Biology of Human Tumors

Clinical Cancer Research

Elevated Serum Angiopoietin-like Protein 2 Correlates with the Metastatic Properties of Colorectal Cancer: A Serum Biomarker for Early Diagnosis and Recurrence

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Abstract

Purpose: Angiopoietin-like protein 2 (ANGPTL2) is a mediator of chronic inflammation and inflammatory carcinogenesis. The biologic and clinical significance of ANGPTL2 remains unknown in human cancer. Therefore, we investigated the function of ANGPTL2 and evaluated its clinical significance in both primary tumors and matched sera in patients with colorectal cancer.

Experimental Design: A colorectal cancer cell line was transfected with *siRNA* against *ANGPTL2* for the assessment of its function. We examined ANGPTL2 expression in colorectal cancer tissues ($n = 195$) by immunohistochemistry. Finally, we screened serum ANGPTL2 levels from 32 colorectal cancers and 23 normal controls (NC), and validated these results in serum samples obtained from 195 colorectal cancers and 45 NCs by ELISA.

Results: Knockdown of *ANGPTL2 in vitro* significantly inhibited cell proliferation, migration, and invasion, whereas it enhanced anoikis. ANGPTL2 was overexpressed in colorectal cancer tissues, and was significantly associated with advanced T stage, lymph node, and liver metastasis. Likewise, serum ANGPTL2 levels in colorectal cancers were significantly higher than NCs ($P < 0.01$), and allowed distinguishing of colorectal cancers from NCs with high accuracy (AUC = 0.837). The subsequent validation step confirmed that serum ANGPTL2 levels in colorectal cancers were significantly higher than in NCs ($P < 0.0001$), and had a high AUC value (0.885) for distinguishing colorectal cancers from NCs. High serum ANGPTL2 was significantly associated with advanced T stage, lymph node and liver metastasis, early relapse, and poor prognosis in colorectal cancers.

Conclusion: Serum ANGPTL2 is a novel diagnostic and recurrence-predictive biomarker in patients with colorectal cancer. *Clin Cancer Res; 20(23); 6175–86. ©2014 AACR.*

Introduction

Colorectal cancer is one of the most common malignancies worldwide and is a major cause of cancer-related deaths (1). Several colorectal cancer screening tests, including fecal occult-blood test and colonoscopy have been available for years (2), and have aided in reducing the mortality associated with this disease (3, 4). However, compliance with these tests has been far from adequate. Patients with metastatic colorectal cancer frequently receive expensive cytotoxic chemotherapeutic regimens coupled with targeted monoclonal antibodies but with relatively modest benefits (5). Without *a priori* knowledge of which patients will experience tumor recurrence, there is inevitable overtreatment of patients with such chemotherapeutic drugs with severe toxic side effects (6). These limitations underscore the need for novel biomarkers, particularly noninvasive biomarkers in serum, for diagnosing, prognosticating, and predicting response to cytotoxic chemotherapy.

There is substantial evidence that inflammatory processes play an important role in colorectal carcinogenesis (7). Studies have shown that individuals with chronic inflammatory bowel disease are at a higher risk of colorectal cancer compared with individuals without such a condition (8, 9). Furthermore, the use of aspirin and other anti-inflammatory drugs is associated with a lower risk of colorectal

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Translational Relevance

New findings

- Angiopoietin-like protein 2 (ANGPTL2) promoted metastatic capacity of colorectal cancer cells, and high expression of this protein was significantly associated with tumor progression in patients with colorectal cancer.
- In this study, serum ANGPTL2 levels demonstrated a sensitivity of 69.7% and a specificity of 95.6% with an AUC value of 0.885 in distinguishing patients with colorectal cancer from normal controls (NC). More importantly, ANGPTL2 expression levels had a sensitivity of 54.2% and a specificity of 93.3% with an AUC value of 0.795 in distinguishing patients with early-stage colorectal cancers (stage I) from NCs.
- Serum ANGPTL2 levels increased in a stage-dependent manner and increased serum ANGPTL2 levels were associated with poor disease-free survival and overall survival among patients with colorectal cancer.

Clinical practice

Serum ANGPTL2 testing could offer a reliable, noninvasive means of screening for colorectal cancer at early stages, and for monitoring of colorectal cancer progression and recurrence.

neoplasia (10, 11). Obesity is associated with chronic low-grade inflammation due to the production of proinflammatory cytokines such as TNFα and IL-6, which induce the hepatic secretion of acute phase proteins such as C-reactive protein (CRP; ref. 12). Weight loss reduces inflammatory processes not only systematically, as seen by reduced CRP levels (13), but also locally in the colorectal mucosa (14). Thus, inflammatory processes might account, in part, for the positive association between obesity and colorectal cancer risk.

Angiopoietin-like protein 2 (ANGPTL2) is a mediator of chronic inflammation in obesity and its related metabolic abnormalities (15). Obese adipose tissue–related ER stress increases ANGPTL2 secretion or expression in adipocytes (15). In addition, *ANGPTL2* mRNA levels in tumor cells are significantly increased under hypoxia and under nutritional deprivation. Furthermore, increased ANGPTL2 expression was detected in tumor cells in hypoxic regions, suggesting that the tumor microenvironment induces ANGPTL2 expression in colorectal cancer. On the other hand, ANGPTL2 expression in tumor cells is closely associated with tumor cell metastasis to lymph nodes and/or distant organs due to increased angiogenesis in the tumor environment, as well as tumor cell invasion and migration associated with the epithelial mesenchymal transition (16, 17). Thus, cancer cell- and/or tumor microenvironment–derived ANGPTL2 is considered a critical factor in inflammation-induced carcinogenesis and cancer progression. Furthermore, ANGPTL2 proteins have signal sequences at their N-termini for protein secretion (15), which have been detected in the systemic circulation under conditions of obesity-associated inflammation (18, 19). These data suggest that quantification of the ANGPTL2 level in serum might be useful as a diagnostic and predictive biomarker in colorectal cancer.

Accordingly, the present study aimed to evaluate the clinical significance of ANGPTL2 in both serum and matched primary tumors in colorectal cancer. In addition, we investigated the functional role of ANGPTL2 in colorectal cancer by RNA interference analysis of cultured colorectal cancer cells. Using multiple approaches, we have for the first time demonstrated that high expression of ANGPTL2 in tumor cells inhibits anoikis and promotes proliferation, invasion, and migratory ability and is significantly associated with tumor progression in patients with colorectal cancer. From a clinical perspective, our data provide novel evidence that serum ANGPTL2 could serve as a biomarker, useful in the diagnosis and prognosis of early recurrence in patients with colorectal cancer.

Materials and Methods

Study design

This study analyzed 490 serum and tissue specimens that were obtained from consecutively enrolled colorectal cancer patients and sex-and age-matched healthy volunteers at the Mie University Medical Hospital (Mie, Japan), between January 1, 2006 and December 31, 2011. Exclusion criteria included inflammatory bowel disease, familial adenomatous polyposis, hereditary nonpolyposis colon cancer, or other rare and complex types of tumors. Healthy volunteers were asymptomatic individuals recruited from a colonoscopy screening program at the Mie University (Mie, Japan). This study undertook a functional analysis of ANGPTL2 in colorectal cancer cells, followed by an evaluation of the associations between ANGPTL2 expression in colorectal cancer tissues ($n = 195$) assessed by immunohistochemistry and clinicopathologic and survival outcomes. We also quantified serum ANGPTL2 levels to analyze their clinical significance as a disease biomarker. Blood samples from preoperative colorectal cancer patients and healthy controls were collected using "red top" serum vacuum blood collection tubes (Becton Dickinson and Company). The samples were centrifuged at 4,000 rpm immediately; the supernatants were aliquoted in 1.5 mL tubes (Eppendorf) and were stored at −80°C conditions until use. In the screening phase, a small set of preoperative serum samples was collected from 16 patients with colorectal cancer with stage I disease and 16 patients with colorectal cancer with stage IV disease. We included 23 sex- and age-matched healthy subjects. To further assess the significance of serum ANGPTL2 in patients with colorectal cancer, a validation survey was conducted using a large, independent cohort of patients. In this group, preoperative sera ($n = 195$) were matched with surgical tissues assessed by immunohistochemistry. Supplementary Table S1 and Supplementary Material and Methods show detailed patient characteristics

in the validation step. All patients were classified according to TNM classification (20). We also assessed another control group consisting of 45 healthy subjects from our institute. Both serum- and tissue-based specimen collection and studies were approved by the Institutional Review Board at the Mie University Hospital in Japan. All participants provided written informed consent and willingness to donate their blood and tissue samples for research.

Cell lines

Human colorectal cancer cell lines Caco2, DLD1, HT29, Lovo, and SW480 were provided by the Cell Resource Center of Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). All cell lines were authenticated by short tandem repeat DNA profiling in 2014. Cell culture conditions are described in Supplementary Material and Methods.

Proliferation, anoikis, invasion, and migration assays

ANGPTL2-specific siRNA (Silencer Select Validated siRNA, standard purity) and negative control siRNA (Silencer Negative Control siRNA) were purchased from Ambion. Transfections were performed by mixing cell suspensions with siRNA oligonucleotides (20 nmol/L), Opti-MEM I (Invitrogen), and Lipofectamine RNAiMAX (Invitrogen) before cell plating. Proliferation, anoikis, invasion, and wound healing assays were performed after 48-hour incubation to assess the function of ANGPTL2. Additional experimental details on these assays are provided in Supplementary Materials and Methods.

Total RNA extraction, cDNA synthesis, and quantitative real-time reverse transcription PCR

Total RNA from cell lines were isolated using an RNeasy Mini Kit (Qiagen Inc.) according to the manufacturer's instructions. cDNA was synthesized by random hexamers using Superscript III reverse transcriptase (Invitrogen). We performed quantitative real-time reverse transcription analysis (qRT-PCR) using the StepOne Real Time PCR System (Applied Biosystems). *ANGPTL2* and *GAPDH* were quantified in duplicate by qRT-PCR, using RNA Assay Kits (Applied Biosystems). The following cycling conditions were used: 95°C, 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. The average expression levels of *ANGPTL2* were normalized against *GAPDH* using the $2^{-\Delta\Delta Ct}$ method.

KRAS/BRAF mutation and tumor microsatellite instability analysis

Formalin-fixed, paraffin-embedded (FFPE) sections (10 μm thick) from 195 colorectal cancer surgical patients were used for mutation analysis. Hematoxylin and eosin–stained FFPE sections were microdissected for DNA extraction from the tumor cells. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's protocol. DNA quantity and quality were assessed by Nanodrop. KRAS (exons 2 and 3) and BRAF (V600E) mutations were analyzed by pyrosequencing using primers listed in Supplementary Table S2. Reactions were run on a PyroMark Q96 ID system (Qiagen).

Microsatellite unstable (MSI) analysis was carried out using five mononucleotide repeat microsatellite markers (BAT-25, BAT-26, NR-21, NR-24 and NR-27) in a pentaplex PCR system. Primer sequences were described previously (21). Tumors with instability at >3 these markers were classified as (MSI) and those showing instability at <2 markers as microsatellite stable (MSS).

Evaluation of immunohistochemistry

FFPE sections (2–3 μm thick) from 195 colorectal cancer surgical patients were used for IHC analysis of ANGPTL2 expression. Further information is provided in Supplementary Material and Methods. The immunoreactivity scoring (IRS) system was based on the intensity and the extent of staining. The criteria were as follows: (i) When the fraction of positively stained cells was 1%–25%, the score was 1. If 26%–50% were positive, the score was 2. If 51%–75% were stained, the score was 3. If >75% stained, the score was 4 (Supplementary Fig. S1A–S1D). (ii) If no staining was observed, the score was 0. When the staining was weak, moderate, or strong, the scores were 1, 2, or 3, respectively (Supplementary Fig. S1E–S1F). Scores obtained from (i) and (ii) were multiplied together to make the staining score reflect the proportion and intensity of positively stained cancer cells. Specimens were rescored if the difference between the scores by the two pathologists was more than 3.

ELISA

In the screening assay, the serum concentrations of ANGPTL2 were quantified with a commercially available ELISA (Uscn Life Science Inc.) according to the manufacturer's protocol, and the values were reported as the optical densities. In contrast, ANGPTL2 concentrations in serum during the validation assay was measured using the human ANGPTL2 ELISA kit (IBL) made by Kumamoto University (Kumamoto, Japan) according to the manufacturer's instructions and values were reported as ng/mL. Further information is provided in the Supplementary Materials and Methods.

Statistical analysis

The significance of ANGPTL2 in both sera and their corresponding tumors was determined by the Mann–Whitney test, Kruskal–Wallis test, or the χ^2 test as appropriate. The association between ANGPTL2 levels in sera and ANGPTL2 immunohistochemical (IHC) scores of matched primary tumors were analyzed by Kendall Tau (22). Receiver operating characteristic (ROC) analysis was performed to determine the diagnostic performance of serum ANGPTL2 levels in distinguishing patients with colorectal cancer or stage I colorectal cancer from the healthy control subjects. Sensitivity against 1 − specificity was plotted at each cutoff threshold, and the area under the curve (AUC) values that reflect the probability of correctly identifying stage I colorectal cancer or patients with colorectal cancer from control subjects were computed. The optimal cutoff thresholds for

diagnosis were obtained by the Youden index (23). In brief, the optimal cutoff threshold values were determined at the point on the ROC curve at which the Youden index (sensitivity + specificity−1) was maximal. A two sided *z* test was used to compare the AUCs of two ROC curves from screening and validation set (24), and reproducibility of diagnostic ability of serum ANGPTL2 levels was evaluated. Finally, a multivariable logistic regression model was used to calculate ORs for age- and sex-adjusted cases associated with colorectal cancer or stage I colorectal cancer according to serum ANGPTL2 levels.

We estimated that 154 patients were needed to achieve 80% power to substantiate more than 20% differences in prognostic outcome. Therefore, we enrolled adequate sample size of serum and tissues from 195 colorectal cancer patients. Overall survival (OS) and disease-free survival (DFS) curves were analyzed using the Kaplan–Meier method, and differences were examined using log-rank tests. ROC curves were established to discriminate patients with or without death for OS and the patients with or without recurrence for DFS. The Youden index (23) was used to determine the optimal cutoff threshold of serum ANGPTL2 levels to predict the OS and DFS. Cox proportional hazard regression test was used to estimate univariate and multivariate HR for OS and DFS. All *P* values were two-sided, and those less than 0.05 were considered statistically significant. All statistical analyses were carried out using MedCalc 12.3 for Windows.

Results

Functional analyses of ANGPTL2 in colorectal cancer cells

ANGPTL2 gene and ANGPTL2 protein expression in selected colorectal cancer cells. We investigated *ANGPTL2* gene expression by qRT-PCR in five established colorectal cancer cell lines (Supplementary Fig. S2A). In the colorectal cancer cell lines, Both SW480 and HT29 showed the highest *ANGPTL2* expression. In the other cell lines, gene expression was distinctly lower or nondetectable. To assess the protein expression levels of ANGPTL2 in the same colorectal cancer cell lines, ELISA was performed using homogenized lysates. The results revealed a pattern consistent with the qRT-PCR data for all cell lines, except Caco2 cells (Supplementary Fig. S2B). On the basis of these results, we selected SW480 and HT29 for further knockdown experiments. Transfection of SW480 and HT29 with *ANGPTL2*-siRNA resulted in a dramatic reduction in *ANGPTL2* mRNA expression compared with negative control siRNA–treated cells 48 hours after transfection (Supplementary Fig. S2C and S2D). In addition, ELISA results were consistent with the real-time PCR data (Supplementary Fig. S2E and S2F). On the basis of these data, we next analyzed ANGPTL2 function *in vitro*.

ANGPTL2 promoted proliferation, invasion and migration but suppressed anoikis in colorectal cancer cells. We assessed various cellular functions such as proliferation, anoikis, migration and invasion after treatments with control siRNA or *ANGPTL2* siRNA. MTT assays revealed that downregulation of *ANGPTL2* resulted in significant inhibition of tumor cell growth 48 and 72 hours after *ANGPTL2* siRNA transfection of SW480 and HT29 cells (Fig. S1A and S1B). Anoikis is known to induce apoptosis after loss of cell adhesion. Thus, we evaluated the number of viable SW480 and HT29 cells that were floating in low-attachment culture plates. The MTT assay showed that transfection of *siANGPTL2* significantly decreased the number of viable SW480 and HT29 cells compared with control cells, indicating that ANGPTL2 inhibits apoptosis even in nonadherent colorectal cancer cells floating in culture medium (Fig. 1C and D). We next performed invasion assays to determine whether attenuated *ANGPTL2* levels might affect cellular invasion. *ANGPTL2* siRNA transfection of SW480 and HT29 weakened the invasive capacity compared with cells transfected with nonsilencing siRNA (Fig. 1E and F). In addition, wound healing assays were performed to compare the migratory potential of SW480 and HT29 transfected with *ANGPTL2* siRNA or nonsilencing siRNA. The number of migratory cells treated with *ANGPTL2* siRNA was markedly decreased compared with control siRNA–treated cells (Fig. 1G and H). Taken together, these results demonstrated that ANGPTL2 expression enhanced cell proliferation, invasion and migration but inhibited anoikis in colorectal cancer cells.

ANGPTL2 protein expression in colorectal cancer tissues was associated with tumor growth and metastasis. IHC analysis was performed to investigate the location and intensity of ANGPTL2 protein expression, and for the evaluation of the associations between protein expression and clinicopathologic data. ANGPTL2 protein expression was observed in both the cytoplasm and nucleus of each colorectal cancer tumor cell (Fig. 2A). In contrast, there was no staining of ANGPTL2 in normal epithelial cells but weak expression in stroma in normal colonic mucosa (Fig. 2B). ANGPTL2 staining scores were significantly increased according to TNM stage ($P = 0.0026$; Fig. 2C). Based upon median expression values, we established a cutoff value of >2 to define the high-staining group [$n = 84/195$ (43%)] and <2 as the low-staining group [$n = 111/195$ (57%)]. The high-staining group was significantly associated with large tumor size ($P = 0.02$), serosal invasion ($P = 0.02$), and distant metastasis ($P = 0.01$; Table 1). No differences in ANGPTL2 staining were found in relationship with tumor KRAS and BRAF mutations and MSI status. Next, we evaluated whether protein expression of ANGPTL2 had prognostic value in patients with colorectal cancer. Kaplan–Meier curves showed no statistically significant differences between high and low expression groups for either OS (Fig. 2D; $P = 0.9$) of DFS (Fig. 2E; $P = 0.44$).

Serum levels of ANGPTL2 in patients with colorectal cancer were significantly higher than those in healthy controls in the screening phase cohort. ANGPTL2 protein is a secreted glycoprotein with homology to the angiopoietins. To examine the feasibility of detecting circulating ANGPTL2 by ELISA assay, serum ANGPTL2 levels were determined in a subset of 16 patients with colorectal cancer in stage I, 16 patients with colorectal cancer in stage IV and 23 healthy volunteers in the screening phase cohort. Serum ANGPTL2 levels in stages I and IV colorectal cancer patients were

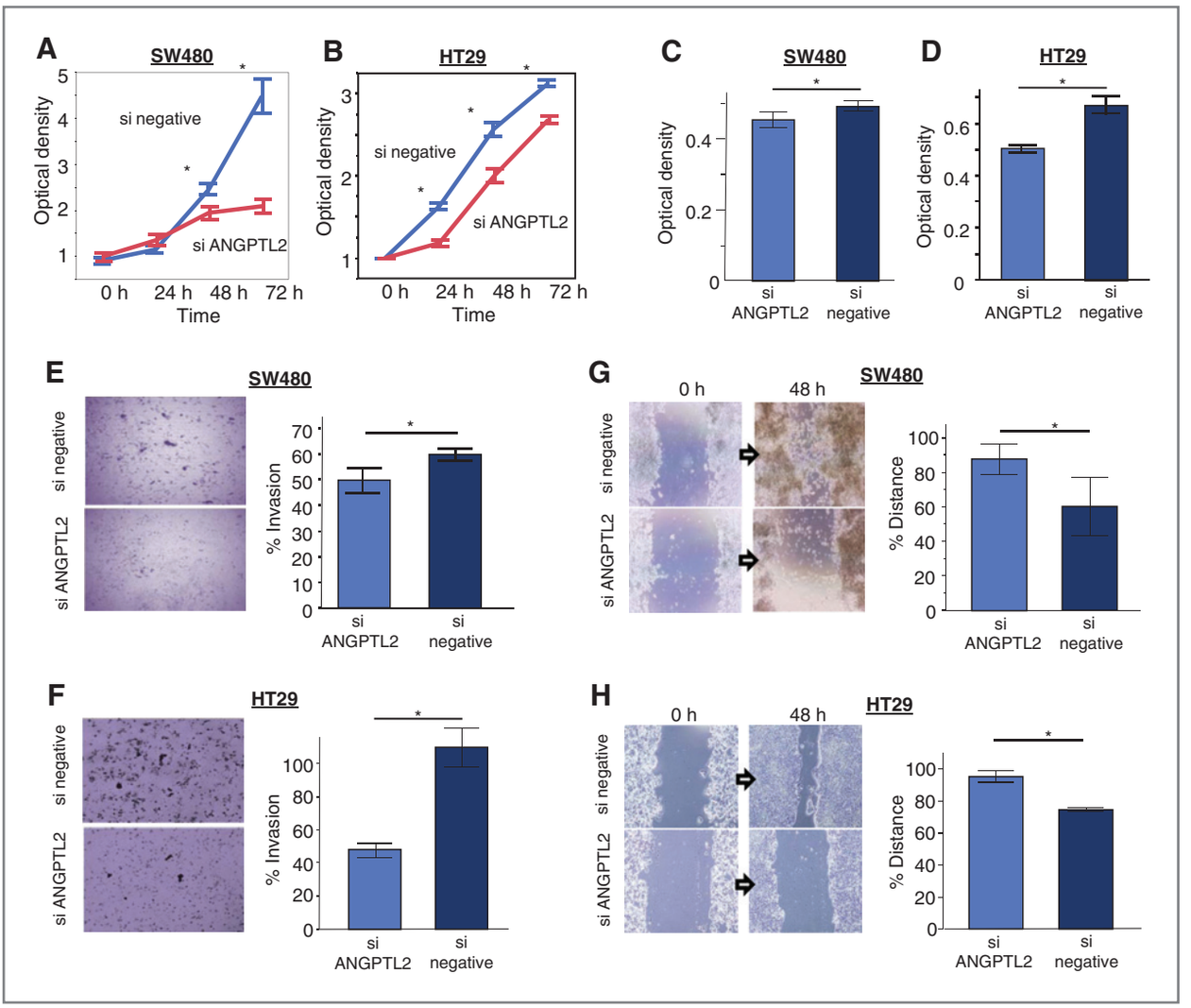

Figure 1. Reduction of ANGPTL2 expression suppressed cancer cell proliferation, invasion and migration, and increased anoikis. MTT assays of SW480 and HT29 cell lines from 0 to 72 hours after *ANGPTL2* siRNA transfection. A and B, cell growth of SW480 and HT29 treated with *ANGPTL2* siRNA (red line) was significantly inhibited compared with controls (blue line) at 48 and 72 hours. Anoikis assay. C and D, after anoikis induction for 24 hours, apoptosis rates were measured by MTT assays to calculate the number of viable floating SW480 and HT29 cells in low-attachment plates. Viable cells in the plates decreased significantly after knockdown of ANGPTL2 in both cell lines. E and F, the Transwell invasion system demonstrated enhanced invasive capacity of SW480 and HT29 after ANGPTL2 knockdown. Images of invading cells were taken by phase contrast microscopy at 100× magnification. Quantitative Transwell invasion assays, in which the *y*-axis represents the number of invading cells. G and H, wound healing assays were performed to investigate migratory potential of SW480 and HT29 after ANGPTL2 knockdown. Quantitative migration assay results, in which the *y*-axis represents migration rates relative to control cells. ANGPTL2 knockdown inhibited migration ability of both cell lines significantly. All assays were replicated and results are presented as mean ± SE; *, $P < 0.05$.

significantly elevated compared with healthy controls ($P < 0.05$ for both; Fig. 3A). In addition, ANGPTL2 expression levels increased with disease progression (Fig. 3A). Next, we generated ROC curves to assess the potential significance of serum ANGPTL2 as a noninvasive biomarker for the diagnosis of colorectal cancer. Our ROC analyses revealed that serum ANGPTL2 levels were robust in discriminating patients with colorectal cancer from control subjects, with AUC values of 0.814 [95% confidence interval (CI): 0.686–0.906; Fig. 3B]. The sensitivity and specificity to identify a patient with colorectal cancer were 59.4% and 95.7%, respectively. In addition, serum ANGPTL2 levels also differentiated early colorectal cancer (stage I) from control subjects with high accuracy [AUC (95% CI) = 0.785 (0.625–0.90); sensitivity = 62.5%; specificity = 91.3; Fig. 3C].

Validation of ANGPTL2 levels in serum

Sensitive quantitation of ANGPTL2 levels in serum specifically identified patients with colorectal cancer. To evaluate the diagnostic potential of ANGPTL2, a total of 240 serum samples, including those from patients with colorectal

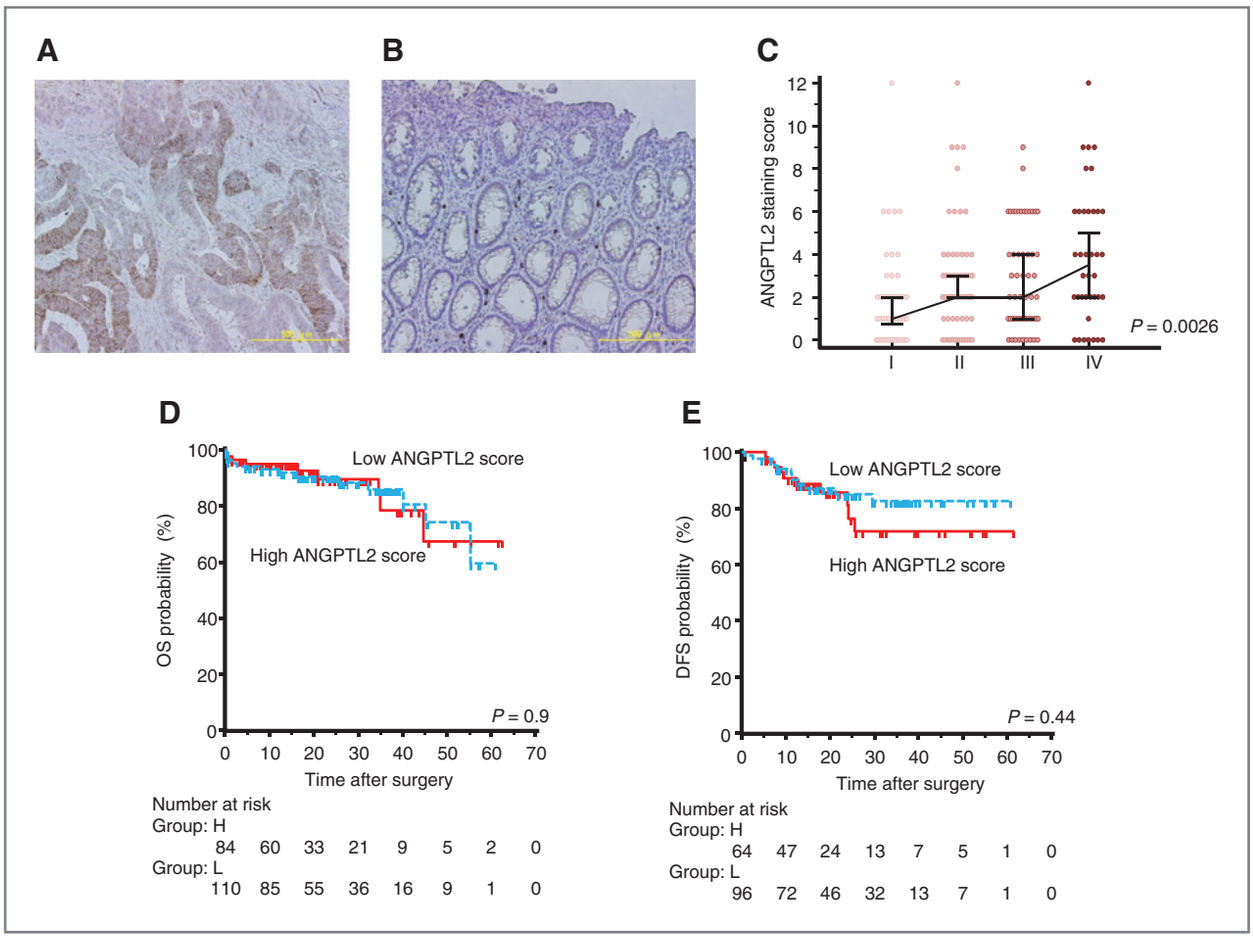

Figure 2. Representative photomicrographs showing IHC analysis of ANGPTL2 expression (original magnification, 100×) in colorectal cancer (A) and adjacent normal mucosa (B). C, ANGPTL2 immunostaining scores in 195 patients with colorectal cancer subdivided by TMN staging. Bar represents SD; line across the box indicates median value. Statistical analysis was performed using Kruskal–Wallis tests. Survival curves of patients with colorectal cancer versus ANGPTL2 protein expression score. There was no significant difference between patients with high ANGPTL2 scores and those with low ANGPTL2 scores versus (D) overall survival (OS) [$P = 0.9$, log-rank test; cutoff value = 2 (median)] or disease-free survival (DFS) [$P = 0.44$, log-rank test; cutoff value = 2 (median)].

cancer ($n = 195$) and normal controls ($n = 45$) were examined. In comparison with healthy controls, serum levels of ANGPTL2 were significantly higher in patients with colorectal cancer (Fig. 3D; $P < 0.0001$). Moreover, serum ANGPTL2 levels clearly increased with TNM stage, such that significantly higher levels were observed in stage IV patients compared with stage I or II patients ($P < 0.05$ for both; Fig. 3E), an observation that was consistent with the screening data.

Next, we performed ROC analyses to validate the potential usefulness of serum ANGPTL2 as a noninvasive biomarker for the diagnosis of colorectal cancer. Our ROC curves revealed that serum ANGPTL2 levels were robust in discriminating patients with colorectal cancer from control subjects, with AUC values of 0.885 (95% CI: 0.838–0.923; Fig. 3F). The sensitivity and specificity to identify a patient with colorectal cancer were 69.7% and 95.6%, respectively. Even more important from a screening perspective, ROC analyses demonstrated that serum ANGPTL2 levels could reliably differentiate early colorectal cancer patients (stage I) from healthy controls, as evidenced by an AUC value of 0.795 (95% CI: 0.699–0.872; Fig. 3G), and the sensitivity and specificity to identify early colorectal cancer patients were 54.2% and 93.3%, respectively. In addition, even if patients with colorectal cancer had carcinoembryonic antigen (CEA) values within normal range, serum ANGPTL2 could still discriminate patients with colorectal cancer or stage I colorectal cancer patients from healthy controls with AUC values of over 0.8, respectively (Supplementary Fig. S3A and S3B).

To further confirm the reproducibility of serum ANGPTL2 as a diagnostic marker in colorectal cancer, we compared ROC curves from validation step in both colorectal cancer patients versus control subjects, and stage I colorectal cancer patients versus control subjects with those from screening set. The results revealed that AUC values obtained from ROC analysis from the screening and validation step for identifying a patient with colorectal cancer were not significantly different (AUC of screening = 0.814, SE = 0.0563; AUC of validation = 0.885, SE = 0.885;

Table 1. Association between ANGPTL2 expression in colorectal cancer tissue or matched serum and clinicopathologic findings

Category	ANGPTL2 high ($N = 84$)	ANGPTL2 low ($N = 111$)	*P*	Serum ANFPTL2 (mean ± SD)	*P*
Age, y					
≤67[a]	42	61	0.58	1.75 ± 0.96	0.94
>67	42	50		1.66 ± 0.74	
Gender					
Male	49	64	0.95	1.75 ± 0.85	0.3
Female	35	47		1.66 ± 0.78	
Histology					
Well and mod	79	97	0.27	1.68 ± 0.84	0.14
Poor and mucinous	4	11		2.11 ± 1.01	
KRAS status					
Wild-type	54	79	0.35	1.70 ± 0.81	0.92
Mutation	30	32		1.75 ± 0.99	
BRAF status					
Wild-type	81	108	0.88	1.74 ± 0.87	0.034
Mutation	3	3		1.07 ± 0.59	
Microsatellite instability					
MSS	80	104	0.80	1.70 ± 0.88	0.078
MSI	4	7		2.02 ± 0.64	
Tumor location					
Proximal	29	35	0.77	1.82 ± 0.79	0.07
Distal	55	76		1.66 ± 0.90	
Tumor size					
≤40 mm[a]	40	72	0.02	1.55 ± 0.74	0.01
>40 mm	44	39		1.92 ± 0.97	
Serosal invasion					
Present	22	48	0.02	1.43 ± 0.79	0.0001
Absent	62	63		1.86 ± 0.87	
Lymph node metastasis					
Present	40	41	0.2	1.51 ± 0.74	0.0004
Absent	43	67		1.97 ± 0.95	
Lymphatic invasion					
Present	62	77	0.5	1.51 ± 0.85	0.0073
Absent	21	34		1.79 ± 0.86	
Venous invasion					
Present	32	34	0.3	1.58 ± 0.78	0.0066
Absent	51	77		1.96 ± 0.92	
Liver metastasis					
Present	12	8	0.16	1.65 ± 0.79	0.0239
Absent	72	103		2.26 ± 1.26	
Peritoneal metastasis					
Present	8	8	0.7	1.65 ± 0.84	0.0060
Absent	76	103		2.31 ± 0.90	
Distant metastasis					
Present	13	13	0.01	1.66 ± 0.79	0.19
Absent	71	98		2.02 ± 1.22	

[a]The median age and tumor size, respectively.

$P = 0.24$; Table S3). In a similar manner, no significant differences were observed in screening and validation step-derived AUC values for discriminating patients with stage I colorectal cancer (AUC of screening = 0.785, SE = 0.0791; AUC of validation = 0.795, SE = 0.0456; $P = 0.91$; Supplementary Table S3).

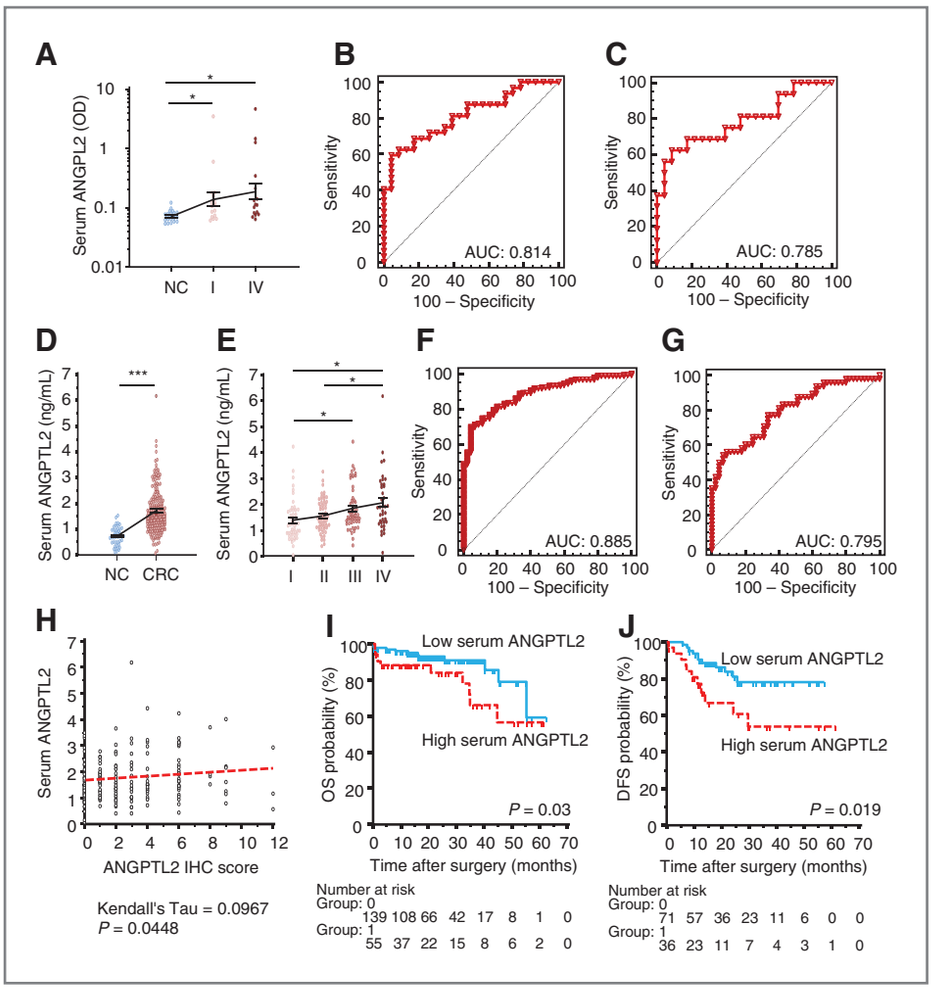

Figure 3. Serum ANGPTL2 levels in the screening phase. A, plots representing serum ANGPTL2 levels using a small subset of serum specimens from NCs ($n = 23$), colorectal cancer patients with stage I ($n = 16$), and colorectal cancer patients with stage IV ($n = 16$). ROC curve analysis using serum ANGPTL2 for distinguishing patients with colorectal cancer from NC. B, serum ANGPTL2 levels yielded an AUC value of 0.814 (95% CI: 0.686–0.906), with 59.4% sensitivity and 95.7% specificity in distinguishing colorectal cancer from NC. C, serum ANGPTL2 levels yielded AUC values of 0.785 (95% CI: 0.625–0.900) with 62.5% sensitivity and 91.3% specificity in discriminating early colorectal cancer patients (stage I) from NC. Serum levels of ANGPTL2 protein in the validation step. D, plots illustrating serum ANGPTL2 protein levels in colorectal cancers ($n = 195$) and NCs ($n = 45$). E, plots showing serum ANGPTL2 levels versus TNM staging. F, ROC curve analysis using serum ANGPTL2 levels to distinguish patients with colorectal cancer from NC. Serum ANGPTL2 levels yielded an AUC value of 0.885 (95% CI: 0.838–0.923), with 69.7% sensitivity and 95.6% specificity in distinguishing colorectal cancer from NC. G, serum ANGPTL2 levels yielded AUC values of 0.795 (95% CI: 0.699–0.872) with 54.2% sensitivity and 93.3% specificity in discriminating early colorectal cancer patients (stage I) from NC. Statistical analysis was performed using Wilcoxon and Mann–Whitney tests. *, $P < 0.05$; ***, $P < 0.0001$. H, scatter plots showing the correlation between ANGPTL2 levels in serum (*y*-axis: pg/μL) and matched tumor tissues (*x*-axis: IHC score) obtained from 195 patients with colorectal cancer. A positive correlation was found by Kendall analysis ($\tau = 0.0967$; 95% CI = −0.0112–0.206; $P = 0.0448$). I, survival curves of patients with colorectal cancer plotted against serum ANGPTL2 levels. Patients with higher serum ANGPTL2 levels showed a significantly poorer OS than those with lower levels ($P = 0.03$, log-rank test; cutoff value = 1.9652). J, patients with higher serum ANGPTL2 levels showed a significantly poorer DFS than those with lower levels ($P = 0.019$, log-rank test; cutoff value = 1.8933).

These results were further strengthened by multivariate logistic regression analyses that included variables such as age, gender and serum ANGPTL2 levels. The results showed that serum ANGPTL2 could be used as a potential diagnostic biomarker for the identification of colorectal cancer or early colorectal cancer patients ($P < 0.0001$ and $P < 0.0001$, respectively; Table 2). The ORs for patients with serum ANGPTL2 cutoff threshold values of >1.2334 being associated with colorectal cancer was 47.9 (95% CI: 11.02–205.58), and for cases with ANGPTL2 levels of >1.2095 being associated with early colorectal cancer was 15.89 (95% CI: 4.25–59.39; Table 2).

To further enhance the specificity of our assay and validate that circulating ANGPTL2 levels accurately reflected concentrations found in colorectal cancer tissues, we determined the relationship between ANGPTL2 intensity scores in primary colorectal cancer tissues and matched serum ANGPTL2 levels from individual patients with colorectal cancer. Interestingly, we observed a significant positive

Table 2. Multivariable logistic analyses of serum ANGPTL2 levels and various diagnostic factors in patients with colorectal cancer in the validation step

Variables	OR (95% CI)	*P*
Colorectal cancer patients versus control subjects		
Age, >65 vs. ≤65 y[a]	2.11 (0.95–4.67)	0.06
Sex, male vs. female	0.92 (0.42–2.01)	0.85
ANGPTL2 in serum, >1.2334 vs. ≤1.2334[b]	47.9 (11.02–205.58)	<0.0001
Early colorectal cancer patients vs. control subjects		
Age, >64 vs. ≤64 y[a]	2.29 (0.87–6.02)	0.09
Sex, male vs. female	0.94 (0.35–2.49)	0.90
ANGPTL2 in serum, >1.2095 vs. ≤1.2095[b]	15.89 (4.25–59.39)	<0.0001

[a]Median age was 65 (colorectal cancer and controls) and 62 (stage I colorectal cancer and controls) years, respectively.
[b]The cutoff values for serum ANGPTL2 in colorectal cancer patients versus control subjects and early colorectal cancer patients versus control subjects were derived by receiver operating characteristic curves with the Youden index.

correlation between ANGPTL2 expression in colorectal cancer lesions and matched serum samples (Fig. 3H; $\tau = 0.0967$, $P = 0.0448$).

Serum levels of ANGPTL2 significantly correlated with tumor progression and recurrence after surgery in patients with colorectal cancer. Next, we asked whether serum

Table 3. Univariate and multivariate analyses of OS and DFS (Cox proportional hazards regression model)

OS	Univariate analysis		Multivariate analysis	
Factors	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age (>67/67 y)[a]	1.5020 (0.6645–3.3953)	0.3307	—	—
Gender (male/female)	0.5560 (0.2440–1.2670)	0.1646	—	—
Histology (poorly and mucinous/well and mod)	5.9841 (2.3230–15.4150)	0.0002	4.1851 (1.4072–12.4468)	0.0104
KRAS (mutation/wild-type)	1.7475 (0.7565–4.0368)	0.2001	—	—
BRAF (mutation/wild-type)	2.0152 (0.2703–15.0213)	0.4964	—	—
Microsatellite instability (MSI/MSS)	0.5457 (0.0738–4.0340)	0.5549	—	—
Tumor location (proximal/distal)	1.3254 (0.5739–3.0610)	0.5116	—	—
Tumor size (>40/40 mm)[a]	2.5785 (1.1218–5.9265)	0.0265	1.2088 (0.4520–3.2325)	0.7069
Serosal invasion (present/absent)	3.9483 (1.1796–13.2157)	0.0266	2.7120 (0.5676–12.9569)	0.2135
Lymph node metastasis (present/absent)	3.3449 (1.3797–8.1096)	0.0078	1.7753 (0.6656–4.7351)	0.2539
Distant metastasis (present/absent)	4.7027 (1.8926–11.6854)	0.0009	4.3562 (1.5842–11.9788)	0.0046
Serum ANGPTL2 (high/low)	2.3287 (1.0254–5.2885)	0.0445	1.0919 (0.4299–2.7734)	0.8541
DFS	**Univariate analysis**		**Multivariate analysis**	
Factors	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age (>67/67 y)[b]	1.217 (0.5298–2.7974)	0.6448	—	—
Gender (male/female)	0.6341 (0.2749–1.4625)	0.2878	—	—
Histology (poorly and mucinous/well and mod)	2.6246 (0.8892–7.7469)	0.0821	—	—
KRAS (mutation/wild-type)	1.5823 (0.6694–3.7403)	0.3055	—	—
BRAF (mutation/wild-type)	1.4523 (0.1959–10.7668)	0.7164	—	—
Microsatellite instability (MSI/MSS)	0.6409 (0.0867–4.7351)	0.6644	—	—
Tumor location (proximal/distal)	1.3994 (0.5993–3.2678)	0.4397	—	—
Tumor size (>40/40 mm)[a]	1.1884 (0.5151–2.7417)	0.6872	—	—
Serosal invasion (present/absent)	5.7067 (0.7748–42.0314)	0.0890	—	—
Lymph node metastasis (present/absent)	2.9743 (1.1671–7.5797)	0.0231	2.7611 (1.0803–7.0573)	0.0348
Serum ANGPTL2 (high/low)	2.6239 (1.1389–6.0451)	0.0242	2.4068 (1.0418–5.5599)	0.0408

[a]The median age and tumor size, respectively.

levels of ANGPTL2 correlated with other clinicopathologic data. Table 1 illustrates that serum ANGPTL2 levels were significantly higher in patients with colorectal cancer with wild-type BRAF status ($P = 0.034$), a large tumor ($P = 0.01$), serosal invasion ($P = 0.0001$), lymphatic invasion ($P = 0.0073$), venous invasion ($P = 0.0066$), and lymph node ($P = 0.0004$), liver ($P = 0.0239$), and peritoneal metastasis ($P = 0.006$). No significant differences in serum ANGPTL2 levels were found for any of the other clinicopathologic features including KRAS mutations and MSI status.

We examined whether serum ANGPTL2 levels in patients with colorectal cancer could serve as a predictor of patient outcome. Toward that end, we performed Kaplan–Meier survival analysis. As anticipated, patients with higher levels of serum ANGPTL2 had significantly worse OS (Fig. 3I; $P = 0.03$). Moreover, increased ANGPTL2 serum concentrations were associated with decreased DFS (Fig. 3J; $P = 0.019$).

In univariate analysis (Table 3), poor OS in patients with colorectal cancer was associated with high levels of ANGPTL2 in serum ($P = 0.0445$), large tumor size (>40 mm; $P = 0.0265$), serosal invasion ($P = 0.0266$), pathologic findings (poorly differentiated or mucinous adenocarcinoma, $P = 0.0002$), lymph node metastasis ($P = 0.0078$), and distant metastasis ($P = 0.0009$). However, multivariate analysis demonstrated that high levels of serum ANGPTL2 did not serve as an independent prognostic marker for OS in patients with colorectal cancer ($P = 0.8541$).

To determine whether serum ANGPTL2 could serve as a predictor for tumor recurrence in potentially curative patients (stages II and III), Cox proportional hazard regression model was utilized (Table 3). Univariate analysis showed that lymph node metastasis ($P = 0.0231$) and high ANGPTL2 in serum ($P = 0.0242$) were significantly associated with DFS. Furthermore, multivariate analysis revealed that a high serum level of ANGPTL2 was an independent predictor for tumor recurrence for colorectal cancer patients in stages II and III (HR = 2.4068; 95% CI = 1.0418–5.5599; $P = 0.0408$; Table 3).

Discussion

The current study presents the first analysis for the function of ANGPTL2 in colorectal cancer cells. Colorectal cancer cells with elevated ANGPTL2 exhibited high metastatic potential through acquisition of enhanced proliferation, invasion, and cell motility and reduced anoikis. Using IHC analysis of clinical specimens, we found that expression of ANGPTL2 protein in colorectal cancer tissues was significantly higher than adjacent normal mucosa. In addition, we observed that high ANGPTL2 expression in colorectal cancer was significantly associated with disease progression, including larger tumor size, advance T stage (T3/T4), and cancer cell metastases to lymph nodes and distant organs.

High ANGPTL2 expression facilitates carcinogenesis through enhanced susceptibility to both premalignant changes and tumor progression. Aoi and colleagues elegantly demonstrated that ANGPTL2 expression is highly correlated with the frequency of carcinogenesis in chemical-induced skin squamous cell carcinoma mouse model (16). In addition, tumor-derived ANGPTL2 drives the metastasis of tumor cells to lymph nodes and distant organs through acquisition of EMT-related invasive and migratory abilities and by promoting angiogenesis (16). A more recent report for the molecular mechanisms of ANGPTL2 revealed that its high expression in osteosarcoma cell lines correlated with increased tumor metastasis and decreased animal survival by promoting tumor cell intravasation mediated by the integrin $\alpha5\beta1$, p38 MAPK, and matrix metalloproteinases (25). Clinically, elevated ANGPTL2 in tumor cells within the primary tumor was associated with lymph node metastasis and a reduction in the period of DFS after surgery in patients with lung cancer (17, 26). These reports are consistent with our data, suggesting a previously unrecognized causal role and clinical significance for ANGPTL2 overexpression in imparting aggressiveness and metastases of colorectal cancer.

Our study also revealed intriguing clinical data regarding serum ANGPTL2 levels in colorectal cancer. Data from the initial screening demonstrated a significant increase of serum ANGPTL2 in colorectal cancer patients compared with healthy controls, and also revealed significantly higher ANGPTL2 levels in colorectal cancer patients with stage I than healthy controls. Increased serum ANGPTL2 levels were thereafter successfully validated in a large independent set of serum samples. Our results are the first to demonstrate that high levels of ANGPTL2 in both primary colorectal cancer tissues and matched serum samples are associated with large tumor size, distant metastasis, and advanced TNM stage. Another interesting feature of our study was the existence of a statistically significant correlation between ANGPTL2 expression in primary lesions and the levels of ANGPTL2 in serum. Recently, Endo and colleagues demonstrated that breast cancer cells secrete ANGPTL2 *in vitro* and *in vivo*, and serum ANGPTL2 levels in patients with metastatic breast cancer were significantly higher compared with healthy controls (27). This report supports our hypothesis that serum ANGPTL2 in patients with colorectal cancer is likely secreted by primary colorectal cancer tissues.

We also demonstrated for the first time the potential role of serum ANGPTL2 in the diagnosis of colorectal cancer. This is supported by the markedly high AUC values derived from comparisons between patients with colorectal cancer and healthy control subjects (screening step, AUC = 0.814; validation step, AUC = 0.885). In general, the 5-year survival rates for patients with colorectal cancer are strikingly different by stage, ranging from greater than 93% for stage I disease to less than 8% for stage IV disease (28). Given the improved survival rates seen with patients with colorectal cancer, the development of a screening test for early diagnosis is extremely important. In this study, we demonstrated that serum ANGPTL2 levels demonstrated promising AUC values for the identification of early stage I colorectal cancer patients (screening step: AUC = 0.785; validation step; AUC = 0.795). It is noteworthy that these values represent some of the highest AUC levels for any serum biomarker aimed at noninvasive identification of stage I

colorectal cancer patients [compared with CEA (0.681) and CA19-9 (0.651); ref. 29]. In fact, our ROC curves revealed that serum ANGPTL2 can predict patients with colorectal cancer or stage I colorectal cancer patients with high AUC values (patients with colorectal cancer: AUC = 0.859, stage I colorectal cancer patients: AUC = 0.804) that have normal range of CEA (< 5 ng/mL). Furthermore, a multivariable logistic regression model was used to calculate the ORs for age- and gender-adjusted cases and their association with colorectal cancer or early-stage colorectal cancer. We found that the ORs for the association of high levels of ANGPTL2 with colorectal cancer or stage I colorectal cancer (early colorectal cancer) were 47.9 and 15.89, respectively. These data are encouraging for a noninvasive biomarker compared with recently reported data for a positive first guaiac FOBT (OR = 7.6; ref. 30).

We also showed that serum ANGPTL2 serves as a biomarker for predicting which patients will experience an early recurrence of colorectal cancer. Our study demonstrated that tissue ANGPTL2 expression scoring based on imunohistochemistry was not a prognostic marker for early relapse in colorectal cancer. In contrast, high levels of serum ANGPTL2 indicated poor OS and DFS, an important step toward the identification of a noninvasive method for predicting disease outcome. The multivariable Cox proportional hazards model illustrated that high level of serum ANGPTL2 was an independent DFS variable, whereas OS could not be predicted as it was significantly compromised by other clinicopathologic factors. Therefore, serum levels of ANGPTL2 might diagnose patients with colorectal cancer as well as predict tumor recurrence after curative surgery.

Evaluation of serum ANGPTL2 might become a promising tool for diagnosis or prediction of early recurrence in patients with colorectal cancer. However, there is a potential limitation of using ANGPTL2 as a single biomarker for colorectal cancer detection, as circulating ANGPTL2 levels have been described in several lifestyle related diseases, including hypertension, diabetes, dyslipidemia, obesity, and cardiovascular disease (18, 19, 31–33). The only available data regarding the colorectal cancer patients' history was the body mass index (BMI). There was no correlation between serum ANGPTL2 and BMI in colorectal cancer patients ($r = -0.0565$; $P = 0.4374$). In addition, we also reported that serum ANGPTL2 may be a potential biomarker for the diagnosis and prognosis of gastric cancer (34). Thus, in future studies, we need to investigate whether circulating ANGPTL2 levels are specifically associated with colorectal cancer itself or if this is a common phenomenon that manifests during cancer occurrence and progression initiated by inflammation in the cancer microenvironment and/or perturbations in the host immune response (35).

In conclusion, this is the first study to demonstrate the biologic and clinical significance of ANGPTL2 expression in colorectal cancer. First, colorectal cancer cells expressing high levels of ANGPTL2 had enhanced ability to proliferate, invade, and migrate and a reduced susceptibility for anoikis. In addition, ANGPTL2 protein expression in tumors was significantly associated with a metastatic phenotype in colorectal cancer. Second, our results provide compelling evidence for the potential usefulness of serum ANGPTL2 as a noninvasive diagnostic tool in patients with colorectal cancer. Moreover, our results suggest that ANGPTL2 expression might be superior to use of CEA or FOBT tests. Third, ANGPTL2 in serum is an independent predictive biomarker of tumor recurrence in curative patients, especially in stages II–III colorectal cancer. Finally, our data suggest that the primary tumor and/or adjacent normal colonic mucosa might be the source of serum ANGPTL2. Collectively, we propose that evaluation of serum ANGPTL2 might be a promising clinical tool for diagnosing early colorectal cancer patients noninvasively and for determining tumor recurrence in curative patients who require intensive monitoring after surgery.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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