant correlation ($P<0.01$) was found between FAP expression percentage, not FAP staining intensity and MVD in malignant tumors where 78.1% of tumors with high FAP percentage were hypervascular and 66.7% of tumors with low FAP percentage were hypovascular (Table 3).

Discussion

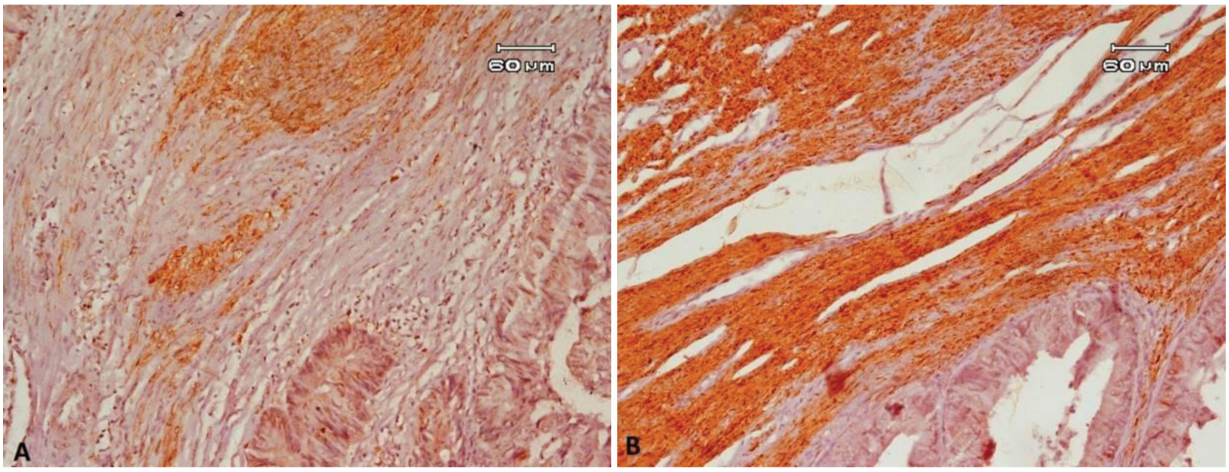
FAP has been shown to play a key role in tissue remolding and help invade the surrounding tissue by the neoplastic cells [15]. Many studies showed that FAP was overexpressed in tumor-associated stromal cells in several epithelial tumors [16]. When FAP radionuclide-based imaging had been done with antibodies and inhibitor molecules, high FAP uptake was found in esophageal cancer, pancreatic cancer, head and neck, and colon cancer [17–19].

Colorectal cancer as one of the commonest types of malignant tumors was shown to express FAP. Because of its stromal distribution, the inhibition of FAP is an attractive avenue of many promising researches [5].

On the other hand, angiogenesis is essential for tumor occurrence and for its local and metastatic growth, where MVD has been proved as a marker that reflects tumor angiogenesis in various neoplasms, including CRC [11].

Our study aimed to investigate the expression of FAP at the protein level by immunohistochemistry in 50

Figure 1



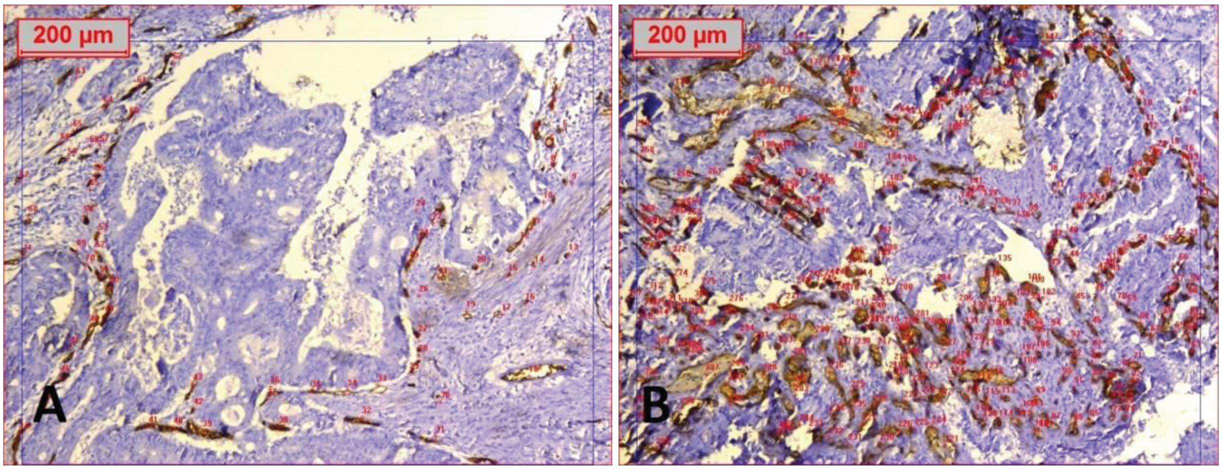
Fibroblast activation protein (FAP) immunohistochemical expression in colorectal carcinoma showing (A) percentage (score 2) and intensity (score 2) and (B) percentage (score 3) and intensity (score 3) (FAP immunostaining, scale bar: 60 μm).

Table 2 Correlation between microvessel density and clinicopathological parameters in colorectal carcinoma

Clinicopathological parameters	No	MVD Hypervas. (31) Hypovas. (19) *P* value
Grades		
I	6	3 (50%) 3 (50%)
II	30	18 (60%) 12 (40%) >0.01
III	14	10 (71.4%) 4 (28.6%)
T-staging		
T1	5	1 (20%) 4 (80%)
T2	12	5 (41.7%) 7 (58.3%) <0.01
T3	14	10 (71.4%) 4 (28.6%)
T4	19	15 (78.9%) 4 (21.1%)
Lymph nodes		
N0	10	3 (30%) 7 (70%)
N1	26	16 (61.5%) 10 (38.5%) <0.01
N2	14	12 (85.7%) 2 (14.3%)

MVD, microvascular density.

Figure 2



CD34 immunohistochemical expression in (A) well-differentiated colorectal carcinoma (CRC) with an overlapping binary image showing hypovascularity and (B) poorly differentiated CRC with an overlapping binary image showing hypervascularity (CD34 immunostaining, scale bar: 200 µm).

Table 3 Correlation between FAP expression and microvessel density in colorectal carcinoma

FAP expression	MVD		*P* value
	Hypervas. (31)	Hypova s. (19)	
FAP frequency			
High (32)	25 (78.1%)	7 (21.9%)	<0.01
Low (18)	6 (33.3%)	12 (66.7%)	
FAP intensity			
High (29)	19 (65.5%)	10 (34.5%)	>0.01
Low (21)	12 (57.1%)	9 (42.9%)	

MVD, microvascular density; FAP, fibroblast activation protein.

cases of CRC and to study its association with angiogenesis and the clinicopathological parameters of the patients.

The present study has shown that FAP was highly expressed in stromal cells of CRC compared with nontumoral tissue with a statistically significant difference ($P<0.01$). High percentage of FAP-positive cells was found in 64% of cases of CRCs, whereas high intensity was observed in 58% of cases. This is in agreement with previous studies by Henry *et al.* [12]. These studies had shown that FAP was detected in more than 93% of cases of CRC, while 30% of cases were shown to have high FAP staining intensity. In gastric cancer, FAP was found to be highly expressed in invasive gastric carcinoma in 61.8% of cases, which is higher than its expression in normal gastric ulcer groups [20]. They suggested that FAP is a matrix cell marker; its proteolytic activity could help the invasion of the extracellular matrix by the neoplastic cells. Most studies on FAP showed that it is upregulated in tumors compared with nontumoral tissues and is associated with poor survival [16].

In our study, we found that the percentage of FAP-positive cells was significantly more frequently found in CRCs with high grade, advanced stage, and lymph node metastases ($P<0.01$). On the other hand, high FAP intensity was significantly associated with tumors showing high stage ($P<0.01$), while its intensity was non-significantly correlated with tumor grade. In line with poor prognosis, the study of Cota-Lterena *et al.* [15] showed that high frequency of FAP-positive cells and high intensity of FAP at both RNA and protein levels were associated with advanced stages. Moreover, FAP immune expression was associated with tumor budding and lymphatic invasion, the last has been used to evaluate the aggressiveness of CRC [21,22]. Taken together, the findings related to the association of FAP expression with clinicopathological features were proved to be associated with tumor aggressiveness and poor prognosis of patients with CRCs [22,23]. Our results and previous studies are in line demonstrating that FAP overexpression was involved in tumor growth, proliferation, invasion, and metastases. Moreover, the molecular role of FAP and its potential value in modulating the tumor microenvironment was proved in oral cell carcinoma [24]. As FAP is expressed on most of activated fibroblasts in the tumor microenvironment and because its expression plays a critical role in tumor growth and progression, FAP-targeting strategies may be more effective and considered excellent target cells for antitumor therapy [4,25,26].

However, FAP expression in CRC lacks sufficient evidence to serve a significant role in angiogenesis. The degree of tumor angiogenic activity was important to evaluate tumor aggressiveness [9,10].

In our study, MVD was evaluated using anti-CD34. MVD was significantly higher in tumor than nontumor tissue ($P<0.01$). Hypervascularity was significantly correlated with T-stage and lymph node metastasis. Hanrahan *et al.* [27] emphasized the association between MVD and T4 depth invasion that contributes to invasion, metastasis, and unfavorable outcome in CRC.

Previous reports implicated MVD as being a prognostic indicator in the case of CRC with a high value and its expression was associated with poor patient survival [26]. In contrast, Hutajulu *et al.* [11] showed that angiogenic markers had no influence on patient outcome of CRC.

Although the association between MVD and clinicopathological parameters and patients' outcome remains controversial, many studies proved that angiogenic markers positively associated with age, sex, tumor grade, clinical stage, tumor depth, and nodal and distant metastases [28,29].

Our study showed a significant correlation between FAP expression, but not staining intensity and MVD in CRC, where 78.1% of tumors with high FAP percentage were hypervascular ($P<0.01$). This is in agreement with the study of Coto-Lterena *et al.* [15], who showed that FAP plays important roles in tumor progression, such as modulation of angiogenesis and immunoregulation in the microenvironment of CRC. They suggested that one of the mechanisms of which FAP promotes carcinogenesis is linked to its ability to recruit endothelial cells and to promote angiogenesis.All these approaches have shown encouraging results for FAP as a prognostic molecular marker in CRC. Furthermore, FAP-targeting radioligands have been used for *in vivo* imaging and targeted therapy for a variety of tumors including CRC [19,30].

Conclusion

Our study concluded that FAP is overexpressed in primary CRC and is associated with poor clinicopathological parameters. We suggested that FAP may be used as a prognostic marker and could be reliable for predicting the angiogenic activity of CRCs. Further studies are recommended applying FAP as a diagnostic marker for CRCs and for evaluating its promising role as an excellent target for antitumor therapy.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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