

Research Article

Cathepsin B Expression and Survival in Colon Cancer:
Implications for Molecular Detection of Neoplasia

Andrew T. Chan^{1,3}, Yoshifumi Baba⁵, Kaori Shima⁵, Katsuhiko Noshō⁵, Daniel C. Chung¹, Kenneth E. Hung^{6,7}, Umar Mahmood², Karen Madden⁸, Kirtland Poss⁸, Audrey Ranieri¹, Daniel Shue¹, Raju Kucherlapati⁷, Charles S. Fuchs^{3,5}, and Shuji Ogino^{3,4}

Abstract

Background and Aims: Proteases play a critical role in tumorigenesis and are upregulated in colorectal cancer and neoplastic polyps. In animal models, cathepsin B (CTSB)-activatable imaging agents show high enzyme activity within intestinal tumors.

Methods: We conducted a prospective cohort study of 558 men and women with colon cancer with tumors that were accessible for immunohistochemical assessment. We used Cox proportional hazards models, stratified by stage, to compute colon cancer-specific and overall mortality according to tumoral expression of CTSB.

Results: Among 558 participants, 457 (82%) had tumors that expressed CTSB (CTSB positive) and 101 (18%) had tumors that did not express CTSB (CTSB negative). CTSB expression was not associated with disease stage ($P = 0.19$). After a median follow-up of 11.6 years, there were 254 total and 155 colon cancer-specific deaths. Compared with participants with CTSB-negative tumors, participants with CTSB-positive tumors experienced a multivariate hazard ratio for colon cancer-specific mortality of 1.99 (95% confidence interval, 1.19-3.34) and overall mortality of 1.71 (95% confidence interval, 1.16-2.50). CTSB expression was independently associated with *KRAS* ($P = 0.01$) and *BRAF* mutation ($P = 0.04$), but not microsatellite instability status, CpG island methylator phenotype status, *PIK3CA* mutation, LINE-1 methylation, TP53 expression, or PTGS2 (cyclooxygenase-2) expression. Among 123 individuals with adenomas, 91% expressed CTSB.

Conclusions: As assessed by immunohistochemistry, CTSB is expressed in the vast majority of colon cancers, independent of stage, and is significantly associated with higher risk of colon cancer-specific and overall mortality.

Impact: These results support the potential of CTSB a target for image detection of neoplastic lesions in humans. *Cancer Epidemiol Biomarkers Prev*; 19(11); 2777-85. ©2010 AACR.

Introduction

Proteases play a critical role in tumorigenesis by facilitating rapid cell cycling, mediating local invasion, fueling angiogenesis, and promoting metastasis (1). Specifically, cathepsin B (CTSB, the Human Genome Organisation-approved official gene symbol), a lysosomal cysteine

protease, has been shown to be involved in tumor initiation, hyperproliferation, and dedifferentiation, and is upregulated in early human colon adenomas, carcinomas, and metastatic lesions (2-7). The central role of CTSB in carcinogenesis suggests that it is not only a promising target for therapy or chemoprevention, but also for molecular detection of neoplasia (8).

Authors' Affiliations: ¹Gastrointestinal Unit and ²Division of Nuclear Medicine and Molecular Imaging, Massachusetts General Hospital and Harvard Medical School, ³Channing Laboratory, Department of Medicine, and ⁴Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, ⁵Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, ⁶Department of Medicine, Tufts Medical Center, and ⁷Harvard-Partners Center for Genetics and Genomics and Harvard Medical School, Boston, Massachusetts; and ⁸VisEn Medical, Bedford, Massachusetts

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A.T. Chan, Y. Baba, K. Shima, C.S. Fuchs, and S. Ogino contributed equally to this work.

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Corresponding Authors: Andrew T. Chan, Gastrointestinal Unit, Massachusetts General Hospital, 55 Fruit Street, GRJ-722, Boston, MA 02114. Phone: 617-726-3212; Fax: 617-726-3673. E-mail: achan@partners.org or Shuji Ogino, Center for Molecular Oncologic Pathology, Dana-Farber Cancer Institute, Brigham and Women's Hospital, 44 Binney Street, Room JF-215C, Boston, MA 02115. Phone: 617-632-3978; Fax: 617-277-9015. E-mail: shuji_ogino@dfci.harvard.edu

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In previous work, we developed a novel class of optical imaging agents that are “smart” near IR (NIRF) protease-activatable agents that become brightly fluorescent in areas of increased CTSB expression, as seen in colorectal neoplasia (9, 10). These agents offer high tumor to background ratio compared with nonspecific agents, due to their selective activation. The agents are optically silent in their native (quenched) state and become highly fluorescent after enzyme-mediated release of fluorochromes, resulting in *in vitro* signal amplification of several hundred-fold. In *Apc^{Min}/+* mice, immunohistochemistry and fluorescent antibody microscopy show that CTSB is expressed throughout the adenoma in epithelial and stromal cells (8). When mice were injected i.v. with the cathepsin-activatable agent, adenomas became highly fluorescent, indicative of high protease activity, and were easily visualized with a target to background ratio of 9:1 using NIRF imaging as opposed to 1:1 for standard white light imaging (8).

Given this promising preclinical data, we examined the importance of CTSB in human colonic carcinogenesis by determining the overall prevalence of CTSB expression in human colon tumors. Furthermore, given the key role of CTSB in the pathogenesis of tumor growth and invasion, we specifically assessed the relationship between CTSB expression on prognosis and other important tumoral molecular markers in colon cancer.

Materials and Methods

Study population

The Nurses' Health Study (NHS) was established in 1976 when 121,701 U.S. female registered nurses, 30 to 55 years of age, completed a mailed questionnaire. The Health Professionals Follow-up Study (HPFS) was established in 1986 as a parallel cohort of 51,529 U.S. male dentists, optometrists, osteopaths, podiatrists,

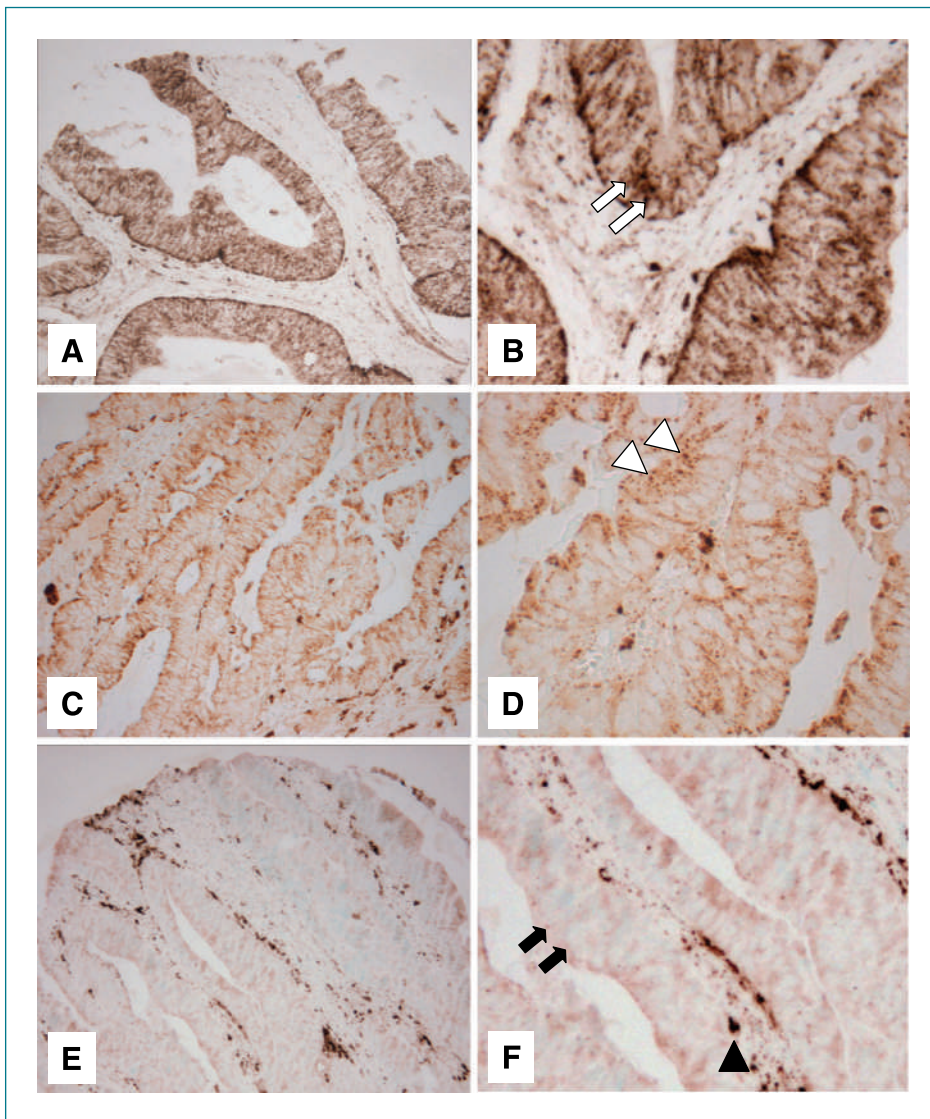


Figure 1. CTSB expression in colon cancer. A and B, strong expression of CTSB in colon cancer cells (white arrows). C and D, weak expression of CTSB in colon cancer cells (white arrowheads). E and F, negative expression of CTSB in colon cancer cells (black arrows). Stromal cells serve as an internal positive control for CTSB expression (arrowhead). A, C, and E, low magnification; B, D, and F, high magnification.

Table 1. Clinical and pathologic features of colon cancer according to cathepsin B expression

Clinical or pathologic feature	Total No. (%)	CTSB (-) No (%)	CTSB (+) No (%)	P
All cases	558	101	457	
Gender				0.02
Male	192 (34)	25 (25)	167 (37)	
Female	366 (66)	76 (75)	290 (63)	
Mean age \pm SD, years	67.2 \pm 8.2	67.8 \pm 8.8	67.1 \pm 8.1	0.43
Body mass index (kg/m ²)				0.58
<30	459 (82)	85 (84)	374 (82)	
\geq 30	99 (18)	16 (16)	83 (18)	
Family history of colorectal cancer in any first-degree relative				0.27
(-)	418 (75)	80 (79)	338 (74)	
(+)	140 (25)	21 (21)	119 (26)	
Tumor location				0.87
Right	344 (62)	63 (62)	281 (61)	
Left	214 (38)	38 (38)	176 (39)	
Stage				0.19
I	108 (19)	21 (21)	87 (19)	
II	194 (35)	28 (28)	166 (36)	
III	150 (27)	36 (36)	114 (25)	
IV	81 (15)	12 (12)	69 (15)	
Missing	25 (4.5)	4 (4.0)	21 (4.6)	
Tumor grade				0.28
Low	500 (90)	87 (87)	413 (91)	
High	56 (10)	13 (13)	43 (9.4)	
Mucinous component				0.54
0%	269 (52)	45 (51)	224 (53)	
>0%	245 (48)	46 (49)	199 (47)	
Signet ring cell component				0.24
0%	428 (88)	76 (84)	352 (89)	
>0%	58 (12)	14 (16)	44 (11)	

pharmacists, and veterinarians, who were 40 to 75 years of age at entry. In each cohort, with a follow-up rate of 92%, we mailed biennial questionnaires to update information and identify new cases of cancer.

On each biennial follow-up questionnaire, participants were asked whether they had had a diagnosis of colon cancer during the prior two years. When a participant reported a diagnosis of colon cancer, we asked for permission to obtain hospital records and pathology reports. Study physicians, blinded to exposure data, reviewed all medical records related to colon cancer, classifying disease stage according to the 6th version of the American Joint Committee on Cancer. We had previously described our procurement of colon cancer paraffin-embedded specimens in detail (Supplementary Materials; ref. 11). For this analysis, we included the 558 participants (366 women from NHS and 192 men from HPFS) with pathologically confirmed colon adenocarcinoma that were diagnosed through 2004 and for whom we were able to obtain sufficient amounts of tumor tissue for immunohis-

tochemistry. Baseline characteristics among participants with colon cancer with available tissue for analysis were largely similar to those without available tissue. We excluded participants if they had reported any cancer (other than nonmelanoma skin) previous to colon cancer diagnosis. We also requested paraffin-embedded tissue samples of colorectal polyps that were \geq 1 cm in diameter on endoscopy among a separate group of 123 men and women enrolled in these cohorts. The institutional review boards at the Brigham and Women's Hospital and the Harvard School of Public Health approved this study.

Immunohistochemical assessment and molecular assays

We did immunohistochemistry of CTSB, PTGS2 (cyclooxygenase-2, COX-2), and TP53 from tissue microarrays of our tumor specimens (Supplementary Methods; Fig. 1; refs. 11, 12). A pathologist (Y.B.), blinded to any other participant data, recorded cytoplasmic CTSB expression as absent, weak, moderate, or strong expression.

Among the 558 tumors, 101 tumors showed no CTSB expression, 253 showed weak expression, 184 showed moderate expression, and 20 showed strong expression. In our initial exploratory analysis, we did not observe a significant relationship between CTSB levels (negative, weak, moderate, or strong) and other molecular and clinical features ($P > 0.05$). In our previous data from murine models, we showed the ability of NIRF cathepsin-specific molecular agents to identify tumors with weak to strong levels of CTSB expression using immunohistochemistry. Thus, for further analysis in this study, we defined tumors with weak to strong cytoplasmic expression of CTSB as CTSB positive and tumors with absent cytoplasmic expression of CTSB as CTSB negative (Fig. 1). A random sample of cancers was reread by a second pathologist and the concordance between readers was 0.92 ($\kappa = 0.62$, $P < 0.001$; $n = 108$) for PTGS2 (COX-2), 0.87 ($\kappa = 0.75$, $P < 0.0001$; $n = 108$) for p53, and 0.87 ($\kappa = 0.62$, $P < 0.001$; $n = 364$) for CTSB. Methylation analyses, sequencing of *KRAS*, *BRAF*, and *PIK3CA*, and microsatellite instability (MSI) analysis have each been previously described (Supplementary Materials; refs. 13-21).

Ascertainment of death

We included deaths that occurred after diagnosis of colon cancer and before June 1, 2008. We identified deaths

through the National Death Index and next of kin. Mortality follow-up was >98% complete (22). For all deaths, we sought information to determine the cause, including death certificates, and, when appropriate, requested permission from next of kin to review medical records.

Statistical analysis

As in our prior analysis (11), we pooled data from both cohorts and tested for heterogeneity using the Q statistic. We observed no heterogeneity between the cohorts regarding the association of CTSB and colon cancer-specific survival ($P = 0.75$ for Cochran's Q test; ref. 23). For categorical data, the χ^2 test was done. To assess independent relations of CTSB expression with other variables, a multivariate logistic regression analysis was carried out (Supplementary Materials). For survival analyses, participants eligible for analysis accrued follow-up time beginning on the month of their diagnosis of colon cancer and ending on the month of death from colon cancer, death from any cause, or June 1, 2008, whichever came first. We categorized participants according to CTSB positive versus CTSB negative. We used Kaplan-Meier curves and the log-rank test to compare colon cancer-specific and overall mortality according to CTSB expression. To assess the effect of CTSB independent of stage, we used Cox proportional hazards modeling with tumor

Table 2. Molecular features of colon cancer according to CTSB expression

Molecular feature	Total No. (%)	CTSB (-) No. (%)	CTSB (+) No. (%)	P
MSI status				0.84
MSI-low/MSS	319 (79)	45 (78)	274 (79)	
MSI-high	87 (21)	13 (22)	74 (21)	
CIMP status				0.49
CIMP-low/0	326 (81)	45 (78)	281 (81)	
CIMP-high	77 (19)	12 (22)	64 (19)	
<i>BRAF</i> mutation				0.43
(-)	340 (84)	50 (88)	290 (84)	
(+)	64 (16)	7 (12)	57 (16)	
<i>KRAS</i> mutation				0.01
(-)	254 (63)	44 (77)	210 (60)	
(+)	152 (37)	13 (23)	139 (40)	
<i>PIK3CA</i> mutation				0.76
(-)	300 (84)	42 (86)	258 (84)	
(+)	56 (16)	7 (14)	49 (16)	
LINE-1 methylation level (mean \pm SD)	60.4 \pm 9.2	61.7 \pm 9.4	60.2 \pm 9.2	0.25
TP53 expression				0.63
(-)	264 (65)	40 (68)	224 (65)	
(+)	142 (35)	19 (32)	123 (35)	
PTGS2 (COX-2) expression				0.72
(-)	156 (38)	21 (36)	135 (39)	
(+)	251 (62)	37 (64)	214 (61)	

Abbreviation: MSS, microsatellite stable.

stage (I, IIA, IIB, IIIA, IIIB, IIIC, IV, unknown) as a stratifying variable, which enabled us to avoid residual confounding and overfitting. To further adjust for other potential confounding variables, we constructed a multivariate, stage-matched Cox proportional hazards model (Supplementary Materials). For all analyses, we used SAS version 9.1.3. All *P* values are two-sided and a level of significance <0.05 was considered statistically significant.

Results

Among the 558 eligible participants with colon cancer, we documented 254 total deaths with 155 deaths due to colon cancer. For participants who are alive, the median time of follow-up from date of diagnosis was 11.6 years (interquartile range, 3.9-26.3 years). A total of 457 (82%) participants had cancers that expressed CTSB (CTSB positive) and 101 (18%) had cancers that did not express CTSB (CTSB negative). Baseline clinical characteristics of the participants are shown in Table 1. Compared with participants with CTSB-negative tumors, participants with CTSB-positive tumors were more likely to be male. Of note, CTSB expression did not vary according to stage ($P = 0.19$). The number and percentage with CTSB expression are as follows: stage I, 87 of 108 (81%); stage II, 166 of 194 (86%); stage III, 114 of 150 (76%); stage IV, 69 of 81 (85%).

A comparison of other molecular features in colon cancers according to expression of CTSB is shown in Table 2. Compared with participants with CTSB-negative tumors, participants with CTSB-positive tumors were more likely to have an activating *KRAS* mutation. In contrast, there did not seem to be a significant difference in MSI status, CpG island methylator phenotype (CIMP) status, *BRAF* mutation, *PIK3CA* mutation, LINE-1 methylation level, TP53 expression, or PTGS2 COX-2 expression. In a multivariate model adjusting for both clinical and molecular features, participants with CTSB-positive cancers had 2.47 (95% confidence interval, 1.25-4.88) higher odds of having a *KRAS* mutation and 2.47 (95% confidence interval, 1.00-5.92) higher odds of having a *BRAF* mutation (Table 3).

CTSB expression was associated with a significant increase in risk of colon cancer-specific mortality (log-rank $P = 0.02$; Fig. 2A) and an increase in overall mortality (log-rank $P = 0.005$; Fig. 2B). This relationship remained largely unchanged even after adjusting for stage or other predictors of cancer recurrence (Table 4). Compared with participants who had CTSB-negative cancers, the multivariate hazard ratio (HR) associated with having a CTSB-positive cancer was 1.99 [95% confidence interval (95% CI), 1.19-3.34] for colon cancer-specific mortality and 1.71 (95% CI, 1.16-2.50) for overall mortality. Additionally adjusting for MSI status, CIMP status, *BRAF* mutation, *PIK3CA* mutation, LINE-1 level, TP53 expression, or PTGS2 (COX-2) expression did not materially alter these findings (multivariate HR, 2.20; 95% CI, 1.15-4.20

Table 3. Multivariate analysis of the relationship of other molecular features with CTSB expression in colon cancer

Variables in the final model for CTSB	Multivariate OR (95% CI)	<i>P</i>
<i>KRAS</i> mutation	2.47 (1.25-4.88)	0.009
<i>BRAF</i> mutation	2.47 (1.00-5.92)	0.049
Male gender	1.85 (0.96-3.56)	0.065
LINE-1 hypomethylation (for a 30% decrease)	2.35 (0.85-6.49)	0.098

NOTE: Multivariate logistic regression analysis initially included age, sex, body mass index, tumor location, stage, tumor grade, mucinous component, signet ring cell component, MSI, CIMP, PTGS2, TP53, LINE-1 methylation, *KRAS*, *PIK3CA*, and *BRAF*. Backward stepwise elimination with threshold of $P = 0.20$ was used to select variables in the final model.

Abbreviation: OR, odds ratio.

for colon cancer-specific mortality, and multivariate HR, 1.89; 95% CI, 1.18-3.00 for overall mortality).

We examined whether the influence of CTSB expression on colorectal cancer-specific survival was modified by any of the clinical, pathologic, and molecular variables. We did not observe a significant interaction between CTSB expression and any of the covariates (all $P_{\text{interaction}} > 0.16$). Notably, the effect of CTSB did not significantly differ between the two independent cohort studies ($P_{\text{interaction}} = 0.71$). In addition, there was no significant interaction between CTSB and tumor stage ($P_{\text{interaction}} = 0.98$) or tumor location ($P_{\text{interaction}} = 0.85$).

Because CTSB is a promising target for molecular imaging agents in murine models of adenomatous polyps, we also examined the prevalence of CTSB expression in human adenomas. Among a separate group of 123 patients enrolled in these cohorts who had polyps >1 cm in diameter on endoscopy, we found that 112 of 123 (91%) cases expressed CTSB. Among the 101 adenomas in which we had complete size and histologic data, we found that CTSB expression was independent of adenoma size ($P = 0.45$) and histology ($P = 0.28$). The number and percentage that were CTSB positive according to size are as follows: 1.0 to 1.5 cm in diameter, 31 of 35 (89%); 1.6 to 2.0 cm in diameter, 21 of 26 (81%); >2.0 cm in diameter, 31 of 40 (78%). The number and percentage that were CTSB positive according to histology are as follows: tubular, 54 of 69 (78%); tubulovillous, 24 of 26 (92%); villous, 5 of 6 (83%).

Discussion

In summary, we observed that the CTSB proteases are overexpressed in the vast majority of human colon adenoma and cancers, independent of stage. CTSB

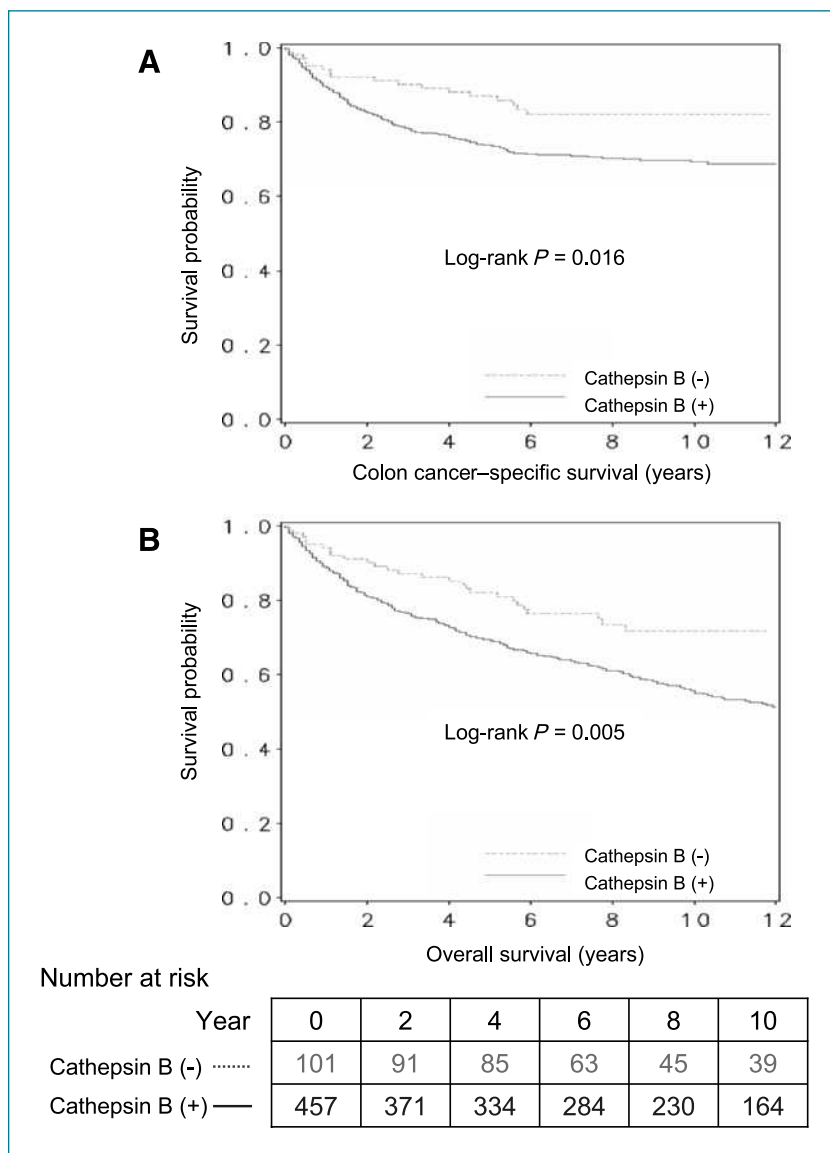


Figure 2. Survival of colon cancer patients according to CTSB expression status. A, colon cancer-specific survival. B, overall survival.

expression was significantly associated with an increased risk of colon cancer-specific and overall mortality. Overall, CTSB was not associated with other molecular features of colon cancer except for the presence of *KRAS* and *BRAF* mutations. Our data support the potential for CTSB as a target for molecular detection of neoplasia and therapeutic intervention.

Our data are supported by other studies that have shown that synthesis and secretion of CTSB is increased in the extracellular environment of colon cancers (2, 24). CTSB has been shown to play an essential role in disrupting the extracellular matrix barriers between tumors and surrounding tissue, thereby facilitating invasion and metastasis (8). A number of observations implicate CTSB in tumor progression: (a) inhibitors of CTSB retard metastases and *in vitro* growth (25); (b) genetic modulation of CTSB favorably alters the invasive properties of tumor

cells (26); (c) CTSB levels correlate with tumor aggressiveness and angiogenesis (8, 27); and (d) serum levels of CTSB are significantly higher in patients with colorectal cancer and adenoma than in tumor-free controls (4, 28, 29). Our results are generally consistent with two previous smaller studies that also showed that increased tissue expression or antigen activity levels of CTSB in colorectal cancer were associated with significantly shorter patient survival (7, 30). In another small study of 60 patients with colorectal cancer, there was a trend toward worsened survival associated with tumor antigen levels of CTSB (5).

Prior studies have observed that CTSB antigen levels or immunohistochemical staining does correlate with advancing Dukes' stage and progression from adenoma to adenocarcinoma (2, 5, 6, 30). However, each of these studies used varying techniques to assay for CTSB levels as well as analytic methodology to relate levels

to clinicopathologic parameters. Thus, it is difficult to directly compare these findings with our study, in which we observed a high prevalence of CTSB expression in all stages of disease, including premalignant adenomas. Previous studies are consistent with our results, showing high levels of CTSB in human colon cancer irrespective of stage (3, 7), as well as in adenomas (2). Moreover, other studies suggest that expression or activity levels may actually peak in early-stage cancer and decline with advanced disease (7, 31, 32). The importance of CTSB across stages of neoplasia is also validated by prior data showing uniform CTSB expression and high enzyme activity in intestinal adenomas generated in mouse models. In a previous study of *Apc^{Min/+}* mice, we observed, using immunohistochemistry and fluorescent antibody microscopy, that CTSB is ubiquitously expressed in intestinal adenomas, even in microscopic lesions that are difficult to visualize through standard visual inspection. Compared with adjacent normal tissue, adenomas had a 36% higher level of CTSB protein by Western blot and 35% higher level of CTSB mRNA by reverse transcriptase-PCR (8). Similarly, in a comprehensive proteomic screening study, we identified CTSB as one of six proteins upregulated in the plasma of tumor-bearing *APC^{Δ580}* mice with concomitant overexpression at the RNA and protein level in adenoma tissue (24). Finally, recent data have shown that genetic ablation of CTSB attenuates polyposis in a hemizygous *APC^{Δ468}* mouse model (33). Taken together, these data support emerging evidence that cathepsins have a role not only in facilitating cancer invasion and metastasis, but also in mediating early, premalignant processes, such as tumor initiation, hyperproliferation, and dedifferentiation (8, 34).

Interestingly, we found significant relations of CTSB expression with *KRAS* and *BRAF* mutations. Experimental studies have shown a correlation between mutations in *KRAS* and upregulation in CTSB expression (35, 36), which are in agreement with our findings. *KRAS* and *BRAF* mutations are not only critical events during carci-

nogenesis, but also important prognostic and/or predictive markers in colon cancer patients. *KRAS* mutational status of stage IV colorectal cancer is a predictive biomarker for anti-EGFR treatment, and *BRAF* mutation identifies a subgroup of patients with unfavorable prognosis (13, 37-41). Further understanding of the relationship between CTSB and mutations in *KRAS* and *BRAF* could potentially provide useful information for refinement of therapeutic strategies. In this respect, our findings may be of clinical interest if confirmed by additional studies.

To exploit the potential role of cathepsins in colonic neoplasia, we have developed biocompatible, optically quenched, NIRF imaging agents that release fluorochromes after enzyme activation by tumor-associated cathepsin proteases (9). In our previous study, when we injected *APC^{Min/+}* mice with this cathepsin-activatable reporter agent and imaged them *ex vivo* with NIRF, intestinal adenomas became highly fluorescent, with a signal intensity consistently higher than background (target to background = 9:1 with NIRF imaging as compared with 1:1 with standard white light imaging; ref. 8). We have confirmed these results with *ex vivo* imaging in tumor-bearing *APC^{Δ580}* mice (24). Using a novel NIRF microendoscope, we and others have shown the ability of cathepsin-activatable agents to image adenoma and carcinomas *in vivo* among *APC^{Min/+}* mice, hemizygous *APC^{Δ468}* mice, mice with orthotopically implanted tumors, and *APC* conditional knockout mice in which an adenovirus expressing cre recombinase infection is focally delivered to the distal colon. Several microscopic lesions that were not obviously detectable by white light imaging were visualized in these animal models using cathepsin-activatable agents with NIRF imaging (33, 42-46). Taken together, these data provide proof of principle of the potential for targeting cathepsins for early detection of colonic neoplasia using NIRF endoscopy (8). Thus, human translation of cathepsin-activatable imaging platforms may provide a unique opportunity to improve the broad detection of adenomas.

Table 4. CTSB expression in colon cancer and overall mortality

CTSB expression	Total	Colon cancer-specific mortality				Overall mortality			
		Deaths/person-years	Univariate HR (95% CI)	Stage-matched HR (95% CI)	Multivariate HR (95% CI)	Deaths/person-years	Univariate HR (95% CI)	Stage-matched HR (95% CI)	Multivariate HR (95% CI)
CTSB (-)	101 (18%)	18/936	1 (referent)	1 (referent)	1 (referent)	32/936	1 (referent)	1 (referent)	1 (referent)
CTSB (+)	457 (82%)	137/3787	1.82 (1.11-2.97)	1.69 (1.03-2.79)	1.99 (1.19-3.34)	222/3787	1.69 (1.17-2.45)	1.56 (1.07-2.27)	1.71 (1.16-2.50)
<i>P</i>			0.018	0.038	0.009		0.0057	0.021	0.0063

NOTE: The multivariate, stage-matched conditional Cox regression model initially included age, year of diagnosis, sex, family history of colorectal cancer, body mass index, tumor location, grade, mucinous component, and signet ring cell. Backward stepwise elimination with threshold of *P* = 0.20 was used to select variables in the final model.

We acknowledge several limitations of our study. Beyond causes of mortality, data on cancer recurrences were not available in this cohort. Nonetheless, because median survival for recurrent (metastatic) colon cancer was approximately 10 to 12 months during much of the time period of this study (47), colon cancer-specific mortality should be a reasonable surrogate for cancer-specific outcome. In this cohort, we also had limited data on chemotherapy. It is unlikely, however, that differential receipt of chemotherapy could explain the observed findings. First, it is unlikely that chemotherapy use differed according to tumoral CTSB expression because such data were not available to patients or treating physicians. Second, the association of CTSB and survival was similar among participants with stage I or II disease, for which surgery alone would be represent a standard of care, and among those with stage III cancer, for which adjuvant chemotherapy would represent a routine approach. Third, because our cohort consisted of health professionals, considerable heterogeneity in use of adjuvant chemotherapy would be unlikely.

We were unable to obtain tumor tissue on all cases of confirmed colon cancer over follow-up. It is unlikely, however, that CTSB or mortality would be differential according to retrieval success. Moreover, an assessment of risk factors did not appreciably differ among those participants for whom we were unable to obtain tumor tissue (11). Finally, we did not assay other proteases which may also be important in colorectal cancer, such as CTSL1 (5). We focused on CTSB given the greater near-term potential to exploit targeting of this specific enzyme using an already developed molecular imaging probe.

Consistent with the well-established role of proteases in facilitating tumor invasiveness and spread, our findings show that CTSB expression is significantly associated with an increased risk of colon cancer-specific and overall mortality. In addition, we show that CTSB

is expressed in the vast majority of human colon adenoma and cancers of all stages, supporting the possible role of CTSB in early alterations leading to tumor formation. These results support the potential for exploiting CTSB as a target for imaging agents with specificity for their enzymatic activity. The feasibility of using such CTSB-specific probes with fluorescent endoscopy has been validated in multiple animal models; adapting this technology for humans may be a promising adjunct to current endoscopic approaches to colon cancer screening and surveillance.

Disclosure of Potential Conflicts of Interest

Dr. Madden and Mr. Poss are employees and equity shareholders in VisEn Medical, which holds the commercial license for the cathepsin sensing agent cited in this work. All other authors have no financial conflicts. A.T. Chan is a Damon Runyon Cancer Research Foundation Clinical investigator. The content is solely the responsibility of the authors and does not necessarily represent the official views of NIH or the Damon Runyon Cancer Research Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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References

1. Keppler D, Sameni M, Moin K, Mikkelsen T, Diglio CA, Sloane BF. Tumor progression and angiogenesis: cathepsin B & Co. *Biochem Cell Biol* 1996;74:799–810.
2. Khan A, Krishna N, Baker SP, Banner BF. Cathepsin B and tumor-associated laminin expression in the progression of colorectal adenoma to carcinoma. *Mod Pathol* 1998;11:704–8.
3. Adenis A, Huet G, Zerimech F, Hecquet B, Balduyck M, Peyrat JP. Cathepsin B, L, and D activities in colorectal carcinomas: relationship with clinico-pathological parameters. *Cancer Lett* 1995;96:267–75.
4. Herszenyi L, Plebani M, Carraro P, et al. Proteases in gastrointestinal neoplastic diseases. *Clin Chim Acta* 2000;291:171–87.
5. Herszenyi L, Plebani M, Carraro P, et al. The role of cysteine and serine proteases in colorectal carcinoma. *Cancer* 1999;86:1135–42.
6. Talieri M, Papadopoulou S, Scorilas A, et al. Cathepsin B and cathepsin D expression in the progression of colorectal adenoma to carcinoma. *Cancer Lett* 2004;205:97–106.
7. Troy AM, Sheahan K, Mulcahy HE, Duffy MJ, Hyland JM, O'Donoghue DP. Expression of cathepsin B and L antigen and activity is associated with early colorectal cancer progression. *Eur J Cancer* 2004;40:1610–6.
8. Marten K, Bremer C, Khazaie K, et al. Detection of dysplastic intestinal adenomas using enzyme-sensing molecular beacons in mice. *Gastroenterology* 2002;122:406–14.
9. Weissleder R, Tung CH, Mahmood U, Bogdanov A, Jr. *In vivo* imaging of tumors with protease-activated near-infrared fluorescent probes. *Nat Biotechnol* 1999;17:375–8.
10. Mahmood U, Wallace MB. Molecular imaging in gastrointestinal disease. *Gastroenterology* 2007;132:11–4.
11. Chan AT, Ogino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. *N Engl J Med* 2007;356:2131–42.
12. Ogino S, Brahmandam M, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS. Combined analysis of COX-2 and p53 expressions reveals synergistic inverse correlations with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Neoplasia* 2006;8:458–64.
13. Ogino S, Noshok K, Kirkner GJ, et al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* 2009;58:90–6.
14. Ogino S, Noshok K, Kirkner GJ, et al. A cohort study of tumoral LINE-1 hypomethylation and prognosis in colon cancer. *J Natl Cancer Inst* 2008;100:1734–8.

15. Nosho K, Kawasaki T, Ohnishi M, et al. PIK3CA mutation in colorectal cancer: relationship with genetic and epigenetic alterations. *Neoplasia* 2008;10:534–41.
16. Ogino S, Kawasaki T, Brahmandam M, et al. Sensitive sequencing method for KRAS mutation detection by pyrosequencing. *J Mol Diagn* 2005;7:413–21.
17. Ogino S, Kawasaki T, Brahmandam M, et al. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. *J Mol Diagn* 2006;8:209–17.
18. Ogino S, Cantor M, Kawasaki T, et al. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut* 2006;55:1000–6.
19. Nosho K, Irahara N, Shima K, et al. Comprehensive biostatistical analysis of CpG island methylator phenotype in colorectal cancer using a large population-based sample. *PLoS One* 2008;3:e3698.
20. Ogino S, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. *J Mol Diagn* 2006;8:582–8.
21. Irahara N, Nosho K, Baba Y, et al. Precision of pyrosequencing assay to measure LINE-1 methylation in colon cancer, normal colonic mucosa, and peripheral blood cells. *J Mol Diagn* 2010;12:177–83.
22. Rich-Edwards JW, Corsano KA, Stampfer MJ. Test of the National Death Index and Equifax Nationwide Death Search. *Am J Epidemiol* 1994;140:1016–9.
23. Cochran WG. The combination of estimates from different experiments. *Biometrics* 1954;10:101–29.
24. Hung KE, Faca V, Song K, et al. Comprehensive proteome analysis of an Apc mouse model uncovers proteins associated with intestinal tumorigenesis. *Cancer Prev Res (Phila)* 2009;2:224–33.
25. Navab R, Mort JS, Brodt P. Inhibition of carcinoma cell invasion and liver metastases formation by the cysteine proteinase inhibitor E-64. *Clin Exp Metastasis* 1997;15:121–9.
26. Krueger S, Haeckel C, Buehling F, Roessner A. Inhibitory effects of antisense cathepsin B cDNA transfection on invasion and motility in a human osteosarcoma cell line. *Cancer Res* 1999;59:6010–4.
27. Bremer C, Tung CH, Bogdanov A, Jr., Weissleder R. Imaging of differential protease expression in breast cancers for detection of aggressive tumor phenotypes. *Radiology* 2002;222:814–8.
28. Herszenyi L, Istvan G, Cardin R, et al. Serum cathepsin B and plasma urokinase-type plasminogen activator levels in gastrointestinal tract cancers. *Eur J Cancer Prev* 2008;17:438–45.
29. Herszenyi L, Farinati F, Cardin R, et al. Tumor marker utility and prognostic relevance of cathepsin B, cathepsin L, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, CEA and CA 19–9 in colorectal cancer. *BMC Cancer* 2008;8:194.
30. Campo E, Munoz J, Miquel R, et al. Cathepsin B expression in colorectal carcinomas correlates with tumor progression and shortened patient survival. *Am J Pathol* 1994;145:301–9.
31. Iacobuzio-Donahue CA, Shuja S, Cai J, Peng P, Murnane MJ. Elevations in cathepsin B protein content and enzyme activity occur independently of glycosylation during colorectal tumor progression. *J Biol Chem* 1997;272:29190–9.
32. Hirai K, Yokoyama M, Asano G, Tanaka S. Expression of cathepsin B and cystatin C in human colorectal cancer. *Hum Pathol* 1999;30:680–6.
33. Gounaris E, Tung CH, Restaino C, et al. Live imaging of cysteine-cathepsin activity reveals dynamics of focal inflammation, angiogenesis, and polyp growth. *PLoS One* 2008;3:e2916.
34. Koblinski JE, Ahram M, Sloane BF. Unraveling the role of proteases in cancer. *Clin Chim Acta* 2000;291:113–35.
35. Cavallo-Medved D, Dosesescu J, Linebaugh BE, Sameni M, Rudy D, Sloane BF. Mutant K-ras regulates cathepsin B localization on the surface of human colorectal carcinoma cells. *Neoplasia* 2003;5:507–19.
36. Kim K, Cai J, Shuja S, Kuo T, Murnane MJ. Presence of activated ras correlates with increased cysteine proteinase activities in human colorectal carcinomas. *Int J Cancer* 1998;79:324–33.
37. Banck MS, Grothey A. Biomarkers of resistance to epidermal growth factor receptor monoclonal antibodies in patients with metastatic colorectal cancer. *Clin Cancer Res* 2009;15:7492–501.
38. Roth AD, Tejpar S, Delorenzi M, et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60–00 trial. *J Clin Oncol* 2010;28:466–74.
39. Samowitz WS, Sweeney C, Herrick J, et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. *Cancer Res* 2005;65:6063–9.
40. French AJ, Sargent DJ, Burgart LJ, et al. Prognostic significance of defective mismatch repair and BRAF V600E in patients with colon cancer. *Clin Cancer Res* 2008;14:3408–15.
41. Zlobec I, Kovac M, Erzberger P, et al. Combined analysis of specific KRAS mutation, BRAF and microsatellite instability identifies prognostic subgroups of sporadic and hereditary colorectal cancer. *Int J Cancer* 2010;127:2569–75.
42. Alencar H, King R, Funovics M, Stout C, Weissleder R, Mahmood U. A novel mouse model for segmental orthotopic colon cancer. *Int J Cancer* 2005;117:335–9.
43. Funovics MA, Alencar H, Montet X, Weissleder R, Mahmood U. Simultaneous fluorescence imaging of protease expression and vascularity during murine colonoscopy for colonic lesion characterization. *Gastrointest Endosc* 2006;64:589–97.
44. Kelly K, Alencar H, Funovics M, Mahmood U, Weissleder R. Detection of invasive colon cancer using a novel, targeted, library-derived fluorescent peptide. *Cancer Res* 2004;64:6247–51.
45. Alencar H, Funovics MA, Figueiredo J, Sawaya H, Weissleder R, Mahmood U. Colonic adenocarcinomas: near-infrared microcatheter imaging of smart probes for early detection-study in mice. *Radiology* 2007;244:232–8.
46. Hung KE, Maricevich MA, Richard LG, et al. Development of a mouse model for sporadic and metastatic colon tumors and its use in assessing drug treatment. *Proc Natl Acad Sci U S A* 2010;107:1565–70.
47. Meyerhardt JA, Mayer RJ. Systemic therapy for colorectal cancer. *N Engl J Med* 2005;352:476–87.

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