Clinical Proteomics Journal

ISSN 1542-6416/04/01:301–311/$25.00

Original Article

Evaluation of Clinical Colon Carcinoma Using Activity-Based Proteomic Profiling

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Abstract

The evaluation of clinical tumor tissues is a valuable approach to discovering novel drug targets because of the direct relevance of human samples. We used activity-based proteomic profiling (ABPP) to study the differences in serine hydrolase activities from 12 matched pairs of clinical normal and tumor colon tissues. Unlike traditional proteomics or measures of mRNA abundance, ABPP actually quantifies enzymatic activities, a characteristic crucial for drug targeting. Several serine hydrolases were differentially active in tumor vs normal tissues, despite a lack of obvious corresponding alterations in protein expression. We identified one tumor-specific activity by mass spectrometry to be fibroblast activation protein (FAP), an integral membrane serine protease that has been reported to be present only in tumor stroma or during wound healing and absent in normal tissues. FAP activity was further found to be approximately twofold higher in stage III relative to stage II colon cancer, suggestive of a role in tumor progression. We were also able to identify other proteins, some of which had not been previously linked to cancer, which had higher activity in tumors. Our results demonstrate the applicability of ABPP for the efficient identification of multiple clinical disease targets.

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Key Words: Tumor; activity; proteomics; colorectal adenocarcinoma; serine hydrolase.

Introduction

The lifetime probability of developing cancer is one in two for men and one in three for women *(1)*. As cancer remains the second leading cause of death behind heart disease, the search for novel drug targets continues. Common methods for evaluating normal vs tumor tissues include two-dimensional (2-D) gel electrophoresis and microarray, which measure protein and mRNA levels, respectively. These approaches search for differences on a global scale; however, mRNA levels do not always correlate to protein abundance, and protein abundance does not always reflect enzyme activity. Zymogen proforms, inhibitors, and protein-protein interactions can all regulate enzymatic activity. In order to increase the probability of finding a druggable cancer target, one approach is to find the differences in the "business end" of proteins, that is, in the protein activities that are upregulated in tumor tissues.

Here, we present an activity-based proteomic profiling (ABPP) approach for drug target discovery in clinical colorectal carcinoma. We used a fluorescently tagged, activity-based chemical probe to comprehensively scan the large serine hydrolase superfamily. This family comprises >1000 members and includes proteases, lipases, esterases, and amidases, among others. In addition, there are still many serine hydrolases whose functions are unknown. The advantage of the ABPP method is that one can use crude tissue lysates to rapidly scan for and quantify alterations in protein activities. Other advantages over traditional protein screening methods include the requirement for only small quantities of tissue (20 μg is used per gel lane), high sensitivity (low femtomole levels of active protein can be detected) *(2)*, and the ability to study both soluble and membrane protein activities. These conditions are well suited for evaluating clinical tissue specimens, which are frequently of limited quantities but provide the most relevant source of human disease targets.

From the analysis of 12 pairs (tumor and adjacent normal) of clinical colon tissues, we found the activities of 12 of 22 soluble serine hydrolases and 3 of 22 membrane serine hydrolases to be significantly increased in tumor vs normal tissues. In contrast, three proteins in the membrane fraction had significantly decreased activity. Following mass spectrometry identification, a number of proteins were found that had previously been studied in relation to cancer. Fibroblast activation protein (FAP) was one such tumor-specific activity, and, interestingly enough, by using ABPP we found a trend of increased FAP activity with more advanced tumors. We also report the identification of other protein activities that had not been previously linked with cancer, such as the intracellular isoforms of platelet activating factor acetylhydrolase (PAF-AH), phospholipases with emerging roles in inflammatory disease. The identification of cancer-associated proteins validates ABPP as a rapid means of tumor target discovery and implicates the other activities as having significant roles.

Materials and Methods

Tissues

Matched primary tumors and adjacent normal colon tissues from 12 patients diagnosed with colorectal adenocarcinoma were selected from samples residing in the Global Repository™ at Genomics Collaborative, Inc. (Cambridge, MA). All samples were obtained from human subjects with full informed consent. The study set consisted of tumor and normal tissue pairs from patients with stage II (n = 6) and stage III (n = 6; lymph node involvement) diagnoses. Staging was established by pathologists at the site of collection

and confirmed by pathologists at Genomics Collaborative Inc. The median percentage of carcinoma in the tumor tissues was 78%, with 0% in the normal tissues. Following procurement from surgical specimens, tissues were flash frozen and stored in liquid nitrogen until used.

Tissues were processed by crushing them into approx 1-mm pieces under liquid nitrogen, using a ceramic mortar and pestle. Approximately 50–100 mg wet weight of each tissue was homogenized in 50 m*M* Tris buffer at pH 7.4, using an Omni mechanical homogenizer (Omni International, Warrenton, VA). Each lysate was briefly sonicated and the unlysed material was pelleted by centrifugation at 1100*g* for 15 min. The crude supernatant was then subjected to centrifugation at 110,000*g* for 1 h at 4°C. The resultant high-speed supernatant containing cytosolic proteins was collected as the soluble fraction. The membrane pellet was solubilized by sonication in 50 m*M* Tris buffer at pH 7.4 with 0.1% Triton X-100. Protein concentrations were assayed using the Dc protein kit (Bio-Rad Laboratories, Hercules, CA). Approximately 1 and 1.5% of the original tissue weight were recovered in the soluble and membrane protein fractions, respectively.

Proteome Labeling and Analysis

The fluorophosphonate TAMRA (AX5070) probe was synthesized as previously described *(2)* and was solubilized in dimethyl sulfoxide (DMSO). To label tissue lysates, we added AX5070 probe to each tissue lysate (1 mg/mL) to a final concentration of 2 μ*M* and incubated it for 1 h at 25°C. One hour has been shown to provide complete end-point labeling. Final DMSO concentrations were <5% of total volume. Membrane proteomes were deglycosylated with PNGase F (New England Biolabs, Beverly, MA) after probe labeling in order to sharpen bands and to get baseline molecular weights. Reactions were quenched by adding an equal volume of 2× reducing SDS-PAGE loading buffer and heating at 95°C for 5 min. Equal protein amounts (20 μg/lane) were loaded onto 12% SDS-PAGE gels. Gels were directly scanned for fluorescence using a 605-nm bandpass filter for detection on the Hitachi FMBIO IIe (Miraibio, Alameda, CA). Fluorescence was measured using the accompanying Image-Analysis software for each distinct activity band on the gel and expressed as fluorescence volume units.

Protein Identification

Tissue lysates (1–3 mg) were labeled with AX5070, subsequently denatured by heating to 65°C for 10 min with 8 *M* urea and 10 m*M* dithiothreitol, and modified with 40 m*M* iodoacetamide. The lysate was then applied to a Bio-Rad PD10 gel exclusion column to eliminate excess free probe and to exchange the buffer for 2 *M* urea and 20 m*M* ammonium bicarbonate. The eluate was adjusted to contain 1% SDS, and heated at 65°C for 5 min before adding an equal volume of 2× binding buffer (2% Triton X-100, 1% Tergitol type NP40, 300 m*M* NaCl, 2 m*M* EDTA, 20 m*M* Tris, pH 7.4). This was then incubated with Affi-Gel Hz Hydrazide Gel (Bio-Rad) coupled to anti-rhodamine monoclonal antibodies custom generated through Rockland Immunochemicals (Gilbertsville, PA). The AX5070-labeled proteins were eluted in nonreducing SDS-PAGE loading buffer and resolved on SDS-PAGE. After scanning for fluorescence, the protein bands of interest were excised and prepared for tryptic digestion. Peptides were analyzed by nano-liquid chromatography/ tandem mass spectrometry (LC/MS/MS), on a combination system of an Agilent 1100 capillary HPLC/Micro Auto-sampler (Agilent Technologies, Palo Alto, CA) and an LCQ DecaXP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). Data generated were analyzed using the Mascot search engine for protein

identification (Matrix Science, London, UK). A positive protein identification was accepted when the following criteria were all met: (1) At least three peptides were assigned to the protein; (2) ranking of the ion match was 1 with the mass error <1; and (3) the probability-based Mowse score was >36 ($p < 0.05$).

For some fluorescent protein bands, peptides matching multiple proteins were found by mass spectrometry. In order to narrow down the identity of the major activity in the band, specific inhibitors, if available, were used in subsequent labeling studies. For those studies, 20 μg of tissue lysate was preincubated with or without inhibitor at various concentrations for 15 min at room temperature. The AX5070 probe was then used to label the active serine hydrolases in the lysate. The prevention of AX5070 labeling by a specific inhibitor, seen as an absence of fluorescence for the protein band on the gel, indicates the protein's identity. Specific inhibitors included prolyl endopeptidase inhibitor (Calbiochem, San Diego, CA), methyl arachidonyl fluorophosphonate (phospholipase inhibitor; Cayman Chemical, Ann Arbor, MI), and 1-*O*-palmitol-2-(*N*-methylcarbamyl)-*sn*-glycero-3-phosphocholine (nonhydrolyzable form of PAF; Sigma, St. Louis, MO).

F19 Antibody and Western Blot

The F19 hybridoma cell line that produces antibodies against human fibroblast activation protein was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI supplemented with 10% fetal bovine serum. Monoclonal antibody F19 was affinity purified from F19 hybridoma-conditioned media using protein A Sepharose.

Tissue lysates labeled with AX5070 were run on SDS-PAGE, scanned for fluorescence, and transferred to nitrocellulose. The blot was blocked in 5% nonfat dried milk/TBS-T (50 m*M* Tris with 0.1% Tween-20) for 1 h at 25°C. For the primary antibody incubation, blots were incubated with monoclonal antibody F19 at 2 μg/mL in 5% milk/TBS-T, 4°C overnight. Following washes in TBS-T, the blot was incubated with 1:5000 horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Pierce Chemical Co., Rockford, IL) in 5% milk/TBS-T for 1 h at 25°C. After washing, the blot was developed in SuperSignal West Pico Substrate (Pierce) and visualized using the FluorChem imaging system (Alpha Innotech Corp., San Leandro, CA).

Statistics

Normal and tumor samples were always run and scanned on the same gel. The paired student's *t*-test was used to analyze the mean fluorescence volume units of individual protein bands from normal and tumor tissues. Significance was accepted if $p < 0.05$ and if at least two of three experiments were consistent statistically.

Results

Serine Hydrolase Activities in Tumor and Normal Colon Tissues

The AX5070 probe was developed to selectively react with the catalytic serine of the large superfamily of serine hydrolase enzymes. This activity-based chemical probe consists of a fluorophosphonate reactive "warhead," an alkyl linker, and a fluorescent tetramethylrhodamine (TAMRA) tag (Fig. 1). The probe covalently links to active enzyme (but not to inactive versions such as proenzyme, denatured protein, and inhibited enzyme), thus allowing for an assessment of the active enzymatic state of a complex protein mixture *(2,3).* In any given sample, only a subset of the total proteins is made up of serine hydrolases, and only a portion of this subset is active proteins that will bind to AX5070. Therefore, as this process results in the fluorescent tagging of only a portion of the proteome, the AX5070-tagged proteins can be easily resolved by one-dimensional SDS-PAGE and visualized using a fluorescence scanner.

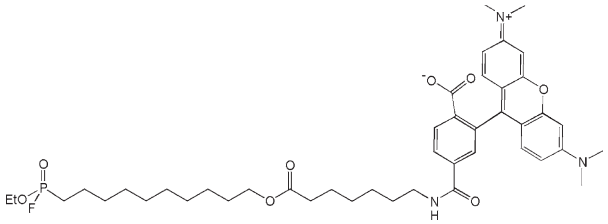

Fig. 1. AX5070, activity-based serine hydrolase probe. AX5070 consisted of a fluorophosphonate reactive group, an alkyl linker, and a fluorescent tetramethylrhodamine tag.

The AX5070 probe was used to assess the serine hydrolase activities in tissue samples from patients with colorectal adenocarcinoma. From the total of 12 paired (tumor and adjacent normal) tissues, 6 were from patients with stage II cancer and 6 were from patients with stage III cancer. Tissue lysates from each patient were fractionated into soluble and membrane fractions by high-speed centrifugation and labeled with AX5070. Equal amounts of proteome were loaded onto SDS-PAGE gels. After resolving the proteins, the gels were scanned for fluorescence and each protein band was quantified for fluorescence volume as a measure of activity. Despite the expected biological variance in enzyme activities among patients, a number of protein bands were found to display statistically significant differences in activity. In the soluble fraction, 12 of 22 fluorescent protein bands were significantly upregulated in tumor vs normal tissues (Fig. 2A). In the membrane fraction, the fluorescence of three protein bands was significantly increased, whereas that of three others was significantly decreased in tumor relative to normal tissue (Fig. 2B). Interestingly, the three membrane protein activities that increased had minimal probe labeling in normal tissues, therefore appearing to be tumor specific. Coomassie blue staining of the gels after fluorescence scanning did not reveal obvious accompanying changes in protein abundance (Fig. 3A,B). This is not unexpected, because activity-based probes are so sensitive that enzyme activities can be easily seen below the picomole range (equivalent to nanogram amounts for a typical protein mass of 50 kDa), which is close to the limit of detection for Coomassie blue staining.

Protein Identifications and Activities

In order to identify the fluorescently labeled, active serine hydrolases from the proteome, the AX5070-tagged proteins were captured by immunoprecipitation using an anti-rhodamine antibody in order to separate them from nonlabeled proteins. The fluorescently labeled proteins were then resolved on SDS-PAGE and the individual bands were excised, digested with trypsin, and subjected to peptide analysis by nano-LC/MS/MS. Resultant peptide identities were searched out using Mascot. For some protein bands, especially in the crowded 25- to 37-kDa molecular weight range, peptides from multiple proteins were identified within a single band and it was not clear which of the proteins were responsible for the observed changes in activity. In those cases where inhibitors were available, we verified the band identity in additional studies by inhibiting probe labeling as described in Materials and Methods (data not shown).

Table 1 lists the identified proteins that exhibited significantly altered activity between tumor and normal tissues. When more than one protein was identified within a band, they were listed as "and/or." The majority of the protein activities that changed were elevated in tumor relative to normal colon tissues. In contrast, the highly related carboxylesterases of 50–60 kDa had distinctly decreased activity in tumors, comprising two of the three protein bands observed to have lower activities. To our knowledge, only five of the differentially active proteins (Table 1, bold highlighted)

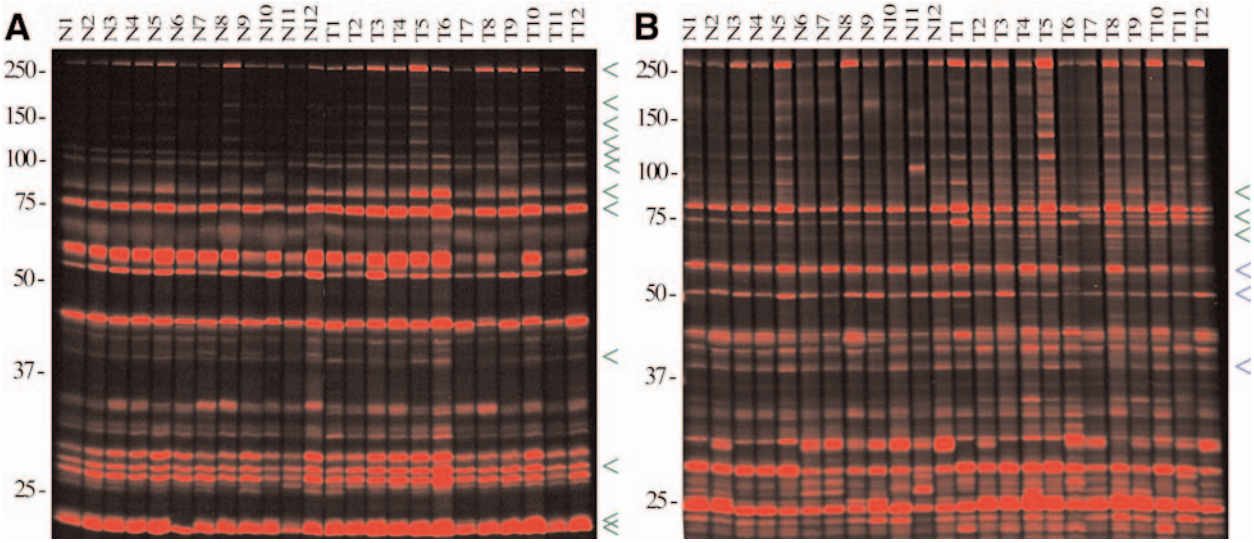

Fig. 2. Serine hydrolase activity profiles of clinical normal (N) and tumor (T) colon tissue proteomes. Representative in-gel fluorescence of serine hydrolase activities from **(A)** soluble and from **(B)** deglycosylated membrane fractions of clinical proteomes labeled with AX5070. The tumor (T) or adjacent normal (N) colon tissues were numbered 1–12, in which patients 1–6 and 7–12 have been diagnosed with stage II and III colorectal adenocarcinoma, respectively. The green arrowheads indicate proteins whose activities were higher in tumors vs normal tissues, whereas blue arrowheads indicate lower activities.

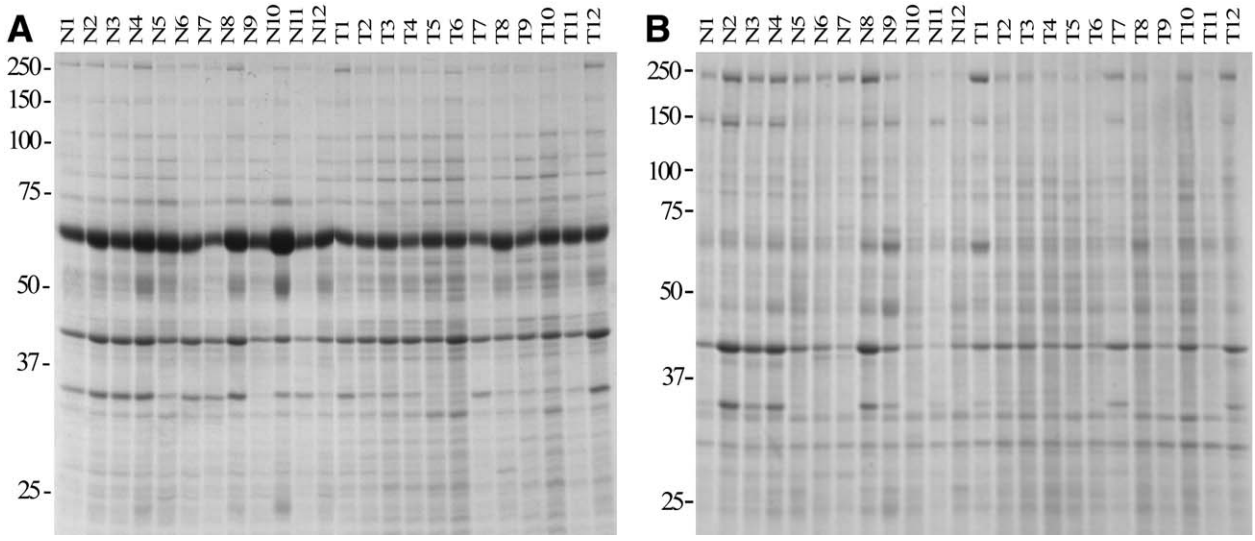

Fig. 3. Total protein staining of clinical normal (N) and tumor (T) colon tissue proteomes. The gels of **(A)** soluble and of **(B)** deglycosylated membrane fractions of clinical proteomes represented in Fig. 2 were stained for total protein expression using Coomassie blue. The tumor (T) or adjacent normal (N) colon tissues were numbered 1–12, in which patients 1–6 and 7–12 have been diagnosed with stage II and III colorectal adenocarcinoma, respectively. Twenty micrograms of total protein were loaded per gel lane.

Table 1
Serine Hydrolases That Exhibited Significant Changes in Activity in Clinical Tumor vs Normal Colon Tissues

Soluble proteins: increased activity in tumor
Fatty acid synthase (4.1)
Dipeptidyl peptidase IV (4.9)
Prolyl endopeptidase (2.3)
N-acylaminoacyl-peptide hydrolase (2.9)
Cytosolic acyl coA thioester hydrolase and/or Platelet-activating factor acetylhydrolase II (2.6)
Platelet-activating factor acetylhydrolase Ib β- and γ-subunits (1.9)
Acylprotein thioestrase I/Lysophospholipase II (2.1)
Membrane Proteins: increased activity in tumor
Fibroblast activation protein (23)
Membrane Proteins: decreased activity in tumor
Carboxylesterase hCE2 (0.7)
Carboxylesterases ACAT, HUMCOXYSB, HSSE1, HUMLCEA, and/or HBR3 (0.5)

Activity was measured as fluorescence volume units in the identified protein bands. The ratio of the average activity values of 12 colon tumors and of 12 matched-patient normal colon tissues is calculated and presented in parentheses (T:N). Proteins that have been previously associated with cancer in the literature are highlighted in bold.

have been previously reported in the literature to play a role in human cancer: fatty acid synthase, dipeptidyl peptidase IV (DPP-IV), prolyl endopeptidase (PEP), FAP, and carboxylesterase 2 (hCE2). We are currently ascertaining the identities of the other protein bands that displayed differential activity. These were proteins that were generally of very low activity that approached the limits of detection (femtomole) for the mass spectrometer, or were identified but could not be distinguished from among other proteins of similar molecular weight.

Fibroblast Activation Protein

Of the proteins that displayed elevated activity in tumor tissues and very low-to-absent activity in patient-matched, adjacent normal tissues, the integral membrane protein FAP was the most distinguishable. Mass spectrometry confirmed that FAP was the only protein in the band of interest. Notably, the average tumor-to-normal activity ratios for FAP were 1.5- to 3.5-fold higher in stage III (patients 7–12) vs stage II (patients 1–6) carcinoma in three repeat experiments, giving an overall increase of 2.3-fold (Fig. 4A). In comparing stages, the number of subjects in each group decreased from $n = 12$ to $n = 6$, resulting in a loss of statistical power. Nevertheless, this trend appears to be the first indication that FAP activity may increase with tumor progression.

The activities of *N*-acylaminoacyl-peptide hydrolase and PEP characteristically flank FAP near 75 kDa when the membrane sample is deglycosylated (Fig. 4B). We have observed and verified this feature in multiple tissue types and cell lines (data not shown), making for a convenient assessment of FAP activity.

We next determined whether the increased FAP activity in the tumor samples was associated with increased protein expression. The low amount of FAP protein was not detectable with Coomassie blue staining; therefore, FAP expression was measured by Western blot using the F19 antibody (Fig. 4C). In initial experiments, we found that the monoclonal F19 antibody recognized deglycosylated FAP very poorly and we therefore used native membrane proteome for the Western blot. Under these conditions, the denatured, glycosylated FAP subunit is 97 kDa. Similar to FAP activity (Fig. 4B), FAP protein expression (Fig. 4C) was found to be higher in stage III tumor samples as compared with stage II, and there was no detectable signal in the normal tissue samples. We were able to correlate FAP activity

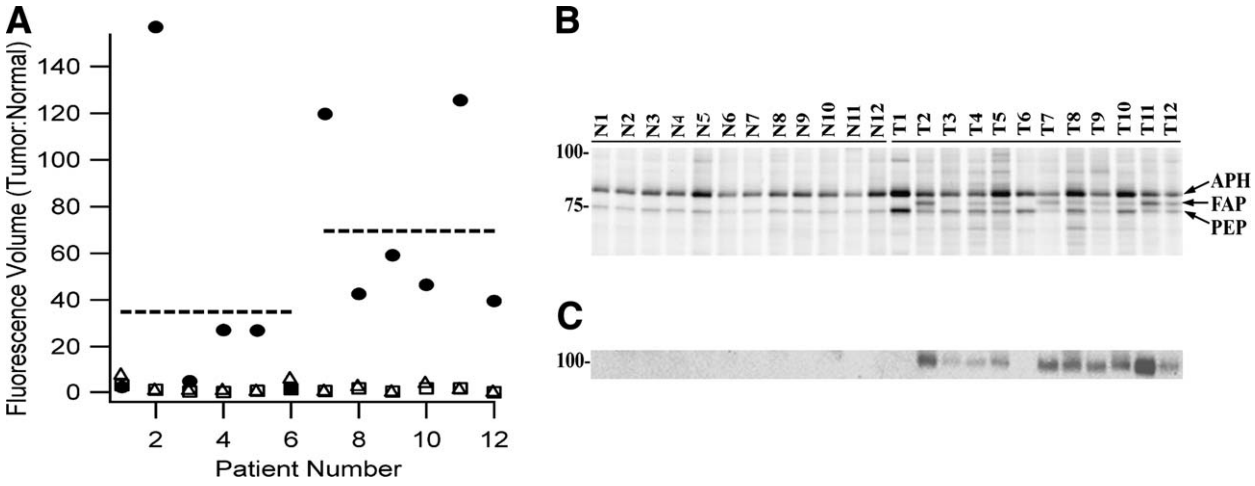

Fig. 4. Fibroblast activated protein (FAP) is increased in tumor (T) relative to normal (N) colon tissues in a stage-dependent manner. Tumor membrane proteomes from patients with stage III cancer (T7–T12) displayed a trend of having higher FAP activity as compared with those with stage II cancer (T1–T6) as measured by AX5070 labeling. **(A)** The relative activity (fluorescence) of FAP is graphically depicted to highlight the approximately twofold higher activity in patients with stage III cancer. Dashed lines indicate the average values. Closed circles represent FAP; open squares and triangles represent N-acylaminoacyl-peptide hydrolase (APH) and prolyl endopeptidase (PEP) activities, respectively, as measured in the membrane proteome fraction. **(B)** Shown is a grayscale depiction of the in-gel fluorescence of the deglycosylated membrane proteome region where FAP characteristically migrates, flanked by APH and PEP. There was minimal FAP activity in the normal tissues. **(C)** The amount of FAP protein expression was determined by Western blot of native membrane proteomes. The amount of FAP protein was found to be increased in patients with more advanced cancer, similar to that seen for FAP activity. Shown are representative data from three repeat experiments.

(by probe labeling) to FAP expression (by Western blot) with $r = 0.94$, indicating that the increase in activity arises entirely from an increase in protein expression.

Discussion

It has become increasingly evident that the development of cancer results from a multitude of different cell types acting in concert to optimize tumor progression and survival *(4)*. Stromal cells, including fibroblasts, have been reported to make up as much as 90% of the tumor mass, whereas other cell types such as leukocytes and macrophages are known to infiltrate tumors and contribute to the tumor environment. All of these cells have dynamic enzymatic activities; for example, activated fibroblasts are known to acquire the expression of proteolytic enzymes such as matrix metalloproteinases and urokinase plasminogen activator (reviewed by Martin *[5]* and Schurch *[6]*). Given such complexities, the search for novel tumor targets must rely on unbiased and global methods of analysis.

ABPP is well suited to sifting through complex proteomes such as tumors. It reports only on protein activity, is exquisitely sensitive, and allows for the analysis of membrane proteins. For screening, only about 20 μg of protein is required for activity analysis. Furthermore, ABPP limits the class of proteins to that classified by the probe (e.g., serine hydrolases, metalloproteinases, kinases), therefore producing a manageable but significant set of results. This aspect is also advantageous for rapidly classifying novel proteins.

In this report, we applied ABPP toward the discovery of possible drug target enzymes in human clinical colorectal adenocarcinoma using a small pilot set of matched tissues. From the large protein superfamily, the serine hydrolases, a number of active enzymes were identified, a subset of which showed differential activity between tumor and normal tissues. One of these proteins, fatty acid synthase, has long been known to be overexpressed in tumors, and inhibitors have been developed that have shown antitumor activity *(7,8).* In contrast, we also identified elevated serine hydrolase activities that are not well associated with cancer, such as DPP-IV and PEP. These enzymes are currently pursued as targets for type 2 diabetes and memory loss, respectively *(9–12)*, with scant reports of their increases in cancer *(13,14)*. Nevertheless, their roles as peptidases make them attractive targets for new indications.

The selective expression of FAP in tumors, specifically in stromal fibroblasts, was initially recognized using qualitative immunohistochemistry methods *(15).* It is not detected in normal adult tissues. Recently, methods have been devised to quantitatively measure FAP's activity by either immunocapturing FAP from a cellular lysate prior to adding a fluorogenic substrate *(16)*, or by measuring FAP's ability to degrade 3H-gelatin *(17).* Using ABPP, we detected a twofold increase in the relative ratio of FAP activity in patients with more advanced clinical disease, a finding that is suggestive of an involvement in cancer progression. We were able to measure the FAP activity directly and quantitatively without prior purification because FAP appears as a very distinct band on SDS-PAGE after deglycosylation. This represents an advance in the detection of FAP and may serve as a paradigm for other proteins. Immunocapture-based methods may not be optimally specific or quantitative. DPP-IV has been demonstrated to form heteromeric dimers with FAP *(18,19).* Therefore, immunoprecipitation of FAP could potentially precipitate DPP-IV as well, resulting in an overestimation of dipeptidyl peptidase activity. Immunocapture may also precipitate other FAP-associated proteins with prolylpeptidase activity. As FAP has been observed in high-molecular-weight complexes of >400,000 kDa, this possibility may exist *(20)*. Finally, in order for the immunocapture methods to be accurate, the antibody must capture all of the FAP in the proteome or at least capture consistently from proteome to proteome. Incomplete immunocapture could lead to an underestimation of activity. Because activity-based probes label at the active site, the activity reflects that of the protein of interest alone. The only circumstance when this is not true occurs when multiple protein activities share the same molecular weight and cannot be discerned by one-dimensional SDS-PAGE. In those infrequent instances, the use of selective inhibitors, two-dimensional gels, or peptide-based profiling *(21)* can resolve the identity.

Of several carboxylesterases, hCE2 has been recognized as the key enzyme that converts the chemotherapy prodrug irinotecan to the active cytotoxic metabolite, SN-38 *(22,23)*. It is also the only carboxylesterase whose gene and protein expressions correlate with irinotecan-hydrolyzing activity *(22,24).* Interestingly, a number of investigators have found hCE2 expression and activity to be lower in tumor relative to normal tissues, despite the use of irinotecan as a common treatment for advanced colorectal cancer *(25,26).* Using activity-based proteomic profiling, we also observed hCE2 activity to be 50% lower in colorectal tumors compared with adjacent normal tissue, corroborating previous reports.

We identified several protein activities that were elevated in tumor compared with normal colon tissues that had not been previously recognized to have an association with cancer. Of these, a particularly interesting finding was

the approximately twofold increase in the activities of PAF-AHs Ib and II, two intracellular phospholipase A_2 enzymes that serve distinct functions (reviewed by Stafforini *[27]* and Arai *[28]*). PAF-AH Ib regulates the levels of PAF, a phospholipid that mediates a broad range of immune and allergic reactions. In contrast, PAF-AH II scavenges oxidatively fragmented phospholipids and can protect against oxidative stress-induced apoptosis. To our knowledge, neither of these has been implicated with a role in cancer, although it is not difficult to imagine an involvement of immune reactivity and oxidative stress within the tumor environment. Intriguingly, the activity of the secreted plasma isoform of PAF-AH (which has homology with intracellular isoform II) was recently reported to be increased in the plasma of patients with colorectal cancer *(29)*.

There were a few protein activities that we did not observe in these samples that may have been expected, for example, urokinase plasminogen activator (uPA). Because we have seen AX5070 label uPA in other samples such as human umbilical vein endothelial cells, it is likely that we did not identify it because it was not very active in the clinical colon samples.

The present study highlights the powerful application of ABPP toward the examination of clinical tumor tissues, complex proteomes that are available in only scarce quantities but that are most informative for target discovery. Protein activities that are increased in tumor relative to normal tissues are possible drug targets, whose inhibition may produce antitumor effects. Approximately half of such protein activities identified in this study have already been validated in the literature to have an association with cancer. It is probable that additional studies will further implicate the other half to also play a role. Because the proteins identified by ABPP are based on reactivity with small molecules, they should prove to be "druggable" via inhibition of the active site. ABPP can further be used to screen for the effectiveness and specificity of drug inhibition, potentially shortening the drug development process.

Acknowledgments

We thank John Kozarich and Jonathan Rosenblum for their critical evaluations of the manuscript and Matthew Patricelli for the development and synthesis of the AX5070 probe.

References

1. American Cancer Society. Cancer facts and figures 2004. 2004. Available at: http:// www.cancer.org. Accessed on 8/19/04.
2. Patricelli, M.P., Giang, D.K., Stamp, L.M., and Burbaum, J.J. (2001). Direct visualization of serine hydrolase activities in complex proteomes using fluorescent active site-directed probes. Proteomics 1:1067–1071.
3. Liu, Y., Patricelli, M.P., and Cravatt, B.F. (1999). Activity-based protein profiling: the serine hydrolases. Proc. Natl. Acad. Sci. USA 96: 14694–14699.
4. Hanahan, D. and Weinberg, R.A. (2000). The hallmarks of cancer. Cell 100:57–70.
5. Martin, M., Pujuguet, P., and Martin, F. (1996). Role of stromal myofibroblasts infiltrating colon cancer in tumor invasion. Pathol. Res. Pract. 192:712–717.
6. Schurch, W. (1999). The myofibroblast in neoplasia. Curr. Top. Pathol. 93:135–148.
7. Kuhajda, F.P., Pizer, E.S., Li, J.N., Mani, N.S., Frehywot, G.L., and Townsend, C.A. Synthesis and antitumor activity of an inhibitor of fatty acid synthase. Proc. Natl. Acad. Sci. USA 97: 3450–3454.
8. Li, J.N., Gorospe, M., Chrest, F.J., Kumaravel, T.S., Evans, M.K., Han, W.F., et al. (2001). Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53. Cancer Res. 61:1493–1499.
9. Holst, J.J. and Deacon, C.F. (1998). Inhibition of the activity of dipeptidyl-peptidase IV as a treatment for type 2 diabetes. Diabetes 47:1663–1670.
10. Weideman, P.E. and Trevillyan, J.M. (2003). Dipeptidyl peptidase IV inhibitors for the

treatment of impaired glucose tolerance and type 2 diabetes. Curr. Opin. Investig. Drugs 4:412–420.
11. Toide, K., Shinoda, M., and Miyazaki, A. (1998). A novel prolyl endopeptidase inhibitor, JTP-4819—its behavioral and neurochemical properties for the treatment of Alzheimer's disease. Rev. Neurosci. 9:17–29.
12. Bellemère, G., Morain, P., Vaudry, H., and Jegou, S. (2003). Effect of S 17092, a novel prolyl endopeptidase inhibitor, on substance P and alpha-melanocyte-stimulating hormone breakdown in the rat brain. J. Neurochem. 84: 919–929.
13. Šedo, A., Křepela, E., and Kasafirek, E. (1991). Dipeptidyl peptidase IV, prolyl endopeptidase and cathepsin B activities in primary human lung tumors and lung parenchyma. J. Cancer Res. Clin. Oncol. 117:249–253.
14. de la Haba-Rodriguez, J., Macho, A., Calzado, M.A., Blazquez, M.V., Gomez, M.A., Munoz, E.E., et al. (2002). Soluble dipeptidyl peptidase IV (CD-26) in serum of patients with colorectal carcinoma. Neoplasma 49:307–311.
15. Garin-Chesa, P., Old, L.J., and Rettig, W.J. (1990). Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. Proc. Natl. Acad. Sci. USA 87:7235–7239.
16. Park, J.E., Lenter, M.C., Zimmermann, R.N., Garin-Chesa, P., Old, L.J., and Rettig, W.J. (1999). Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. J. Biol. Chem. 274:36505–36512.
17. Kelly, T. (1999). Evaluation of seprase activity. Clin. Exp. Metastasis 17:57–62.
18. Scanlan, M.J., Raj, B.K., Calvo, B., Garin-Chesa, P., Sanz-Moncasi, M.P., Healey, J.H., et al. (1994). Molecular cloning of fibroblast activation protein alpha, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers. Proc. Natl. Acad. Sci. USA 91:5657–5661.
19. Wesley, U.V., Albino, A.P., Tiwari, S., and Houghton, A.N. (1999). A role for dipeptidyl peptidase IV in suppressing the malignant phenotype of melanocytic cells. J. Exp. Med. 190:311–322.
20. Rettig, W.J., Su, S.L., Fortunato, S.R., Scanlan, M.J., Raj, B.K., Garin-Chesa, P., et al. (1994). Fibroblast activation protein: purification, epitope mapping and induction by growth factors. Int. J. Cancer 58:385–392.
21. Okerberg, E.S., Wu, J., Zhang, B., Samii, B., Blackford K., Winn D.T., et al. (2005). High-resolution functional proteomics by active-site peptide profiling. Proc. Natl. Acad. Sci. USA 102:4996–5001.
22. Sanghani, S.P., Quinney, S.K., Fredenburg, T.B., Sun, Z., Davis, W.I., Murry, D.J., et al. (2003). Carboxylesterases expressed in human colon tumor tissue and their role in CPT-11 hydrolysis. Clin. Cancer Res. 9:4983–4991.
23. Humerickhouse, R., Lohrbach, K., Li, L., Bosron, W.F., and Dolan, M.E. (2000). Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. Cancer Res. 60:1189–1192.
24. Xu, G., Zhang, W., Ma, M.K., and McLeod, H.L. (2002). Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of irinotecan. Clin. Cancer Res. 8:2605–2611.
25. Guichard, S., Terret, C., Hennebelle, I., Lochon, I., Chevreau, P., Fretigny, E., et al. (1999). CPT-11 converting carboxylesterase and topoisomerase activities in tumour and normal colon and liver tissues. Br. J. Cancer 80:364–370.
26. Tobin, P.J., Dodds, H.M., Clarke, S., Schnitzler, M., and Rivory, L.P. (2003). The relative contributions of carboxylesterase and beta-glucuronidase in the formation of SN-38 in human colorectal tumours. Oncol. Rep. 10: 1977–1979.
27. Stafforini, D.M., McIntyre, T.M., Zimmerman, G.A., and Prescott, S.M. (1997). Platelet-activating factor acetylhydrolases. J. Biol. Chem. 272:17895–17898.
28. Arai, H., Koizumi, H., Aoki, J., and Inoue, K. (2002). Platelet-activating factor acetylhydrolase (PAF-AH). J. Biochem. 131:635–640.
29. Denizot, Y., Desplat, V., Drouet, M., Bertin, F., and Melloni, B. (2001). Is there a role of platelet-activating factor in human lung cancer? Lung Cancer 33:195–202.