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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New findings

- Angiopoietin-like protein 2 (ANGPTL2) promoted metastatic capacity of colorectal cancer (CRC) cells, and high expression of this protein were significantly associated with tumor progression in patients with CRC.

- 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 CRC patients from normal controls (NCs). 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 CRCs (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 CRC.

Clinical practice

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

Purpose: Angiopoietin-like protein 2 (ANGPTL2) is a mediator of chronic inflammation and inflammatory carcinogenesis. The biological 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 (CRC).

Experimental Design: A CRC cell line was transfected with siRNA against ANGPTL2 for the assessment of its function. We examined ANGPTL2 expression in CRC tissues (n = 195) by immunohistochemistry (IHC). Finally, we screened serum ANGPTL2 levels from 32 CRCs and 23 normal controls (NCs), and validated these results in serum samples obtained from 195 CRCs 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 CRC tissues, and was significantly associated with advanced T stage, lymph node and liver metastasis. Likewise, serum ANGPTL2 levels in CRCs were significantly higher than NCs (p < 0.01), and allowed distinguishing of CRCs from NCs with high accuracy (AUC=0.837). The subsequent validation step confirmed that serum ANGPTL2 levels in CRCs were significantly higher than in NCs (p < 0.0001), and had a high AUC value (0.885) for distinguishing CRCs from NCs. High serum ANGPTL2 was significantly associated with advanced T stage, lymph node and liver metastasis, early relapse and poor prognosis in CRCs.

Conclusion: Serum ANGPTL2 is a novel diagnostic and recurrence-predictive biomarker in patients with CRC.
Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide and is a major cause of cancer-related deaths (1). Several CRC 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 CRC 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 CRC 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 neoplasia (10, 11). Obesity is associated with chronic low-grade inflammation due to the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6), which induce the hepatic secretion of acute phase proteins such as C-reactive protein (CRP) (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 CRC 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 CRC. 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 CRC.

Accordingly, the present study aimed to evaluate the clinical significance of ANGPTL2 in both serum and matched primary tumors in CRC. In addition, we investigated the functional role of ANGPTL2 in CRC by RNA interference analysis of cultured CRC 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 CRC patients. 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 CRC patients.
Methods

Study Design

This study analyzed 490 serum and tissue specimens that were obtained from consecutively enrolled CRC patients and sex- and age-matched healthy volunteers at the Mie University Medical Hospital, Japan, between January 1, 2006 and December 31, 2011. Exclusion criteria included inflammatory bowel disease, familial adenomatous polyposis, hereditary non-polyposis 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. This study undertook a functional analysis of ANGPTL2 in CRC cells, followed by an evaluation of the associations between ANGPTL2 expression in CRC tissues (n = 195) assessed by immunohistochemistry (IHC) and clinicopathological and survival outcomes. We also quantified serum ANGPTL2 levels to analyze their clinical significance as a disease biomarker. Blood samples from preoperative CRC patients and healthy controls were collected using ‘red top’ serum vacuum blood collection tubes (Becton Dickinson and Company, UK). The samples were centrifuge at 4,000 rpm immediately; the supernatants were aliquoted in 1.5 ml tubes (Eppendorf, Germany) and were stored at −80°C conditions until use. In the screening phase, a small set of preoperative serum samples was collected from 16 CRC patients with stage I disease and 16 CRC patients with stage IV disease. We included 23 sex- and age-matched healthy subjects. To further assess the significance of serum ANGPTL2 in CRC patients, 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 IHC. 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 (IRB) 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 CRC 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 (Austin, TX). Transfections were performed by mixing cell suspensions with siRNA oligonucleotides (20 nM), 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 material 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., Valencia, CA) according to the manufacturer’s instructions. cDNA was synthesized by random hexamers using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA).
performed quantitative real-time reverse transcription analysis (qRT-PCR) using the StepOne™ Real Time PCR System (Applied Biosystems, Foster City, CA). 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 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. The average expression levels of ANGPTL2 were normalized against GAPDH using the 2-ΔΔCt method.

**KRAS/BRAF Mutation and Tumor Microsatellite Instability Analysis**

FFPE sections (10 μm thick) from 195 CRC 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 analysed by pyrosequencing using primers listed in Table S2. Reactions were run on a PyroMark Q96 ID system (Qiagen).

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 microsatellite unstable (MSI) and those showing instability at <2 markers as microsatellite stable (MSS).

**Evaluation of immunohistochemistry**

Formalin-Fixed Paraffin-Embedded (FFPE) sections (2 to 3 μm thick) from 195 CRC surgical patients were used for immunohistochemical 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. (A) 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 (Figure S1A-D). (B) 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 (Figure S1E-F). Scores obtained from A and B 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 2 pathologists was more than 3.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

In the screening assay, the serum concentrations of ANGPTL2 were quantified with a commercially available enzyme-linked immunosorbent assay kit (Uscn Life Science Inc., Wuhan) 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 enzyme-linked immunosorbent assay kit (IBL, Japan) made by Kumamoto University according to the manufacturer’s instructions and values were reported as ng/ml. Further information is provided in the Supplementary material and methods.

**Statistical Methods**

The significance of ANGPTL2 in both sera and their corresponding tumors was determined by the Mann–Whitney test, Kruskal-Wallis test or the χ² test as appropriate. The association between ANGPTL2 levels in sera and ANGPTL2 IHC scores of matched primary tumors was analyzed by Kendall's Tau (22). Receiver operating characteristic (ROC) analysis
was performed to determine the diagnostic performance of serum ANGPTL2 levels in distinguishing patients with CRC or stage I CRC 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 CRC or CRC patients from control subjects were computed. The optimal cutoff thresholds for diagnosis were obtained by Youden’s index (23). In brief, the optimal cutoff threshold values were determined at the point on the ROC curve at which Youden’s 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 odds ratios (ORs) for age- and sex-adjusted cases associated with CRC or stage I CRC according to serum ANGPTL2 levels.

We estimated that 154 patients were needed to achieve 80% power to substantiate more that 20% differences in prognostic outcome. Therefore, we enrolled adequate sample size of serum and tissues from 195 CRC 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. Youden’s index (23) was used to determine the optimal cutoff threshold of serum ANGPTL2 levels to predict the OS and DFS. Cox’s proportional hazard regression test was used to estimate univariate and multivariate hazard ratios for OS and DFS. All P values were 2-sided, and those less than 0.05 were considered statistically significant. All statistical analyses were carried out using MedCalc 12.3 for Windows (Broekstraat 52, 9030, Mariakerke, Belgium).
Results

Functional analyses of ANGPTL2 in CRC cells

ANGPTL2 gene and ANGPTL2 protein expression in selected CRC cells

We investigated ANGPTL2 gene expression by quantitative RT-PCR in 5 established CRC cell lines (Figure S2A). In the CRC cell lines, Both SW480 and HT29 showed the highest ANGPTL2 expression. In the other cell lines, gene expression was distinctly lower or non-detectable. To assess the protein expression levels of ANGPTL2 in the same CRC cell lines, ELISA was performed using homogenized lysates. The results revealed a pattern consistent with the quantitative RT-PCR data for all cell lines, except Caco2 cells (Figure S2B). Based on 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 to negative control-siRNA-treated cells 48 h post-transfection (Figure S2C, D). In addition, ELISA results were consistent with the real-time PCR data (Figure S2E, F). Based on these data, we next analyzed ANGPTL2 function in vitro.

ANGPTL2 promoted proliferation, invasion and migration but suppressed anoikis in CRC 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 h after ANGPTL2 siRNA transfection of SW480 and HT29 cells (Figure 1A, B). 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
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SW480 and HT29 cells compared to control cells, indicating that ANGPTL2 inhibits apoptosis even in non-adherent CRC cells floating in culture medium (Figure 1C, 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 to cells transfected with non-silencing siRNA (Figure 1E, F). In addition, wound healing assays were performed to compare the migratory potential of SW480 and HT29 transfected with ANGPTL2 siRNA or non-silencing siRNA. The number of migratory cells treated with ANGPTL2 siRNA was markedly decreased compared to control siRNA-treated cells (Figure 1G, H). Taken together, these results demonstrated that ANGPTL2 expression enhanced cell proliferation, invasion and migration but inhibited anoikis in CRC cells.

**ANGPTL2 protein expression in CRC tissues was associated with tumor growth and metastasis**

Immunohistochemical analysis was performed to investigate the location and intensity of ANGPTL2 protein expression, and for the evaluation of the associations between protein expression and clinicopathological data. ANGPTL2 protein expression was observed in both the cytoplasm and nucleus of each CRC tumor cell (Figure 2A). In contrast, there was no staining of ANGPTL2 in normal epithelial cells but weak expression in stroma in normal colonic mucosa (Figure 2B). ANGPTL2 staining scores were significantly increased according to TNM stage (P = 0.0026) (Figure 2C). Based upon median expression values, we established a cut-off 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 CRC patients. Kaplan-Meier curves showed no statistically significant differences between high and low expression groups for either OS (P = 0.9, **Figure 2D**) of DFS (P = 0.44, **Figure 2E**).

**Serum levels of ANGPTL2 in CRC patients 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 CRC patients in stage I, 16 CRC patients in stage IV and 23 healthy volunteers in the screening phase cohort. Serum ANGPTL2 levels in stages I and IV CRC patients were significantly elevated compared to healthy controls (P < 0.05 for both) (**Figure 3A**). In addition, ANGPTL2 expression levels increased with disease progression (**Figure 3A**). Next, we generated ROC curves to assess the potential significance of serum ANGPTL2 as a noninvasive biomarker for the diagnosis of CRC. Our ROC analyses revealed that serum ANGPTL2 levels were robust in discriminating CRC patients from control subjects, with AUC values of 0.814 (95% CI: 0.686 – 0.906; **Figure 3B**). The sensitivity and specificity to identify a patient with CRC were 59.4% and 95.7%, respectively. In addition, serum ANGPTL2 levels also differentiated early CRC (stage I) from control subjects with high accuracy (AUC [95% CI] = 0.785 [0.625 - 0.90]; sensitivity = 62.5%; specificity =91.3; **Figure 3C**).

**Validation of ANGPTL2 levels in serum**

**Sensitive quantitation of ANGPTL2 levels in serum specifically identified CRC patients.**
To evaluate the diagnostic potential of ANGPTL2, a total of 240 serum samples, including those from patients with CRC (n = 195) and normal controls (n = 45) were examined. In comparison to healthy controls, serum levels of ANGPTL2 were significantly higher in CRC patients (\(P < 0.0001\); Figure 3D). Moreover, serum ANGPTL2 levels clearly increased with TNM stage, such that significantly higher levels were observed in stage IV patients compared to stage I or II patients (\(P < 0.05\) for both) (Figure 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 CRC. Our ROC curves revealed that serum ANGPTL2 levels were robust in discriminating CRC patients from control subjects, with AUC values of 0.885 (95% CI: 0.838 – 0.923; Figure 3F). The sensitivity and specificity to identify a patient with CRC 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 CRC patients (stage I) from healthy controls, as evidenced by an AUC value of 0.795 (95% CI: 0.699 – 0.872; Figure 3G), and the sensitivity and specificity to identify early CRC patients were 54.2% and 93.3%, respectively. In addition, even if CRC patients had CEA values within normal range, serum ANGPTL2 could still discriminate CRC patients or stage I CRC patients from healthy controls with AUC values of over 0.8, respectively (Figure S3A,B).

To further confirm the reproducibility of serum ANGPTL2 as a diagnostic marker in CRC, we compared ROC curves from validation step in both CRC patients vs. control subjects, and stage I CRC patients vs. 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 CRC were not significantly different (AUC of screening = 0.814, Standard Error (SE) = 0.0563; AUC of validation = 0.885, SE = 0.885; 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 CRC (AUC of screening = 0.785, SE = 0.0791; AUC of validation = 0.795, SE = 0.0456; P = 0.91) (Table S3).

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 CRC or early CRC patients (P < 0.0001 and P < 0.0001, respectively; Table 2). The odds ratio (OR) for patients with serum ANGPTL2 cut-off threshold values of >1.2334 being associated with CRC was 47.9 (95% CI: 11.02 – 205.58), and for cases with ANGPTL2 levels of >1.2095 being associated with early CRC 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 CRC tissues, we determined the relationship between ANGPTL2 intensity scores in primary CRC tissues and matched serum ANGPTL2 levels from individual CRC patients. Interestingly, we observed a significant positive correlation between ANGPTL2 expression in CRC lesions and matched serum samples (Tau=0.0967, P=0.0448; Figure 3H).

Serum levels of ANGPTL2 significantly correlated with tumor progression and recurrence after surgery in CRC patients

Next, we asked whether serum levels of ANGPTL2 correlated with other clinicopathological data. Table 1 illustrates that serum ANGPTL2 levels were significantly higher in CRC patients 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 clinico-pathological features including KRAS mutations and MSI status.

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

In univariate analysis (Table 3), poor OS in CRC patients 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), pathological 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 CRC patients (p = 0.8541).

To determine if serum ANGPTL2 could serve as a predictor for tumor recurrence in potentially curative patients (stages II and III), Cox’s 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 CRC patients in stages II and III (HR = 2.4068, 95% CI = 1.0418 - 5.5599, p = 0.0408; Table 3).
Discussion

The present study presents the first analysis for the function of ANGPTL2 in CRC cells. CRC 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 CRC tissues was significantly higher than adjacent normal mucosa. In addition, we observed that high ANGPTL2 expression in CRC 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 et al. 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 α5β1, p38 mitogen-activated protein kinase, 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 CRC.
Our study also revealed intriguing clinical data regarding serum ANGPTL2 levels in CRC. Data from the initial screening demonstrated a significant increase of serum ANGPTL2 in CRC patients compared to healthy controls, and also revealed significantly higher ANGPTL2 levels in CRC 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 CRC 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 et al. demonstrated that breast cancer cells secrete ANGPTL2 in-vitro and in-vivo, and serum ANGPTL2 levels in metastatic breast cancer patients were significantly higher compared to healthy controls (27). This report supports our hypothesis that serum ANGPTL2 in CRC patients is likely secreted by primary CRC tissues.

We also demonstrated for the first time the potential role of serum ANGPTL2 in the diagnosis of CRC. This is supported by the markedly high AUC values derived from comparisons between CRC patients and healthy control subjects (screening step, AUC = 0.814; validation step, AUC = 0.885). In general, the 5-year survival rates for CRC patients 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 CRC, 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 CRC 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 CRC patients.
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(compared to CEA (0.681) and CA19-9 (0.651)) (29). In fact, our ROC curves revealed that serum ANGPTL2 can predict CRC patients or stage I CRC patients with high AUC values (CRC patients: AUC = 0.859, stage I CRC patients: AUC = 0.804) that have normal range of CEA (< 5ng/ml). Furthermore, a multivariable logistic regression model was used to calculate the odds ratios (ORs) for age- and gender-adjusted cases and their association with CRC or early stage CRC. We found that the ORs for the association of high levels of ANGPTL2 with CRC or stage I CRC (early CRC) 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) (30).

We also showed that serum ANGPTL2 serves as a biomarker for predicting which patients will experience an early recurrence of CRC. Our study demonstrated that tissue ANGPTL2 expression scoring based on IHC was not a prognostic marker for early relapse in CRC. 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 clinico-pathological factors. Therefore, serum levels of ANGPTL2 might diagnose patients with CRC 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 CRC patients. However, there is a potential limitation of using ANGPTL2 as a single biomarker for CRC detection, since circulating ANGPTL2 levels have been described in several life-style related diseases, including hypertension, diabetes, dyslipidemia, obesity, and cardiovascular disease (18, 19, 31-33). The only available data regarding the CRC patients’ history was the body mass index (BMI). There was no correlation between serum ANGPTL2 and BMI in CRC 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 CRC 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 biological and clinical significance of ANGPTL2 expression in CRC. First, CRC 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 CRC. Second, our results provide compelling evidence for the potential usefulness of serum ANGPTL2 as a noninvasive diagnostic tool in patients with CRC. 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 CRC. 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 CRC patients noninvasively and for determining tumor recurrence in curative patients who require intensive monitoring after surgery.
Figure Legends

**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 0-72 h after ANGPTL2 siRNA transfection. (A, B) Cell growth of SW480 and HT29 treated with ANGPTL2 siRNA (red line) was significantly inhibited compared to controls (blue line) at 48 and 72 h. Anoikis assay. (C, D) After anoikis induction for 24 h, 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, 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 100x magnification. Quantitative Transwell invasion assays, in which the Y-axis represents the number of invading cells. (G, 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 ± standard error (SE) *, \( P < 0.05 \)

**Figure 2.** Representative photomicrographs showing immunohistochemical analysis of ANGPTL2 expression (original magnification, x100) in (A) colorectal cancer and (B) adjacent normal mucosa. (C) ANGPTL2 immunostaining scores in 195 CRC patients subdivided by TMN staging. Bar represents standard deviation; line across the box indicates median value. Statistical analysis was performed using Kruskal-Wallis tests. Survival curves of CRC patients 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; cut-off value = 2 (median) or (E) disease-free survival (DFS) \( P = 0.44 \), Log-rank test; cut-off value = 2 (median).

**Figure 3.** Serum ANGPTL2 levels in the screening phase. (A) Plots representing serum ANGPTL2 levels using a small subset of serum specimens from normal controls (NC) \( n = 23 \), CRC patients with stage I \( n = 16 \) and CRC patients with stage IV \( n = 16 \). Receiver operating characteristics (ROC) curve analysis using serum ANGPTL2 for distinguishing patients with CRC from NC. (B) Serum ANGPTL2 levels yielded an Area under curve (AUC) value of 0.814 (95% CI: 0.686 – 0.906), with 59.4% sensitivity and 95.7% specificity in distinguishing CRC 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 CRC patients (stage I) from NC. Serum levels of ANGPTL2 protein in the validation step. (D) Plots illustrating serum ANGPTL2 protein levels in CRCs \( n = 195 \) and normal controls (NC; \( n = 45 \)). (E) Plots showing serum ANGPTL2 levels versus TNM staging. (F) ROC curve analysis using serum ANGPTL2 levels to distinguish patients with CRC 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 CRC 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 CRC 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/uL) and matched tumor tissues (x-axis: IHC score) obtained from 195 CRC patients. A positive correlation was found by Kendall’s analysis \( (\text{Tau} = 0.0967; 95\% \text{ CI} = -0.0112 - 0.206; P = 0.0448) \). (I) Survival curves of CRC patients plotted against serum ANGPTL2 levels Patients with higher serum ANGPTL2 levels showed a significantly poorer overall survival (OS) than
those with lower levels ($P = 0.03$, Log-rank test; cut-off value = 1.9652) (J) Patients with higher serum ANGPTL2 levels showed a significantly poorer disease free survival (DFS) than those with lower levels ($P = 0.019$, Log-rank test; cut-off value = 1.8933).
References

**Figure 1**

**A**

SW480

- Optical density
- si negative
- si ANGPTL2
- Time: 0h, 24h, 48h, 72h

**B**

HT29

- Optical density
- si negative
- si ANGPTL2
- Time: 0h, 24h, 48h, 72h

**C**

SW480

- Optical density
- si ANGPTL2
- si negative

**D**

HT29

- Optical density
- si ANGPTL2
- si negative

**E**

SW480

- % invasion
- si negative
- si ANGPTL2

**G**

SW480

- % distance
- 0h, 48h
- si negative
- si ANGPTL2

**F**

HT29

- % invasion
- si negative
- si ANGPTL2

**H**

HT29

- % distance
- 0h, 48h
- si negative
- si ANGPTL2
Figure 2

A

B

C

D

E

Low ANGPTL2 score

High ANGPTL2 score

Low ANGPTL2 score

High ANGPTL2 score

$\text{p} = 0.0026$

$\text{p} = 0.9$

$\text{p} = 0.44$

Number at risk

Group: H

84

60

33

21

9

5

2

0

Group: L

110

85

55

36

16

9

1

0

Number at risk

Group: H

64

47

24

13

7

5

1

0

Group: L

96

72

46

32

13

7

1

0
Figure 3

A

B

C

D

E

F

G

H

I

J

Serum ANGPTL2 (ng/ml)

NC I IV

AUC: 0.814

100-Specificity

Sensitivity

AUC: 0.785

100-Specificity

Serum ANGPTL2 (ng/ml)

NC CRC

AUC: 0.885

100-Specificity

Sensitivity

AUC: 0.795

100-Specificity

Serum ANGPTL2 IHC score

Kendall's Tau = 0.0967

p = 0.0448

Low serum ANGPTL2

High serum ANGPTL2

p = 0.03

Low serum ANGPTL2

High serum ANGPTL2

p = 0.019

Number at risk
Group: 0
139 108 66 42 17 8 1 0
Group: 1
71 57 36 23 11 6 0 0
Number at risk
Group: 0
55 37 22 15 8 6 2 0
Group: 1
36 23 11 7 4 3 1 0

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Table 1: Association between ANGPTL2 expression in CRC tissue or matched serum and clinicopathological findings.

<table>
<thead>
<tr>
<th>Category</th>
<th>ANGPTL2 high (N=84)</th>
<th>ANGPTL2 low (N=111)</th>
<th>p-Value</th>
<th>serum ANGPTL2 (mean ±SD)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>≤67 y*</td>
<td>42</td>
<td>61</td>
<td>0.58</td>
<td>1.75±0.96</td>
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<tr>
<td></td>
<td>&gt; 67 y</td>
<td>42</td>
<td>50</td>
<td></td>
<td>1.66±0.74</td>
</tr>
<tr>
<td>Gender</td>
<td>male</td>
<td>49</td>
<td>64</td>
<td>0.95</td>
<td>1.75±0.85</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>35</td>
<td>47</td>
<td></td>
<td>1.66±0.78</td>
</tr>
<tr>
<td>Histology</td>
<td>Well and mod</td>
<td>79</td>
<td>97</td>
<td>0.27</td>
<td>1.68±0.84</td>
</tr>
<tr>
<td></td>
<td>Poor and muc</td>
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<td>11</td>
<td></td>
<td>2.11±1.01</td>
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<td>KRAS status</td>
<td>wild type</td>
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<td>79</td>
<td>0.35</td>
<td>1.70±0.81</td>
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<td></td>
<td>mutation</td>
<td>30</td>
<td>32</td>
<td></td>
<td>1.75±0.99</td>
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<tr>
<td>BRAF status</td>
<td>wild type</td>
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<td>108</td>
<td>0.88</td>
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<tr>
<td></td>
<td>mutation</td>
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<td>MSS</td>
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<td>104</td>
<td>0.80</td>
<td>1.70±0.88</td>
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<td>MSI</td>
<td>4</td>
<td>7</td>
<td></td>
<td>2.02±0.64</td>
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<td>Tumor location</td>
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<td>35</td>
<td>0.77</td>
<td>1.82±0.79</td>
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<td></td>
<td>distal</td>
<td>55</td>
<td>76</td>
<td></td>
<td>1.66±0.90</td>
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<tr>
<td>Tumor size</td>
<td>≤40 mm*</td>
<td>40</td>
<td>72</td>
<td>0.02</td>
<td>1.55±0.74</td>
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<tr>
<td></td>
<td>&gt; 40 mm</td>
<td>44</td>
<td>39</td>
<td></td>
<td>1.92±0.97</td>
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<tr>
<td>Serosal invasion</td>
<td>present</td>
<td>22</td>
<td>48</td>
<td>0.02</td>
<td>1.43±0.79</td>
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<tr>
<td></td>
<td>absent</td>
<td>62</td>
<td>63</td>
<td></td>
<td>1.86±0.87</td>
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<tr>
<td>Lymph node metastasis</td>
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<td>40</td>
<td>41</td>
<td>0.2</td>
<td>1.51±0.74</td>
</tr>
<tr>
<td></td>
<td>absent</td>
<td>43</td>
<td>67</td>
<td></td>
<td>1.97±0.95</td>
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<tr>
<td>Lymphatic invasion</td>
<td>present</td>
<td>62</td>
<td>77</td>
<td>0.5</td>
<td>1.51±0.85</td>
</tr>
<tr>
<td></td>
<td>absent</td>
<td>21</td>
<td>34</td>
<td></td>
<td>1.79±0.86</td>
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<tr>
<td>Venous invasion</td>
<td>present</td>
<td>32</td>
<td>34</td>
<td>0.3</td>
<td>1.58±0.78</td>
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<tr>
<td></td>
<td>absent</td>
<td>51</td>
<td>77</td>
<td></td>
<td>1.96±0.92</td>
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<tr>
<td>Liver metastasis</td>
<td>present</td>
<td>12</td>
<td>8</td>
<td>0.16</td>
<td>1.65±0.79</td>
</tr>
<tr>
<td></td>
<td>absent</td>
<td>72</td>
<td>103</td>
<td></td>
<td>2.26±1.26</td>
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<tr>
<td>Peritoneal metastasis</td>
<td>present</td>
<td>8</td>
<td>8</td>
<td>0.7</td>
<td>1.65±0.84</td>
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<tr>
<td></td>
<td>absent</td>
<td>76</td>
<td>103</td>
<td></td>
<td>2.31±0.90</td>
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<tr>
<td>Distant metastasis</td>
<td>present</td>
<td>13</td>
<td>13</td>
<td>0.01</td>
<td>1.66±0.79</td>
</tr>
<tr>
<td></td>
<td>absent</td>
<td>71</td>
<td>98</td>
<td></td>
<td>2.02±1.22</td>
</tr>
</tbody>
</table>

*The median age and tumor size, respectively. SD: Standard Deviation, MSI: Microsatellite unstable, MSS: Microsatellite stable.
Table 2: Multivariable logistic analyses of serum ANGPTL2 levels and various diagnostic factors in patients with colorectal cancer (CRC) in the validation step

<table>
<thead>
<tr>
<th>Variables</th>
<th>OR (% CI)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRC patients vs control subjects</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, &gt; 65 y vs ≤ 65 y†</td>
<td>2.11 (0.95 - 4.67)</td>
<td>0.06</td>
</tr>
<tr>
<td>Sex, male vs female</td>
<td>0.92 (0.42 – 2.01)</td>
<td>0.85</td>
</tr>
<tr>
<td>ANGPTL2 in serum, &gt; 1.2334 vs. ≤ 1.2334‡</td>
<td>47.9 (11.02 – 205.58)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Early CRC patients vs control subjects</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, &gt; 64 y vs ≤ 64 y†</td>
<td>2.29 (0.87 – 6.02)</td>
<td>0.09</td>
</tr>
<tr>
<td>Sex, male vs female</td>
<td>0.94 (0.35 - 2.49)</td>
<td>0.90</td>
</tr>
<tr>
<td>ANGPTL2 in serum, &gt; 1.2095 vs. ≤ 1.2095‡</td>
<td>15.89 (4.25 – 59.39)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* CI = confidence interval; OR = odds ratio.
† Median age was 65 (CRC and controls) and 62 (stage I CRC and controls) years, respectively.
‡ The cut-off values for serum ANGPTL2 in CRC patients vs. control subjects and early CRC patients vs. control subjects were derived by receiver operating characteristic curves with Youden’s index.
Table 3: Univariate and multivariate analyses of overall survival (OS) and disease free survival (DFS)
(Cox proportional hazards regression model)

<table>
<thead>
<tr>
<th>Factors</th>
<th>OS</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>p-Value</td>
</tr>
<tr>
<td>Age (&gt; 67 y/67 y) *</td>
<td>1.5020</td>
<td>0.6645 to 3.3953</td>
<td>0.3307</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>0.5560</td>
<td>0.2440 to 1.2670</td>
<td>0.1646</td>
</tr>
<tr>
<td>Histology (poorly and mucinous/well and mod)</td>
<td>5.9841</td>
<td>2.3230 to 15.4150</td>
<td>0.0002</td>
</tr>
<tr>
<td>KRAS (mutation/wild type)</td>
<td>1.7475</td>
<td>0.7565 to 4.0368</td>
<td>0.2001</td>
</tr>
<tr>
<td>BRAF (mutation/wild type)</td>
<td>2.0152</td>
<td>0.2703 to 15.0213</td>
<td>0.4964</td>
</tr>
<tr>
<td>Microsatellite instability (MSI/MSS)</td>
<td>0.5457</td>
<td>0.0738 to 4.0340</td>
<td>0.5549</td>
</tr>
<tr>
<td>Tumor location (proximal/distal)</td>
<td>1.3254</td>
<td>0.5739 to 3.0610</td>
<td>0.5116</td>
</tr>
<tr>
<td>Tumor size (&gt; 40 mm/40 mm) *</td>
<td>2.5785</td>
<td>1.1218 to 5.9265</td>
<td>0.0265</td>
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<tr>
<td>Serosal invasion (present/absent)</td>
<td>3.9483</td>
<td>1.1796 to 13.2157</td>
<td>0.0266</td>
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<tr>
<td>Lymph node metastasis (present/absent)</td>
<td>3.3449</td>
<td>1.3797 to 8.1096</td>
<td>0.0078</td>
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<tr>
<td>Distant metastasis (present/absent)</td>
<td>4.7027</td>
<td>1.8926 to 11.6854</td>
<td>0.0009</td>
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<tr>
<td>Serum ANGPTL2 (high/low)</td>
<td>2.3287</td>
<td>1.0254 to 5.2885</td>
<td>0.0445</td>
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</table>

<table>
<thead>
<tr>
<th>Factors</th>
<th>DFS</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>p-Value</td>
</tr>
<tr>
<td>Age (&gt; 67 y/67 y) **</td>
<td>1.217</td>
<td>0.5298 to 2.7974</td>
<td>0.6448</td>
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<tr>
<td>Gender (male/female)</td>
<td>0.6341</td>
<td>0.2749 to 1.4625</td>
<td>0.2878</td>
</tr>
<tr>
<td>Histology (poorly and mucinous/well and mod)</td>
<td>2.6246</td>
<td>0.8892 to 7.7469</td>
<td>0.0821</td>
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<td>1.5823</td>
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<td>BRAF (mutation/wild type)</td>
<td>1.4523</td>
<td>0.1959 to 10.7668</td>
<td>0.7164</td>
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<tr>
<td>Microsatellite instability (MSI/MSS)</td>
<td>0.6409</td>
<td>0.0867 to 4.7351</td>
<td>0.6644</td>
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<tr>
<td>Tumor location (proximal/distal)</td>
<td>1.3994</td>
<td>0.5993 to 3.2678</td>
<td>0.4397</td>
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<td>Tumor size (&gt; 40 mm/40 mm) *</td>
<td>1.1884</td>
<td>0.5151 to 2.7417</td>
<td>0.6872</td>
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<tr>
<td>Serosal invasion (present/absent)</td>
<td>5.7067</td>
<td>0.7748 to 42.0314</td>
<td>0.0890</td>
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<td>Lymph node metastasis (present/absent)</td>
<td>2.9743</td>
<td>1.1671 to 7.5797</td>
<td>0.0231</td>
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<tr>
<td>Serum ANGPTL2 (high/low)</td>
<td>2.6239</td>
<td>1.1389 to 6.0451</td>
<td>0.0242</td>
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</tbody>
</table>

CI=confidence interval; HR=Hazard Ratio. MSI= Microsatellite unstable; MSS= Microsatellite stable
*The median age and tumor size, respectively.
Elevated serum angiopoietin-like protein 2 correlates with the metastatic properties of colorectal cancer: a serum biomarker for early diagnosis and recurrence

Yuji Toiyama, Koji Tanaka, Takahito Kitajima, et al.

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