Clinical Implications of Fibroblast Activation Protein in Patients with Colon Cancer

Leonard R. Henry,[1] Hyung-Ok Lee,[2] John S. Lee,[1] Andres Klein-Szanto,[3] Perry Watts,[4] Eric A. Ross,[4] Wen-Tien Chen,[5] and Jonathan D. Cheng[2]

Abstract

Purpose: Human fibroblast activation protein (FAP)/seprase is a 97-kDa surface glycoprotein expressed on tumor associated fibroblasts in the majority of epithelial cancers including colon adenocarcinomas. FAP overexpression in human tumor cells has been shown to promote tumor growth in animal models, and clinical trials targeting FAP enzymatic activity have been initiated. The primary objective of this study was to evaluate the clinical significance of stromal FAP in human colon cancers by immunohistochemisty.

Experimental Design: Sections of paraffin-embedded resected primary human colon cancer specimens from 1996 through 2001 within the Fox Chase Cancer Center tumor bank were stained with D8 antibody directed against FAP/seprase. Xenotransplanted human colorectal tumors in mice were examined similarly for stromal FAP in tumors of different sizes. Overall percentage of stromal FAP staining of the primary tumor was assessed semiquantitatively (0, 1+, 2+, 3+) and staining intensity was also graded (none, weak, intermediate, strong). Survival time and time to recurrence data were analyzed using Kaplan-Meier plots, log-rank tests, and Cox proportional hazards models.

Results: One hundred thirty-eight patients with resected specimens were available for study (mean follow-up, 1,050 days) with 6 (4%) stage I, 52 (38%) stage II, 43 (31%) stage III, and 37 (27%) stage IV patients. FAP was detected in >93% of specimens. Semiquantitative staining was scored as 1+ in 28 (20%), 2+ in 52 (38%), and 3+ in 49 (35%). FAP staining intensity was graded as weak in 45 (33%), intermediate in 48 (35%), and dark in 36 (26%). Stromal FAP was found to correlate inversely with tumor stage (semiquantitative, $P = 0.01$; intensity, $P = 0.009$) and with tumor size of the tumor xenograft model (correlation coefficient, -0.61; $P = 0.047$), suggesting that stromal FAP may have a greater role in the early development of tumors. Furthermore, greater stromal FAP for patients with known metastatic disease was associated with a decreased survival.

Conclusion: Our data indicate that patients whose colon tumors have high levels of stromal FAP are more likely to have aggressive disease progression and potential development of metastases or recurrence. This study affirms the rationale for ongoing clinical investigations using FAP as a therapeutic target to disrupt FAP-driven tumor progression in patients with metastatic disease. It also suggests that the effects of FAP inhibition should be investigated in earlier-stage tumors, given its high levels and potential effect earlier in the course of the disease.

Background

Colorectal cancer is the third most frequent cancer with regard to incidence and lethality for both males and females in the United States, with more than 55,000 deaths estimated to occur in 2006 (1). Recent clinical advances in metastatic disease have generated significant optimism and enthusiasm for the efficacy of novel therapeutics (2, 3) aimed at treating colorectal cancer. One of these novel therapies, bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor, has been shown to have antiangiogenic effects (4). The survival benefit seen with bevacizumab treatment in patients with colorectal cancer (2) lends credence to the therapeutic strategy of targeting the tumor microenvironment as an effective anticancer approach.

Human fibroblast activation protein (FAP) is a 97-kDa cell-surface glycoprotein with gelatinase and dipeptidyl peptidase

Authors' Affiliations: Departments of [1]Surgical Oncology, [2]Medical Oncology, [3]Pathology, and [4]Biostatistics, Fox Chase Cancer Center, Philadelphia, Pennsylvania and [5]Department of Medicine, Stony Brook University, Stony Brook, New York

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Note: L.R. Henry and H-O. Lee contributed equally to this work. L.R. Henry's current address: Department of Surgery, National Naval Medical Center, Bethesda, MD.

Requests for reprints: Jonathan D. Cheng, Department of Medical Oncology, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111-2497. Phone: 215-728-2450; Fax: 215-728-3639; E-mail: j_cheng@fccc.edu.

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activity that is expressed on tumor stromal fibroblasts (5, 6). The FAP distribution is unique in its selectivity for tumor fibroblasts, in contrast to other tumor fibroblast products such as matrix metalloproteinase inhibitors or adhesion molecules, which are more ubiquitous in their expression pattern (7). FAP is not expressed by normal fibroblasts except transiently in tissues of healing wound (8) or in chronic inflammatory conditions such as cirrhosis (9), making FAP an attractive objective for both the study of tumor-stromal cell biology and as a potential therapeutic target.

We provided the first direct evidence that FAP overexpression in human tumor cells confers an advantage for tumor growth (10). Mice inoculated with FAP-transfected HEK293 cells were two to four times more likely to develop tumors compared with vector-transfected HEK293 controls, with a 10- to 40-fold enhancement in tumor growth. Abrogation of FAP enzymatic activity by site-directed mutagenesis of FAP was shown *in vivo* to result in a significant reduction in FAP-driven tumor growth (11).

FAP is known to be expressed in the stroma of human colorectal tumors and in the stroma of other epithelial malignancies (12). Because of its stromal distribution and promising preclinical research results, the inhibition of FAP is an attractive avenue of clinical research. Welt et al. (13) first used antibodies targeting FAP to allow imaging of colorectal cancers and suggested the possible diagnostic and therapeutic use of humanized monoclonal antibodies directed against FAP. Additional small phase 1 and phase 2 trials using monoclonal antibodies against FAP have been conducted (14–16). Although safety of administration has been shown, no clinical responses have been observed to monoclonal antibody treatment targeting FAP. The antibodies evaluated thus far do not inhibit FAP enzymatic activity. More recently, however, the administration of the small-molecule inhibitor of FAP, Val-boro-Pro (Talabostat), has been shown to attenuate and reject tumor growth in a variety of tumor models in mice (11, 17, 18). Clinical trials using talabostat have been done for patients with stage IV melanoma (both as monotherapy and in conjunction with chemotherapy) and non–small-cell lung cancer (19–21). Clinical responses have been observed.

To date, there have been relatively few studies examining the clinical effect of stromal FAP in archived human tumors (22, 23). We thus aimed to evaluate the distribution of stromal FAP in archived human colon cancer and relate these findings to clinical outcome. Based on our preclinical results (10, 11), we hypothesized that increased levels of stromal FAP in archived human colon cancer tissue may serve as a negative prognostic marker portending a worse clinical outcome.

Materials and Methods

Immunohistochemistry. This study was approved by the Fox Chase Cancer Center institutional review board. Paraffin-embedded resected primary colon cancer specimens from 1996 through 2001 (n = 138) were retrieved from the Fox Chase Cancer Center Tumor Bank Facility. Rat hybridoma serum-free supernatant containing monoclonal antibody D8 directed against human FAP was applied at 1:40 dilution to paraffin-embedded sections after microwave antigen retrieval for 10 min in 0.01 mol/L citrate buffer (pH 6.0). Specimens were treated with 0.3% hydrogen peroxide in methanol for 15 min after incubation with the primary antibody to block endogenous peroxidase activity and blocked with human AB serum to minimize background reactivity. The secondary antibody of horseradish peroxidase–labeled $F(ab')_2$ fragment antirat antibody (Amersham, Aylesbury, United Kingdom) was applied at 1:500 dilution with 10% human serum and incubated for 1 h.

Semiquantitative analysis of immunohistochemistry. Slides were reviewed under light microscopy with 40× power by two independent observers (L.R.H. and J.S.L.) blinded to each others assessment. Semiquantitative analysis of stromal staining was assessed as 0, 1+, 2+, and 3+ (51-100% stromal staining) as previously established (22, 23). Grade 0 was defined as the complete absence or weak FAP immunostaining in <1% of the tumor stroma; grade 1+ was focal positivity in 1% to 10% of stromal cells; grade 2+ was positive FAP immunostaining in 11% to 50% of stromal cells; and grade 3+ was positive FAP immunostaining in >50% of stromal cells. A global assessment of the entire tumor was made without selection for the invasive front or areas of active tumor growth. An additional assessment of maximal staining intensity was done and graded as none, weak, intermediate, or strong as previously reported (24). Groups scored with 0 or 1 staining by semiquantitative analysis or intensity were then compared with those with greater (2 or 3) staining for differences in clinical outcomes. The staining intensity of FAP was also confirmed using the Automated Cellular Imaging System (ACISII, Chromavision Medical Systems, Inc., San Juan Capistrano, CA). Image analysis was done to quantify the intensity of FAP staining. The scanning was done at ×20 magnification. A color threshold for brown (FAP positive) and blue (nuclear stain) staining was set for every slide analyzed. Thirty to fifty regions of interest (radius of each region, 80 μm; each circular area, 2×10^4 μm^2) were manually selected and each selected region was individually calculated for percent of positive cells. The average percentage of positive cells for all regions was calculated as mean ± SE for statistical comparisons.

Patients. The medical records of corresponding patients were retrospectively reviewed. Demographic, clinicopathologic factors surrounding the primary tumor and management were documented. Tumor location was derived from the operative report. Rectal cancers below 10 cm from the anal verge were excluded to avoid the confounding variable of adequacy of surgical resection given the technically demanding nature of such operations. Rectosigmoid cancers above 10 cm from the anal verge were included in this study.

HT29 xenografted tumor tissue and immunohistochemical analysis. Animal studies were done according to institutional guidelines for animal care and under Fox Chase Cancer Center Laboratory Animal Facility protocols. HT-29 colorectal cancer cells (2×10^6) were s.c. inoculated in C.B17/Icr-scid mice. Eleven tumors of different sizes were dissected at distinct time points and weighed. Tissues were fixed with 4% paraformaldehyde overnight at 4°C and washed with PBS. The tissues were equilibrated in 30% sucrose overnight at 4°C for cryosections. The tissues were embedded in optimum cutting temperature solution and stored at −80°C before sectioning with a cryostat. The tissue sections were stained with rabbit polyclonal anti-FAP antibody as previously described (10). Image analysis was done on the HT29 xenograft sections to quantify FAP staining using the Automated Cellular Imaging System (ACISII) as described above.

Statistics. The outcomes of the study were overall and disease-free survival as measured from the time of surgery and estimated by Kaplan-Meier methods. Patient disease-free or alive at the time of last follow-up was censored for disease-free survival and overall survival, respectively. Univariate and multivariate analyses were conducted with log-rank tests and Cox proportional hazards models. Generalized Fisher's exact test was used to test for independence of stage of disease and binary categorization of FAP staining intensity and percent FAP-positive cells. Pearson correlation was used to measure the association between tumor size and FAP expression. Kruskal-Wallace test was used to assess the distribution of the ASCI FAP stain score across the variables of stage and semiquantitative FAP intensity. All tests were conducted with a 5% type I error. All analyses were done using SAS statistical software version 9.1 (Cary, NC).

Results

Patient results. One hundred thirty-eight tumor-banked specimens were available for review between the specified time period 1996-2001. Median age at cancer diagnosis in the corresponding patients was 68.5 years (range, 28-90). Table 1 summarizes the patient and tumor characteristics.

Immunohistochemistry results. FAP was detected in 129 (93%) specimens. Semiquantitative staining was scored as 1+ in 28 (20%), 2+ in 52 (38%), and 3+ in 49 (35%; examples given in Fig. 1). Maximal staining intensity in the specimen was graded as weak in 45 (33%), intermediate in 48 (35%), and strong in 36 (26%). Thus, FAP was expressed in the vast majority of specimens and was well distributed in these samples across the spectrum of semiquantitative levels and staining intensities. There was no correlation of FAP staining with tumor grade (Table 2), and FAP staining appeared uniform throughout the stroma irrespective of the invasive edge.

FAP is more prominent in early-stage disease. The mean follow-up for all patients was 1,050 days, at which time 89 patients were alive. Overall median survival for the study has not been reached. Stromal FAP intensity and semiquantitative analysis were inversely correlated to cancer stage, with specimens obtained from patients with earlier-stage disease showing significantly increased amounts and intensity of stromal FAP staining (Table 2). Thus, high (intermediate, strong) staining intensity of FAP was associated with earlier-stage disease and consequently improved overall median survival compared with low (none, weak) FAP intensity (median survival not reached versus 1,415 days; $P = 0.0235$). This was confirmed using the ASCI computer image analysis system. The ASCI score showed a significant association with the semiquantitative FAP stain intensity with ASCI scores of 1.3, 21.0, 41.6, and 60.9 for none, weak, intermediate, and strong FAP staining, respectively ($P < 0.0001$). The ASCI quantification of FAP intensity confirmed the finding that FAP staining was greater in early-stage disease, with ASCI scores of 44, 43.5, 39.5, and 25 for stages I, II, III, and IV respectively ($P = 0.008$). Thus, although almost all tumors have stromal FAP, it is more prominently seen in earlier-stage disease.

Table 1. Patient and tumor characteristics

	n (%)
Total	138
Sex	
Female	71 (51)
Male	67 (49)
Tumor location	
Right	62 (45)
Transverse	9 (7)
Left	13 (9)
Sigmoid	37 (27)
Rectosigmoid	17 (12)
Tumor grade	
Well differentiated	7 (5)
Moderately differentiated	110 (80)
Poorly differentiated	20 (15)
Stage	
I	6 (4)
II	52 (38)
III	43 (31)
IV	37 (27)
Chemotherapy use	
None recorded	52 (38)
Adjuvant	56 (41)*
Metastatic	33 (24)*
Unknown	9 (7)

*Twelve patients had chemotherapy in both the adjuvant and metastatic setting.

Immunohistochemistry of xenotransplanted human tumor cells. Similar parallels of stromal FAP levels were observed in an animal model of HT29-xenografted tumors of various sizes. Greater FAP staining was observed in the stroma of smaller HT-29 tumors, with larger tumors also showing stromal FAP, although at a consistently lower level than smaller tumors. The proportion of FAP-positive cells and the intensity of staining were quantified using the ASCI image analysis system (25, 26). There was a significant difference in FAP-positive cells inversely associated with tumor size, with a Pearson correlation coefficient of -0.61 ($P = 0.047$). An example of three different tumor sizes is shown in Fig. 2 (0.047-g tumor, 72.4% FAP stromal cells; 0.32-g tumor, 38.1% FAP cells; and 0.98-g tumor, 19.7% FAP cells). Stromal FAP was thus inversely associated with tumor size in this tumor xenograft model, suggesting that stromal FAP may have a greater role early in the course of tumor growth.

FAP is associated with worse patient survival. The effect of stromal FAP on overall patient survival within each clinical stage was examined. Relatively few mortality events precluded identifying median survivals for stages I through III. An exploratory analysis of disease-free survival in early stage I and II patients was conducted to determine if low FAP expression as determined semiquantitatively (<10% of stromal cells) might portend a better disease-free survival. It is interesting to note that 0 of 8 (0%) patients with low FAP expression had recurred (minimum follow-up 6 years); however, 9 of 50 (18%) patients with high FAP expression had recurred (median follow-up 2.5 years). These results, however, were not statistically significant ($P = 0.23$), potentially due to the small sample size.

Within stage IV, median overall survival also seemed to be worse for patients whose tumors had higher amounts (2+, 3+) of stromal FAP (median survival, 378 versus 672 days; $P = 0.39$), although not statistically significant. In an exploratory analysis to identify all patients with metastatic disease, patients who were initially not identified as having metastatic disease (i.e., stages I-III) but later developed metastasis were included. Thus, evaluating all patients with known metastatic disease (i.e., staged initially as stage IV or "understaged" patients initially diagnosed as stage I-III but later developed metastatic disease) showed a median overall survival significantly longer for patients with low (none, weak) FAP staining intensity compared with greater (intermediate, strong) FAP intensity (671 versus 428 days, respectively; $P = 0.042$; Fig. 3). Similarly, median overall survival trended longer for this group of patients with low (0, 1+) semiquantitative staining versus those with higher (2+, 3+) semiquantitative staining (751 versus 393 days). This suggests that high stromal FAP may be a negative prognostic factor in patients with metastatic disease.

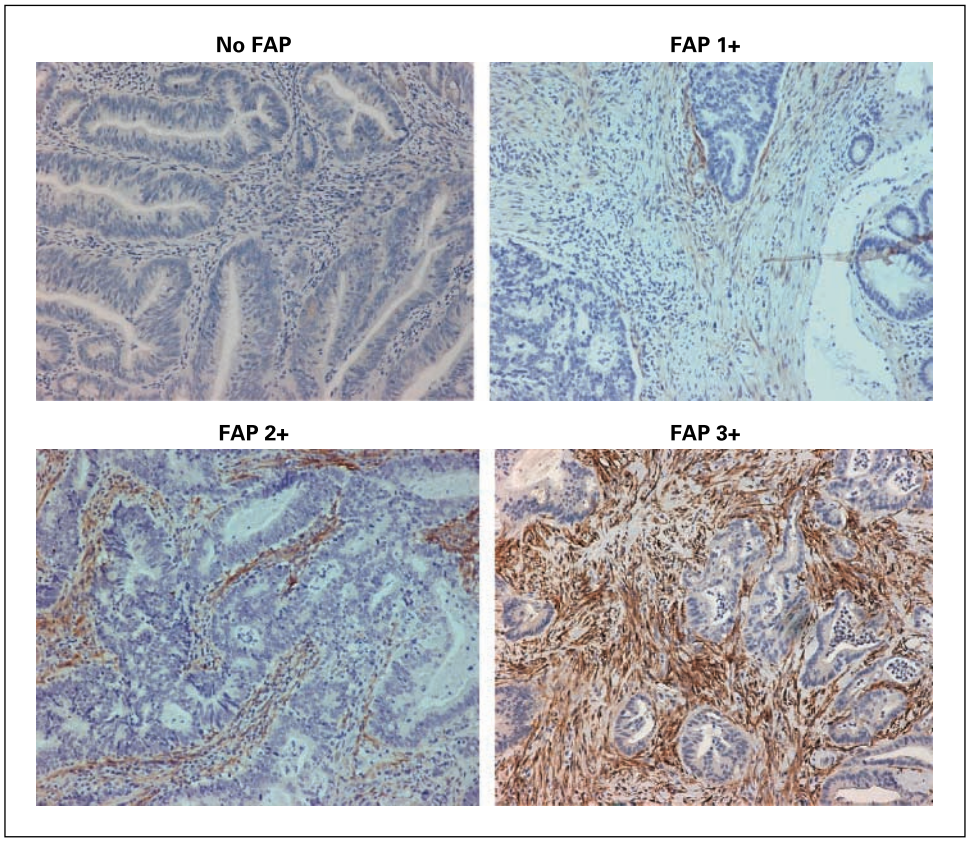

Fig. 1. Representative examples of FAP expression by immunohistochemistry. Semiquantitative analysis of stromal expression of FAP was graded as 0, 1+ (1-10% stromal staining), 2+ (11-50% stromal staining), and 3+ (51-100% stromal staining). Representative examples of the various categories of FAP staining.

Discussion

FAP expression has been described to be present predominantly in the tumor stroma of epithelial malignancies (12, 23), and its presence has been associated with increased microvessel density (27). FAP activity has also been suggested to participate in the growth of human colorectal tumor xenografts because inhibition of FAP enzymatic activity in these tumors resulted in tumor growth attenuation (10, 11). These reports suggest that FAP-dependent pathways may play an important role in epithelial cancer invasion, tumor angiogenesis, and subsequent growth and metastasis.

The current study is the largest series to date examining stromal FAP in patients with colorectal cancer and its correlation with clinical parameters, and the first investigation evaluating patients in North America. We show that stromal FAP is more prominent in early-stage colorectal cancer and smaller colorectal tumor xenografts. Furthermore, we also show that increased FAP is an adverse prognostic indicator in patients with advanced metastatic disease. We have found a statistically significant association between increased FAP intensity and a shortened survival time in patients with known metastatic disease (patients who present with stage IV disease or later develop metastatic disease) and a trend towards decreased survival time in patients who presented with stage IV disease.

Five prior studies assessing FAP in archival tissue with pathologic or clinical end points have been reported (22, 23, 28–30). Although Iwasa et al. (22) suggested that FAP/seprase in

Table 2. FAP expression in relation to cancer stage

FAP expression	No. of specimens (*N* = 138)	FAP semiquantitatively high, *n* (%)	FAP semiquantitatively low, *n* (%)	*P*	FAP intensity high, *n* (%)	FAP intensity low, *n* (%)	*P*
Stage							
I	6	5 (83)	1 (17)	0.01	4 (67)	2 (33)	0.009
II	52	45 (87)	7 (13)		37 (71)	15 (29)	
III	43	30 (70)	13 (30)		29 (67)	14 (33)	
IV	37	21 (57)	16 (43)		14 (38)	23 (62)	
Grade							
Well differentiated	7	5 (71)	2 (29)	0.75	4 (57)	3 (43)	0.94
Moderately differentiated	110	79 (72)	31 (28)		66 (60)	44 (40)	
Poorly differentiated	20	16 (80)	4 (20)		13 (65)	7 (35)	
Unknown	1	1 (100)			1 (100)		

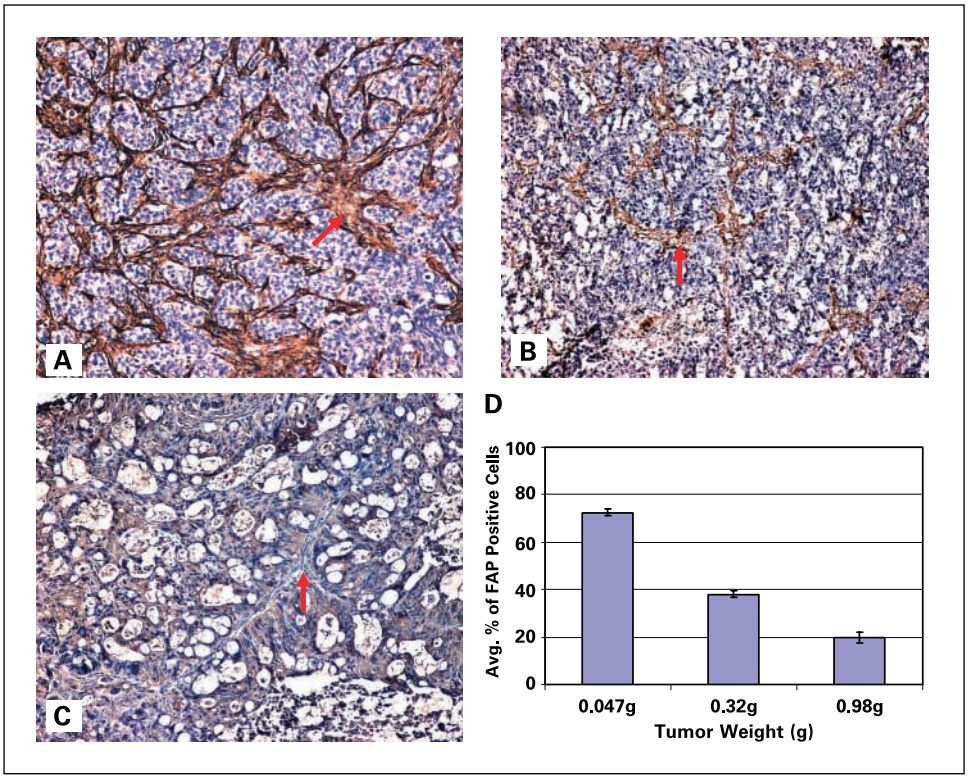

Fig. 2. FAP expression in representative HT29 xenografted tumor tissues. *A* to *C*, immunohistochemical analysis of FAP expression of different tumor sizes (*A*, 0.047 g; *B*, 0.32 g; *C*, 0.98 g). Arrows, stromal area in the tumor. *D*, measurement of area and intensity of FAP expression by ACIS. Histogram shows the average percent of FAP expression [% = brown area / brown (FAP staining) + blue (nuclear staining) × 100].

human colorectal cancer resulted in a more aggressive biological phenotype, Ariga et al. (23) suggested that increased stromal FAP in women with breast cancer was paradoxically associated with prolonged survival. Jin et al. correlated FAP/seprase with increased dysplasia and carcinoma development in cervical cancers and concluded that it may prove useful as a marker of early invasion. Two additional studies have described FAP staining characteristics and patterns in human gastric carcinoma (29, 30). Although FAP staining of the epithelial neoplasm has been seen in some of the above studies as well as occasionally in our study (data not shown), we intentionally focused exclusively on the stromal contribution of FAP for this study.

The two principal findings of our study are that stromal FAP is increased in early-stage cancer and yet is an adverse prognostic marker in advanced metastatic disease. The prognostic value of FAP in early-stage patients is confounded by the relatively few mortality events seen in the clinically stage I-III patients in this study. Given the controversies surrounding the benefit of adjuvant chemotherapy in early-stage disease, an exploratory analysis of disease-free survival in early stage I and II patients was conducted to determine if low FAP expression as determined semiquantitatively might portend a better disease-free survival. Although the results were not statistically significant (P = 0.23), it suggests the possibility that high FAP expression in early-stage patients might portend a worse prognosis and can be further studied to determine its usefulness in guiding adjuvant chemotherapy decisions.

Our findings lead us to hypothesize that perhaps FAP pathways may predominate early in the course of smaller tumors to facilitate tumor invasion and tumor motility that are required for metastases to occur, thus leading to the enhanced induction of FAP expression in early-stage disease. However, in patients whose tumors are larger and more advanced, FAP-dependent pathways may be diluted and diminished as other growth promoting pathways predominate, leading to our finding of decreased overall levels in this group. However, even in these advanced tumors, relatively persistent elevation in FAP levels may still signify a more aggressive tumor and a continued additive contribution of FAP-dependent pathways to tumor invasion and growth. Thus, high FAP may confer a worse prognosis even in those with advanced disease.

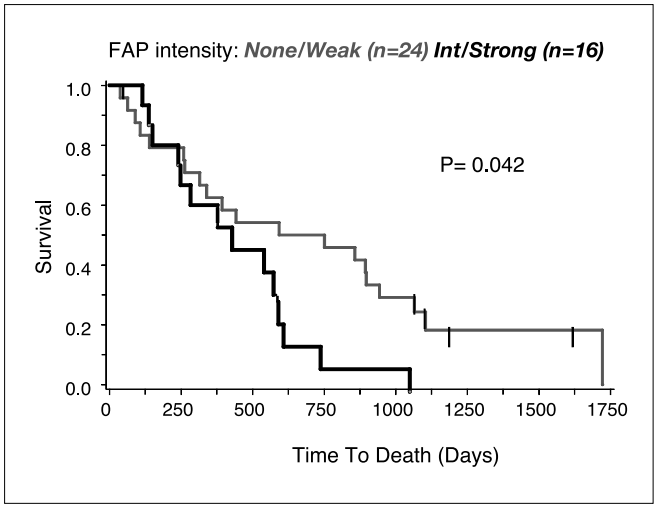

Fig. 3. Kaplan-Meier curve of patients with known metastases. Survival was estimated using Kaplan-Meier methods, which showed greater FAP intensity resulted in worse survival.

In conclusion, we find that stromal FAP is inversely related to clinical stage in human colon cancer and that increased intensity of FAP in the setting of advanced disease is associated with a decreased survival time. Clinical trials have been done using agents to inhibit FAP-dependent pathways in patients. The trials initiated to date have all been in the setting of metastatic disease. Further clinical research is needed in the early-stage setting given the possibility of greater FAP effects in that clinical scenario.

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