Development of autoantibody signatures as biomarkers for early
detection of colorectal carcinoma

Running title: New serum diagnostic markers for early CRC

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Statement of translational relevance

The study provided a 5-phage-peptide classifier that had > 90.0% sensitivity and specificity in discriminating groups between colorectal carcinoma (CRC) patients at early stage (I+II) and healthy controls. The 5-phage-peptide classifier had a high accuracy in diagnosing CRC in those with serum carcinoembryonic antigen < 5ng/ml. The 5-phage-peptide classifier was able to discriminate all of the 9 patients with serrated adenoma who have high tendency to develop CRC from healthy controls. The 5-phage-peptide classifier can be used as a novel method for noninvasive diagnosis and screening of early CRC in high-risk populations. However, the 5-phage-peptide classifier unlikely served as serum biomarkers for discriminating CRC from some autoimmune diseases because of overlapping seroreactivity. Serum samples of patients with autoimmune diseases may be indispensable in the validation of autoantibody biomarkers of CRC before clinical application.
ABSTRACT

Purpose: To select autoantibody signatures for early detection of colorectal cancer (CRC).

Experimental Design: A phage cDNA expression library was constructed with fresh tumors from 30 CRC patients and biopanned using serum pools of 20 CRC patients and 20 healthy controls. A classifier was discovered in the training set of 30 CRC patients at stages I-II and 30 matched healthy controls and then blindly validated in an independent set of 60 CRC patients, 60 healthy controls, 52 polyps patients, and 30 autoimmune diseases patients. Expression of proteins was examined using immunohistochemistry.

Results: Five phage-peptide clones showing higher discriminatory power than others in training set were selected for validation. The 5-phage-peptide classifier was able to discriminate between early CRC patients and healthy controls with sensitivities of 90.0-92.7% and specificities of 91.7-93.3%. In those with serum carcinoembryonic antigen <5ng/mL, the classifier was efficient in discriminating CRC from healthy controls, with an area under the curve of 0.975. The classifier was able to discriminate all of the 9 patients with serrated adenoma from healthy controls. Thirteen (43.3%) of the patients with autoimmune diseases were misclassified. Of the 5 phage-peptides, one encoded a peptide identical to IgG heavy-chain constant region. IgG immunostaining was stronger in mesenchymal cells than in cancer cells in the tumors and was apparent in serrated adenoma.

Conclusions: The 5-phage-peptide classifier stands out as promising early diagnostic
biomarkers for CRC but it is unsuitable for discriminating CRC from autoimmune diseases. Truncated IgGs generated from the tumors might be novel CRC-associated antigens.
Colorectal cancer (CRC) is the third most common cancer in the world. There are about one million new cases diagnosed annually and half a million people died of this malignancy worldwide every year (1). Death from CRC is preventable. Most of the patients can be cured by surgery if CRC is detected at early stage. Early detection and intensive surveillance for the high risk population are important for improving therapeutic responses of CRC patients. Colonoscopy and sigmoidoscopy are reliable for early detection of CRC and colorectal polyps, but the invasive, unpleasant and inconvenient nature limits their widespread use. Fecal occult blood tests (FOBT) and fecal immunochemical test have the advantage of screening early CRC (2). However, feces-based CRC examinations can be difficult to standardize in clinical practice. CRC progresses from normal tissue to carcinoma through an accumulation of genetic alterations, such as mutations and loss of heterozygosity that affect oncogenes such as K-ras and tumor suppressor genes including p53, MCC, APC, and DCC (3). The detection of the genetic materials in blood is limited by the technical challenge of DNA recovery and by the limited sensitivity of any single genetic alteration due to the extremely low abundance of mutated DNA in plasma or serum. Serological markers have advantages in screening or diagnosing early CRC. Colon cancer-specific antigen 2 in sera has overall specificity of 78.4% and sensitivity of 97.3% in separating individuals with advanced adenomas and CRC from normal, hyperplastic, and nonadvanced adenoma populations at a cutoff of 10.8μg/mL (4). Carcinoembryonic antigen (CEA) is of proven benefit as a prognostic marker, but has a limited validity for early CRC. Other biomarkers, such as carbohydrate antigen 19-9 (CA19-9) and
tissue inhibitor of metalloproteinase type 1, are lack of validity for CRC (5). Hence, there is great need for novel serum markers for early detection of this malignancy.

Autoantibody is a hallmark feature of systemic or organ-specific autoimmune diseases (6). Autoantibodies to known or unknown tumor-associated antigens (TAA) are present in sera of patients with some malignancies (7-14). Various technologies such as serologic analysis of recombinant cDNA expression libraries (SEREX), phage display, and protein arrays have been used to identify autoantibody signatures for cancer diagnosis (14-19). The presence of autoantibodies may precede onset of symptoms and clinical findings of malignancies by months or years (7, 20). Profiling of circulating autoantibodies is therefore very attractive in diagnosing cancers at early stage. With the use of SEREX, protein array, and enzyme-linked immunosorbent assay (ELISA), autoantibodies to TAAs have been found in sera of the patients with CRC (14, 21-23). Although a panel of phage-peptides developed by SEREX has high sensitivity and specificity in discriminating colon cancer patients with healthy controls, TAAs have not been fully identified (14). Serum autoantibodies to some known TAAs have been documented to have relative low validity in discriminating CRC patients from healthy controls (22, 23). Furthermore, the specificity of circulating autoantibodies for the detection of CRC has not been evaluated using sera of the patients with precancerous polyps and those with autoimmune diseases.

In this study, we developed a 5-phage-peptide classifier that was able to discriminate
CRC patients at early stage from healthy controls and also able to indicate the presence of precancerous polyps. We demonstrated, for the first time, that some of the phage-peptides had overlapping seroreactivities in the patients with CRC and the patients with autoimmune diseases.

Materials and Methods

Study subjects and samples

Sera of 110 newly diagnosed, pathologically confirmed CRC patients before receiving any treatment, 52 newly diagnosed, pathologically confirmed patients with colorectal non-cancerous polyps, 30 patients with autoimmune diseases (10 patients with systemic lupus erythematosus, 10 patients with rheumatoid arthritis, and 10 patients with ankylosing spondylitis), and 110 healthy controls were collected at Departments of Colorectal Surgery, Oncology, Rheumatology, and Physical Examination Center, the 1st affiliated hospital of this university from August 2008 to October 2010, respectively. All the specimens of tumors and polyps were pathologically identified by a pathologist (Y.Y.). CRC patients with other concurrent malignant diseases and autoimmune diseases were excluded. Age-, sex-matched healthy controls whose sera were used for discovery and training set were free of CRC and colorectal polyps as examined by colonoscopy following the same examination protocol including fasting and colonic preparation as the patients with CRC and the patients with colorectal
polyps. The age-, sex-matched healthy controls whose sera were used for validation were those who received routine physical examination in the hospital and were proven to be free of fecal occult blood as examined by guaiac-based FOBT, free of family history of CRC and colorectal polyps and have normal serum CEA levels (< 2.5ng/mL for all), but refused to receive a colonoscopy. Serum from 5mL fasting blood was separated by centrifugation at 4°C and stored in a sterile tube at -80°C within 4h of sample collection. Serum CEA levels were measured by the CEA-RIA Mab kit (Abbott Laboratories, Inc., Chicago, IL). Fresh primary CRC tissues from 30 patients who had not received any treatment before surgery were collected at Department of Colorectal Surgery of this hospital from March 2008 to May 2009 and were frozen immediately in liquid nitrogen after surgical resection until used for phage library construction. Clinical characteristics of the participants are presented in Table 1. The study protocol conformed to the ethical guideline of the 1975 Declaration of Helsinki, and was approved by the Institutional Review Board of this university. All participants gave informed consents.

Construction of T7 phage display cDNA expression library

Message RNA from fresh tumors was prepared as previously described (24). Equal amounts of mRNA from the samples of the 30 CRC patients were pooled, reversely transcribed to cDNA with random primers, and cloned into T7 select 10-3b vectors arms of phage in T7 Select® system (Novagen, Madison, WI) according to the
manufacturer instructions. After in vitro packaging, a phage display library was titrated and amplified as previously described (15).

**Enrichment and screening of phage-peptide clones**

To enrich phage clones that bind specifically to circulating antibodies of the patients with CRC, we performed 5 cycles of biopanning with pooled sera of 20 patients with CRC and 20 healthy donors, as previously described (15). Selection of independent phage clones, PCR identification and calculation of normalized reactivity (seroreactivity) were performed as previously described (24). We examined the seroreactivity of each selected clone with the samples of another 30 CRC patients at stages I-II and 30 age-, sex-matched healthy controls in training set by using ELISA. The clones with higher discriminatory power than others were validated in an independent set of 120 cases (60 patients with CRC and 60 age-, sex-matched healthy controls). Another set of 82 patients (52 patients with colorectal polyps and 30 patients with autoimmune diseases) was also included in the validation study. The flow chart is shown in Fig. 1.

**Identification of exogenous peptides of the phage clones**

Insert of each phage clone was sequenced using the flanking primers of the vector. DNA sequencing, identification of phage identity and prediction of the polypeptide
epitope were carried out as previously described (24). A known protein identical to an exogenous peptide of the phage clone was selected for immunohistochemistry.

**Immunohistochemistry**

Rabbit polyclonal antibody to human IgG and monoclonal mouse anti-human CD20cy antibody (DAKO, Glostrup, Danmark) were used for immunohistochemical analyses of an independent set of the tumor specimens and adjacent pathologically normal specimens from 54 patients with CRC according to a previous protocol (24). The deparaffinized sections were incubated with 20% goat sera to block nonspecific binding and then incubated with 1:400 diluted primary antibody to human IgG or with 1:100 diluted primary antibody to human CD20. All of the samples were analyzed by three observers (W.C., Y.Y., and G.C.) who were blinded to the clinical information. Scoring of intensity of the immunostaining was done semiquantitatively (negative, weak-moderate staining, and strong staining). There was a close agreement (> 90%) among the investigators. Disagreements were resolved by consensus.

**Statistical analysis**

Independent samples t test was used to compare the seroreactivities between CRC patients and healthy controls. We then ranked the discriminatory power of each phage-peptide clone by using Random Forest analysis with digeR package in training.
set (25). A metaclone classifier with the first 5 phage-peptide clones was developed using Bayesian binary regression method in BinReg 2.0 package (http://www.duke.edu/~dinbarry/) (26, 27). A probit function enabled us to generate a probability of CRC for each sample, referred to as the “classification score.” The classifier was then examined using leave one-out cross-validation (LOOCV) within all the serum samples in training set. An optimal threshold classification score of 0.5 was chosen based on a receiver operating characteristic (ROC) analysis and used as the predefined “cut-point” to dichotomize samples into cancer (classification score > 0.50) and non-cancer (classification score < 0.50). This threshold was then applied in validation set of 60 CRC patients and 60 healthy controls. We also evaluated the validity of serum CEA levels and calculated the area under the curve (AUC) and their 95% confidence interval (CI) of the classification scores and CEA levels in the validation set. Wilcoxon rank-sum test was used to evaluate positive degrees of the immunostaining for IgG in cancer cells and mesenchymal cells of CRC tissues and adjacent colorectal tissues. All statistical tests were two-sided and done with Statistical Program for Social Sciences (SPSS 16.0 for Windows, SPSS, Chicago, IL). $P < 0.05$ was considered as statistically significant.

Results

Screening of phage-peptide clones exhibiting high reactivity with pooled sera of the CRC patients
With the use of mRNA of tumors from the 30 patients with CRC, we constructed a phage-display library of CRC containing $4.0 \times 10^6$ primary recombinants. After 5 rounds of the biopannings, we randomly picked up 2,000 phage clones for PCR identification. A total of 1,247 of the 2,000 clones contained exogenous DNA inserts. In order to decrease the complexity of subsequent studies and develop a specific phage-peptide predictor, we firstly screened the 1,247 clones with the serum pools by using ELISA. Twenty-one clones that had high reactivity with pooled sera of the CRC patients (normalized reactivity > 2.0) but low reactivity with pooled sera of healthy controls (normalized reactivity < 1.5) were selected for subsequent study.

**Selection of phage-peptide clones for the detection of early CRC in the training set**

The 21 clones were subjected to serologic analysis with the training set of 30 CRC patients at stages I-II and 30 age- and sex-matched healthy controls. The discriminatory power of each clone was ranked by using Random Forest analysis. Combination of the first 5 phage clones (no.95, no.149, no.174, no.396, and no.1009) had the higher discriminatory power than others by using the Bayesian binary regression model, as shown in Fig. 2. ROC curve analysis indicated that the 5-phage-peptide classifier had a satisfactory discriminatory power with an AUC of 0.951 (95% CI, 0.894-1.000; $P < 0.001$). Leaving-one-out validations revealed the
sensitivity and specificity of the 5-phage-peptide classifier were 90.0% (95% CI, 0.74-0.98) and 93.3% (95% CI, 0.78-0.99) at a cutoff classification score of 0.50.

Validation of the 5-phage-peptide clones in an independent set of CRC patients and healthy controls

The 5 phage-peptides were subjected to serologic analysis in an independent set of 120 subjects (60 CRC patients and 60 age- and sex-matched healthy controls) in a completely blinded manner. Each of the 5 clones showed a significant difference between cases and controls ($P < 0.001$ for each). With the use of the BinReg classifier with the 5 phage-peptide clones built in training set, the classification score was assigned to each of the 120 subjects based on their seroreactivities. We correctly classified 54 of 60 CRC patients and 55 of 60 healthy controls at a cut-off classification score of 0.50. The misclassified ones contained 2 of 11 (18.2%) patients at stage I, 1 of 30 (3.3%) patients at stage II, and 3 of 19 (15.8%) patients at stage III. The sensitivity and specificity of the 5-phage-peptide classifier were 90.0% (95% CI, 0.82-0.98) and 91.7% (95% CI, 0.85-0.99) for CRC in the validation set, respectively. Odds ratio (OR) for cases with the classification score $> 0.50$ being associated with CRC was 99.0 (95% CI, 28.5-343.7). Importantly, the sensitivity and specificity of the 5-phage-peptide classifier was 92.7% (95% CI, 0.85-1.00) and 91.7% (95% CI, 0.85-0.99) for CRC at early stage (I+II), respectively.
The CEA medians were 3.19 ng/mL (range, 0.20-165 ng/mL) in the patients with CRC and 1.37 ng/mL (range, 0.05-7.26 ng/mL) in the healthy controls. ROC for CEA was generated with serum values of CEA in the 120 subjects. ROC for the seroreactivity of the 5-phage-peptide classifier was generated with the classification score of each sample. Logistic regression analysis with LOOCV revealed that the 5-phage-peptide classifier (AUC, 0.966; 95% CI, 0.935-0.997) performed with better accuracy than CEA (AUC, 0.761; 95% CI, 0.671-0.851). AUCs for clones no.95, no.149, no.174, no.396, and no.1009 were 0.825 (95% CI, 0.752-0.899), 0.812 (95% CI, 0.735-0.890), 0.887 (95% CI, 0.826-0.948), 0.846 (95% CI, 0.778-0.915), and 0.888 (95% CI, 0.830-0.947), respectively. Multivariate logistic regression analysis with CEA levels and the classification score of the 5-phage-peptide classifier indicated that the classifier was a unique significant biomarker ($P < 0.001$) for the detection of CRC. Addition of CEA did not improve the power of the classifier in discriminating the patients with CRC from the healthy controls, indicating overlapping diagnostic values of the classifier and CEA. There were 99 subjects (59 healthy controls and 40 CRC patients) with serum CEA < 5ng/ml. The classifier also performed with better accuracy (AUC, 0.975; 95% CI, 0.952-0.999; $P < 0.001$) than CEA (AUC, 0.653; 95% CI, 0.535-0.772; $P = 0.010$) for the detection of CRC.

The presence of the autoantibodies in the patients with colorectal polyps

Sera from 52 patients with colorectal polyps including 9 patients with serrated
adenoma were applied to analyze the seroreactivities of the 5 phage-peptide clones. The seroreactivities were significantly up-regulated in the patients with colorectal polyps than in the healthy controls ($P < 0.005$ for each). Whereas, the seroreactivities of the 4 phage-peptide clones except clone no.1009 were significantly lower in the patients with colorectal polyps than in the CRC patients ($P < 0.005$ for each), as shown in Fig. 3. The classification scores of 27 patients with colorectal polyps (51.9%, 27/52) were higher than the cutoff value of 0.50. All of the 9 patients with serrated adenoma had the classification scores of $> 0.50$. Thus, the classifier might indicate the presence of some colorectal adenoma.

The presence of the autoantibodies in the patients with autoimmune diseases

The seroreactivities of the 5 phage-peptide clones were analyzed in 30 patients with autoimmune diseases and then compared with those in the CRC patients and those in healthy controls. The seroreactivities of clones no.95, no.149, no.174, and no.396 were significantly lower in the patients with autoimmune diseases than in the CRC patients ($P < 0.001$ for each). However, the seroreactivity of clone no.1009 was significantly higher in the patients with autoimmune diseases than in the CRC patients ($P < 0.005$). The seroreactivities of clones no.174 and no.1009 were significantly higher in the patients with autoimmune diseases than in the healthy controls ($P < 0.001$ for each). The details are shown in Fig. 3. The classification score of each patient with autoimmune disease was evaluated by using the classifier, 43.3% (13/30)
of patients with autoimmune diseases had the classification score $> 0.50$. Thus, the classifier is inefficient in discriminating CRC and autoimmune diseases.

**CRC-associated antigens displayed on the 5 phage-peptide clones**

Table 2 presents amino acid sequences and potential antigenic epitopes of the inserts of the 5 phage-peptides. Two translated polypeptides (no.174 and no.95) showed no homology to any known proteins. A polypeptide (no.149) showed 60% homology to hCG2038983, an uncharacterized protein. Clone no.1009 displayed a polypeptide sharing 80% homology to TAS2R39, taste receptor type 2 member 39. One polypeptide with 29 amino acids (no.396) was identical to human IGHG1, IGHG2, IGH@, and IGHV4-31, the C-terminal constant region of heavy-chain of human immunoglobulin G (IgG).

**Expression of IgG and B cell marker CD20 in CRC and adjacent tissues**

Expression of IgG and CD20 in the 54 tumors and 14 available adjacent tissues of 54 patients with CRC were examined using immunohistochemistry. In the tumors, IgG immunostaining was strongly evident in the mesenchymal cells surrounding cancer cells, whereas in the adjacent colorectal tissues, IgG immunostaining was strong in serrated adenoma (Fig. 4A). Table S1 summarized IgG immunostaining in the tumors of 54 CRC patients and adjacent colorectal tissues of 14 CRC patients. IgG
immunostaining was significantly stronger in the mesenchymal cells than in the cancer cells \( (P < 0.001) \) in the tumors and was significantly stronger in the epithelial cells in the adjacent colorectal tissues than in the cancer cells in the tumors \( (P = 0.008) \).

Furthermore, IgG immunostaining in mesenchymal cells was significantly stronger in tumors than in the adjacent colorectal tissues \( (P = 0.027) \). To investigate if the tumor or mesenchymal cells generated IgG via cell fusion with mature B cells, we performed immunohistochemistry using anti-human CD20 antibody. It was found that CD20 staining was evident in the infiltrating lymphocytes, but absent in epithelial cancer cells (Fig. 4B).

**Discussion**

To ensure the reliability of selected recombinant phage-peptide clones as promising biomarkers of CRC, we used sera of newly diagnostic CRC patients and age- and sex-matched healthy controls without colorectal polyps in the discovery and training stages. The first 5 phage-peptide clones with higher discriminatory power than others were selected for the establishment of the classifier in the training set. The 5-phage-peptide classifier had a strong discriminatory power with an AUC of 0.951. At the classification score of 0.50, the sensitivity was 90.0% and the specificity was 93.3% in discriminating early CRC patients and the healthy controls. The 5-phage-peptide classifier was then validated in an independent set of 60 patients with CRC and 60 healthy controls in a completely blinded manner, and similar sensitivity
and specificity were obtained. Importantly, the sensitivity and specificity of the classifier was 92.7% and 91.7% in discriminating the CRC patients at early stage (I+II) from the healthy controls in the validation set, respectively. We also demonstrated that the classifier was able to characterize an individual with a high probability of being a CRC patient. Thus, the 5-phage-peptide classifier should be efficient in diagnosing CRC at early stage.

With the use of an independent set of validation samples, we demonstrated that each of the 5 phage-peptide clones had a higher power than CEA in discriminating the CRC patients and the healthy controls. In the subjects with CEA level below 5ng/ml, the classifier had a strong power in discriminating the CRC patients and the healthy controls, with an AUC of 0.975, indicating that the classifier might be able to fill in gaps of CEA in diagnosing early CRC. The classifier also has a higher validity than the reported arrays of some known CRC-associated antigens for the detection of CRC (21-23). Furthermore, OR for cases with the classification score > 0.50 being associated with CRC was 99.0, which was much higher than an OR of 7.6 for G-FOBT and an OR of 18.7 for serum miR-92, the two methods recently reported for the early detection of CRC (28, 29). Thus, the classifier compares favorably with some non-invasive biomarkers for the detection of CRC.

More than 95% of CRC arises from adenomas (30). Adenomatous polyps are tumors of benign neoplastic epithelium with variable potential for malignancy. Serrated
adenoma and hyperplastic polyps have a greater malignant potential (31). Transformation from serrated polyps to invasive CRC can be rapid (32). In this study, the seroreactivities of the 5 phage-peptide clones were significantly higher in the patients with colorectal polyps than in the healthy controls, while the seroreactivities of 4 of the 5 phage-peptide clones were significantly higher in the CRC patients than in those with colorectal polyps. Furthermore, all of the 9 patients with serrated adenoma had the classification score of > 0.50. These data indicate that the 5 phage-peptides are not only applicable for early detection of CRC, but also indicative of serrated adenoma, a well-known precancerous change.

It is largely unknown if the immunoreactivity of circulating autoantibodies in the patients with autoimmune diseases overlap with that in cancer patients. In this study, we found that the seroreactivities of clones no.1009 and no.174 were significantly higher in the patients with autoimmune diseases than in the healthy controls, furthermore the seroreactivity of clone no.1009 was significantly higher in the patients with autoimmune diseases than in the patients with CRC (Fig. 3). Clone no.1009 is also a likely marker for autoimmune diseases. The overlapping seroreactivities of clones no.1009 and no.174 greatly reduce the power of the 5-phage-peptide classifier in discriminating CRC from autoimmune diseases and also imply that CRC and autoimmune diseases might share some immunopathologic mechanisms. The 5-phage-peptide classifier in combination with other markers might be of value in discriminating CRC from autoimmune diseases.
Clone No.1009 displayed an exogenous peptide sharing 80% homology to taste receptor type 2 member 39. This receptor may play a role in the perception of bitterness and in sensing the chemical composition of the gastrointestinal content (33). The role of this receptor in human disease has not reported. According to this study, this receptor might be associated with CRC and autoimmune diseases. Clone No.149 displayed a peptide sharing 60% homology to an uncharacterized human protein hcg2038983. The peptide of clone no.396 was identical to Fc terminal of IgG heavy-chain constant region. IgG and IgM autoantibodies against Fc terminal of IgG have been found in some autoimmune diseases and termed as rheumatoid factors (RF). The high rate of RF seropositivity in patients with malignancies had been documented in 1960s. Elevation of RF has been detected in sera of patients with solid cancers at diagnosis and after radiation and/or chemotherapy, possibly because antigenic materials released from tumors elicit an immune response (34). Isotype of RF has been found to be associated with cancer prognosis (35). Recently, Ig heavy-chain proteins and their corresponding genes have been found in epithelial cancer cells including colon cancer cells (36-40). IgG secreted by cancer cells has some capacity in maintaining the growth and survival of malignant cells, furthermore the aberrant expression of Ig heavy-chain constant region may indicate the tumor grades (37, 41, 42). In this study, the seroreactivity of clone no.396 was higher in the CRC patients and the polyp patients but not higher in the patients with autoimmune diseases than in the healthy controls, indicating that the IgG fragment might generate
in neoplastic tissues of colorectal origin. However, our immunohistochemistry demonstrated that IgG immunostaining was strong in the mesenchymal cells in CRC tissues and also strong in serrated adenoma, which is not consistent with previous findings (36-40). In human CRC tissues, the expression of matrix metalloproteinases (MMPs), especially MMP3 and MMP7, is specifically up-regulated, as compared to the normal tissues (43-45). MMP-3 and MMP-7 preferentially cleave IgGs in the lower hinge (46, 47). The truncated IgG heavy-chain constant region released from the tumors might be conformationally altered, thereby eliciting CRC-related humoral immune responses.

Since mature B lymphocyte is traditionally believed to be unique source of Igs and B lymphocytes are required for establishing chronic inflammation that promotes de novo carcinogenesis (48), we investigated if CRC or mesenchymal cells generated IgG via cell fusion with mature B cells in the inflammation-carcinogenesis process. We found that CD20 immunostaining was apparent in lymphocytes infiltrated in the inflammatory stroma rather than in epithelial cancer cells, indicating that mature B cells are involved in the inflammation process but not fuse with epithelial cancer cells that produce IgG.

Weakness of this study should be mentioned. Firstly, the use of Random Forest analysis runs the risk of over fitting the multiple data. Secondly, the healthy controls whose sera used for validation did not receive a colonoscopy, possibly leading to
misclassification of the study subjects. Thirdly, more data are needed to confirm if IgG heavy-chain constant region is a CRC-associated antigen. Fourthly, the sample size was small, further studies with large sample size in different populations are necessary to validate the 5-phage-peptide classifier.

In conclusion, the 5-phage-peptide classifier can be applied to discriminate early CRC patients from healthy controls and to indicate serrated adenoma with high potential to develop CRC. Colorectal tumors might generate truncated IgG via MMPs in situ. The truncated IgG might elicit humoral immune response and generate autoantibodies that indicate CRC. Further prospective studies are needed to confirm if the polyp patients with high seroreactivities to the classifier have higher incidence of CRC.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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Figure legends

Fig. 1. Flow diagram of this study. CRC, colorectal carcinoma; ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristic.

Fig. 2. Development of the 5-phage-peptide classifier for discriminating CRC patients and healthy controls in training set. (A) One-at-a-time cross-validation predictions of classification probabilities. The corresponding values on the vertical axis were estimated classification probabilities with corresponding 90% probability intervals marked as lines to indicate uncertainty about these estimated values. (B) Receiver operating characteristics curves (ROC) of the 5 phage peptide clones in training set. AUC, area under the curve.

Fig. 3. Comparison of the seroreactivities of the 5 phage-peptide clones among 90 healthy controls, 52 patients with colorectal polyps, 90 CRC patients, and 30 patients with autoimmune diseases. The box area contains 50% of the data samples, and 99.3% of the samples are within the upper and lower boundary markers. N, healthy controls; P, colorectal polyps; C, colorectal cancer; AD, autoimmune disease.

Fig. 4. Immunostaining of IgG and B lymphocyte biomarker CD20 in colorectal tumors and adjacent colorectal tissues. (A) Representative examples of immunostaining using polyclonal antibody to IgG. (a) Weak expression in cancer cells
and strong expression in mesenchymal cells in the tumors. (b) Strong expression in papillary colorectal carcinoma. (c) Strong expression in signet-ring cell colorectal carcinoma. (d) and (e) Strong expression in mesenchymal cells and in epithelial cells of adjacent colorectal tissues. (f) Strong expression in mesenchymal cells and in epithelial cells of colorectal serrated adenoma. (B) CD20 immunostaining is negative in epithelial cancer cells (a,b) and non-cancerous epithelial cells but is positive in mature B cells infiltrated in inflammatory stroma (c). Scale bar, 20µm.
Phase I: Discovery

- cDNA pool of 30 CRC tissues
- Cloned into T7 select 10-3b
- Primary phage library
- Biopanned with sera pools
- PCR for 2000 clones randomly selected
- ELISA with sera pools
- 1247 recombinant phages
- 21 CRC-specific clones

Phase II: Selection and training

- ELISA with individual serum of 30 CRC patients and 30 healthy controls
- Primary phage library select 10-3b
  1. Rank discriminatory power of each clone by RandomForest analysis;
  2. Best combination of phage clones by Binregression and ROC analysis.

Phase III: Validation

- A panel of 5 phage-peptide clones with high sensitivity and specificity
- ELISA with individual serum of:
  - 60 CRC patients
  - 60 healthy controls
  - 52 patients with colorectal polyps
  - 30 patients with autoimmune diseases
- DNA sequencing, BLASTp and epitope prediction
AUC = 0.951

Sensitivity

1 - Specificity

Healthy controls (n=30)
CRC patients (n=30)

Training samples

Classification score

Figure 2

P < 0.001
Figure 3
Figure 4
<table>
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<th>Characteristic</th>
<th>Discovery and Training Set (n=100)</th>
<th>Validation Set (n=202)</th>
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<td>Age (years)</td>
<td>Mean 59</td>
<td>Median (range) 61 (35-82)</td>
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<tr>
<td>Gender</td>
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<td>Female 25 (50%)</td>
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<td>TNM stage</td>
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<td>II 7 (23%)</td>
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<tr>
<td>Site of primary lesion</td>
<td>Colon 12 (40%)</td>
<td>Rectum 12 (40%)</td>
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Table 2. Sequence identity of the inserts in the 5 phage-peptide clones

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<tr>
<th>Clone ID</th>
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<th>Protein Sequences†</th>
<th>Size of translated peptides (aa)</th>
<th>Protein identity</th>
<th>E value</th>
<th>BLASTp Identities</th>
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<td><strong>MSFY</strong></td>
<td>4</td>
<td>no</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>No.396</td>
<td>286</td>
<td><strong>QGNVFSCSVMHEALHNHYTQKSL</strong></td>
<td>29</td>
<td>IGHG1, IGHG2,</td>
<td>5.00E-19</td>
<td>29/29(100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>LSPGK</strong></td>
<td></td>
<td>IGH@, IGHV4-31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.396</td>
<td>286</td>
<td><strong>QGNVFSCSVMHEALHNHYTQKSL</strong></td>
<td>29</td>
<td>IGHG3, IGHG4</td>
<td>3.00E-19</td>
<td>28/29(97%)</td>
</tr>
<tr>
<td>No.1009</td>
<td>346</td>
<td>AGAISFIFLS<strong>ACSHKNTTL</strong>NSIAP*</td>
<td>24</td>
<td>TAS2R39</td>
<td>8.3</td>
<td>8/10(80%)</td>
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<tr>
<td>No.149</td>
<td>196</td>
<td><strong>WRCGP</strong></td>
<td></td>
<td><strong>PATREAEADCLDPGR</strong>G</td>
<td>47</td>
<td>hcg2038983</td>
</tr>
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† Epitope sequences marked with bold and underlined. * stop codon.
Development of autoantibody signatures as biomarkers for early detection of colorectal carcinoma

Wen-Jun Chang, Ling-Ling Wu, Fuao Cao, et al.

*Clin Cancer Res* Published OnlineFirst July 19, 2011.

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