Macrophage Migration Inhibitory Factor May Be Used as an Early Diagnostic Marker in Colorectal Carcinomas

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Abstract

Recent genetic studies have identified many differentially expressed genes in colorectal carcinomas. For validation of up-regulated genes in colorectal carcinomas, we performed an enzyme-linked immunosorbent assay. Candidate markers were selected from gene expression data for 40 colorectal cancers and 35 matched normal mucosal samples. Based on intensive filtering, 9 genes were selected for the further evaluations. Among them, macrophage migration inhibitory factor (MIF), inhibin βA, and chemokine ligand 10 were screened, and the results were compared with carcinoembryonic antigen (CEA) in serum samples of 129 patients with colon cancer and 53 healthy control subjects. We found that the serum MIF level was significantly increased in patients with colorectal cancer. Compared with CEA, MIF was more sensitive in early cancer detection (47.3% vs 29.5%). However, the specificity was not as high as that of CEA (90.6% vs 100.0%). Our findings indicate that MIF may be used as a diagnostic marker in colorectal carcinomas.

Molecular genetic changes in colorectal carcinomas are among the best understood of such changes in common human cancers. This is a complex and dynamic process that is expected to involve many genetic changes and altered gene expressions. At the genetic level, inactivation of the *APC*/β-catenin genes, followed by activation of oncogenes and inactivation of additional tumor suppressor genes, is characteristic of colon cancers with chromosomal instability, and these changes are frequent in the majority of colon cancers. Inactivation of 1 of the genes responsible for DNA nucleotide mismatch repair, which leads to extensive mutations in the genes containing repetitive DNA sequences, is characteristic of colon cancers with high microsatellite instability.[1,2] In addition to these causative genetic changes in colon cancers, many differentially expressed genes have been identified by functional genomic studies. Frequently reported up-regulated genes in colon cancer from DNA array studies include *NME1, GNB2L1, CSE1L, SOX9, CCNB2, LAMR1, RAN, SLP1*, and *STK1*.[3-10] According to proteomic analysis, inorganic phosphatase, aldolase A, annexin 3 and 4, calgranulin B, and calreticulin are frequently reported to be up-regulated in colon carcinoma.[11-20]

Identification of altered gene expression profiles in colon cancers and the evaluation of these markers in body fluid could allow for early and efficient detection of colon cancer. To study this issue, we performed a serologic test for molecules up-regulated in colorectal carcinomas and known to be secreted in body fluid. We demonstrated the macrophage migration inhibitory factor (MIF) level as an early marker for the detection of colorectal carcinoma.

Materials and Methods

Case Selection

A total of 40 colorectal carcinomas were included in this study for the discovery of up-regulated molecular markers in colon cancers. In 35 cases, grossly normal mucosa remote from the tumors was included as a control sample. All cases were selected randomly from consecutively identified cases at the Gastrointestinal Tumor Working Group Tissue Bank, Yonsei University Medical Center, Seoul, Korea, between December 1996 and December 2004, and from the Liver Cancer Specimen Bank (supported by the National Research Bank Program of the Korea Science and Engineering Foundation in the Ministry of Science & Technology). Authorization for the use of these tissues for research purposes was obtained from the institutional review board of Yonsei University College of Medicine. For validation of the selected markers in body fluid, fresh blood samples were collected from 129 patients with colorectal cancer and 53 people without colorectal cancers between January 2005 and February 2006. Informed consent was received from each patient, and authorization of the study was obtained from the institutional review board. All of the patients with cancer and healthy control subjects underwent colonoscopy. In patients with colon cancer, blood samples were collected 1 day before surgery, and in control subjects, blood samples were collected in the outpatient clinic. All blood samples were delivered to the pathology laboratory within 30 minutes after collection, and the serum was separated. For the blood preparation, 3 mL of blood was collected in a serum separation tube, and the serum was prepared as previously described.[21] The mean age of the patients with cancer was 63 years and of the control subjects was 60 years. Tumor stage and other data are listed in **Table 1**.

Gene Expression Analysis in Colon Cancer

RNA was extracted from fresh frozen tissue samples. Tumor specimens were microdissected on a cryostat and fractionated to enrich the tumor cell population. Microarray formulation and RNA preparation and hybridization have been previously reported.[10] Briefly, 20 μg of total RNA extracted from microdissected frozen tissue samples was used as input for complementary DNA targets. The targets and Universal Human Reference (UHR) RNA (Stratagene, La Jolla, CA) were hybridized to an oligonucleotide microarray containing 19,200 probe sets representing 18,664 unique genes (LEADS, Compugen, Rockville, MD), and the array was scanned using GenePix scanners (Molecular Devices, Toronto, Canada). Expression values for each gene were calculated by using GenePix Pro 4.0 analysis software. Of the 40 colorectal carcinomas and 35 matched normal mucosal samples, gene expression data from 17 colorectal carcinomas and 15 corresponding normal mucosal samples have been previously reported.[10] The remaining 23 tumors

Table 1
Comparison of Clinicopathologic Features of 129 Patients With Colorectal Cancer by Serum MIF and CEA Levels*

	Serum MIF Level (ng/mL)			Serum CEA Level (ng/mL)		
Feature	**>34.8**	**≤34.8**	***P***	**>6.3**	**≤6.3**	***P***
Age (y)			.5785			.1174
<60	24	25		10	39	
≥60	37	43		28	52	
Sex			.7651			.3694
M	36	38		19	55	
F	25	30		19	36	
Stage			.0100			.0048
I	15	6		1	20	
II	21	21		11	31	
III	15	24		12	27	
IV	10	17		14	13	
Location			.4722			.6623
Colon	34	42		25	51	
Rectum	27	26		13	40	
Size (cm)			.9295			.9462
<5	27	33		18	42	
≥5	34	35		20	49	
MSI status			.9659			.1032
High	8	6		1	13	
MSS and low	53	62		37	78	

CEA, carcinoembryonic antigen; MIF, macrophage migration inhibitory factor; MSI, microsatellite instability; MSS, microsatellite stable.
* Data are given as number of cases. Système International units for CEA are micrograms per liter; the conversion factor is 1.0.

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and 20 normal mucosal samples were analyzed by the same method and used in the subsequent data analysis.

Selection of Up-regulated Molecular Markers

Scanned images from each slide were initially analyzed by using GenePix, version 6.0, and spots of low quality were flagged out manually. Spot intensities were corrected for background and normalized for dye bias using LOWESS regression with the print-tip group correction. Then data were normalized with regard to the distribution of the ratios in each respective data set.

Because relative gene expression levels were measured with respect to UHR RNA, the data were restructured into relative gene expression levels in carcinomas with respect to normal mucosa by calculating the logarithmic difference between the ratio of each carcinoma vs UHR data and the average of the ratios from normal data vs UHR data. Gene expression ratios from data for 15 normal cases vs UHR data points from the first experiment were averaged into a representative normal level in data set A, and gene expression ratios from 20 normal cases vs UHR data points from the second experiment were averaged into a representative normal level in data set B. These relative gene expression levels were then averaged. The same method was used to calculate a representative tumor level of the first and second experiments: expression levels in 17 carcinomas (first experiment) and 23 carcinomas (second experiment), respectively. Genes were considered up-regulated in carcinomas if the representative tumor level was greater than the representative normal level by at least 1.6-fold.

Enzyme-Linked Immunosorbent Assay

*S*erum concentrations of MIF, chemokine ligand 10 (CXCL10), and inhibin A were measured by enzyme-linked immunosorbent assay (ELISA) using the Human MIF Quantikine ELISA kit (R&D Systems, Minneapolis, MN), Human CXCL10/IP-10 Quantikine ELISA kit (R&D Systems), and Activin A assay kit (Oxford Bio-Innovation, Kidlington, England). All ELISAs were performed according to the manufacturers' instructions.

Statistical Analysis

Statistical analysis was performed using MedCalc for Windows, version 9.3.3.0 (MedCalc Software, Mariakerke, Belgium). We constructed receiver operating characteristic (ROC) curves for each biomarker and combination of biomarkers to assess their diagnostic accuracy in distinguishing patients with colon cancer from control subjects. By using the ROC method, we calculated sensitivity, specificity, error rate, and area under the curve to determine the diagnostic accuracy of our findings. Analysis of a combination of carcinoembryonic antigen (CEA) and MIF was performed by logistic regression. We also calculated *P* values to determine whether serum levels of MIF and CEA depend on the histopathologic features of each case. The *P* values were calculated by using the χ^2 test.

Results

Identification of Up-regulated Genes Encoding Secreted Proteins in Colorectal Carcinomas

We examined the relative expressions of each gene in the tumors in 2 ways. Relative gene expression in 40 carcinomas was compared first with respect to UHR RNA and then with respect to the average expression in 35 matched normal mucosal tissue samples. In 40 gene expression profiles, 655,929 values of 768,000 (19,200 spots × 40 samples) remained valid after data processing. Representative tumor levels of 456 genes were differentially expressed by at least 1.6-fold, and 93 genes were predicted to be secreted by using the secreted protein database. Candidate genes were further narrowed by selecting those that were up-regulated by 1.6-fold in all 40 tumor samples. Only 69 genes met these criteria, and 9 genes, including MIF, inhibin A, and CXCL10, were predicted to encode secreted proteins. These genes were finally selected as candidate tumor biomarkers in serum **Table 2**.

Validation of MIF, Inhibin A, and CXCL10 and Up-regulation of MIF in Serum of Patients With Colon Cancer

To examine the diagnostic values of up-regulated genes encoding secreted proteins, serum levels of *MIF, CXCL10,* and inhibin A were evaluated. These 3 genes were selected because commercial ELISA kits are available and known gene function is related to tumor development. Serum samples from 129 patients with colon carcinoma and 53 control subjects were analyzed. MIF showed significantly increased serum levels in patients with colon cancer; the mean serum concentration was 35.2 ng/mL in patients with colon cancer and 21.3 ng/mL in control subjects **Figure 1A**. There was no statistically significant difference in serum levels of CXCL10 and inhibin A between patients with colon cancer and control subjects. The mean serum value of CXCL10 was 0.16 ng/mL in patients with cancer and 0.15 ng/mL in control subjects, and the mean value of inhibin A was 0.35 ng/mL in patients with cancer and 0.28 ng/mL in control subjects **Figure 1B** and **Figure 1C**.

Comparison of CEA and MIF as Diagnostic Markers

To evaluate the diagnostic value of MIF expression levels, we measured CEA levels in serum samples **Figure 1D**. When compared with levels of MIF in serum samples, MIF was more sensitive but its specificity was not as good as that of CEA. We determined cutoff values according to

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Table 2
Nine Candidate Genes for Biomarker Validation*

UniGene	Minimum	Fold	Maximum	Symbol	Gene Name	Biologic Process
Hs.128553	1.2	18.9	233.9	*WNT2*	Wingless-type MMTV integration site family member 2	Multicellular organismal development
Hs.413924	1	5.13	33.36	*CXCL10*	Chemokine (C-X-C motif) ligand 10	Cell surface receptor–linked signal transduction
Hs.28792	1.1	5.1	59.3	*INHBA*	Inhibin βA	Cell surface receptor–linked signal transduction
Hs.407995	1	2.03	4.5	*MIF*	Macrophage migration inhibitory factor	Cell proliferation; cell surface receptor–linked signal transduction
Hs.521171	1	2.01	5.06	*HIG2*	Hypoxia-inducible protein 2	Response to stress
Hs.368131	1.1	1.89	3.92	*ST7*	Suppression of tumorigenicity 7	Unknown
Hs.84113	1.1	1.87	4.11	*CDKN3*	Cyclin-dependent kinase inhibitor 3	G_1/S transition of mitotic cell cycle
Hs.507769	1.1	1.85	5.82	*ALG5*	Asparagine-linked glycosylation 5 homolog	Protein amino acid glycosylation
Hs.517356	1.2	1.77	3.23	*COL18A1*	Collagen, type XVIII, α 1	Organ morphogenesis

MMTV, mouse mammary tumor virus.
* UniGene is an organized view of the transcription. Minimum represents the lowest fold change of expression level in colon cancer patient tissues compared with normal mucosal tissues from our microarray data. Fold represents the average fold change of expression level in colon cancer patient tissues compared with normal mucosal tissues from our microarray data. Maximum represents the highest fold change of expression level in colon cancer patient tissues compared with normal mucosal tissues from our microarray data.

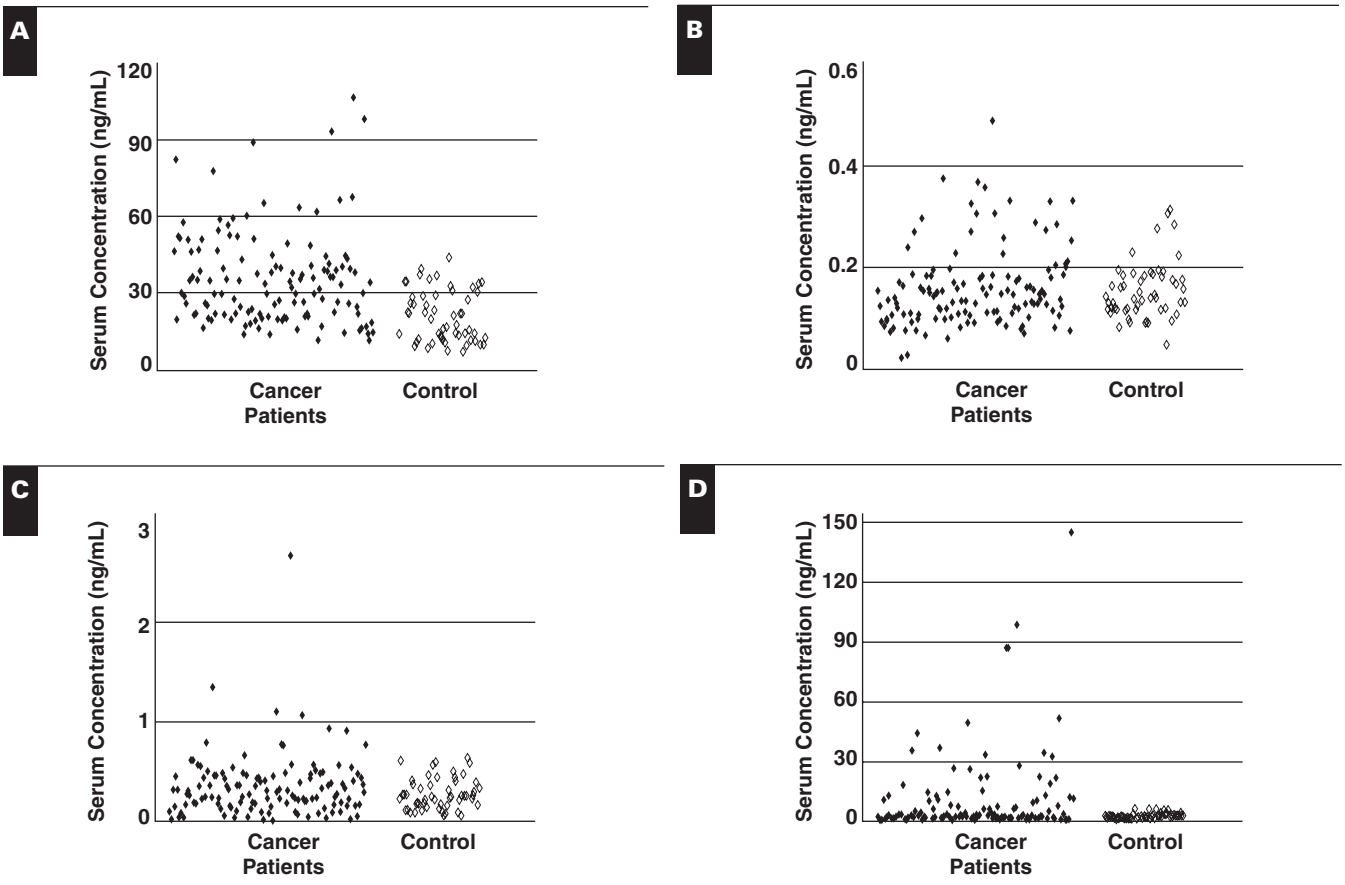

Figure 1 Serum concentrations of macrophage migration inhibitory factor (MIF), CXCL10, inhibin A, and carcinoembryonic antigen (CEA) in 129 patients with colon cancer and 53 healthy control subjects by enzyme-linked immunosorbent assay. Black diamonds represent patients with cancer, and white diamonds represent control subjects. **A**, Serum MIF level. Mean concentration, 35.2 ng/mL in patients with cancer; 21.3 ng/mL in control subjects. **B**, Serum CXCL10 level. Mean concentration, 0.16 ng/mL in patients with cancer; 0.15 ng/mL in control subjects. **C**, Serum inhibin A level. Mean concentration, 0.35 ng/mL in patients with cancer; 0.28 ng/mL in control subjects. **D**, Serum CEA level. Mean concentration, 10.1 ng/mL (10.1 μg/L) in patients with cancer; 2.5 ng/mL (2.5 μg/L) in control subjects.

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the ROC curve. When MIF and CEA levels were increased in cancer patients by a cutoff value of 34.8 ng/mL and 6.3 ng/mL, respectively, the diagnostic sensitivity of MIF was 47.3% and specificity was 90.6% **Figure 2A** and **Table 3**. For CEA, the diagnostic sensitivity was 29.5% and specificity was 100% **Figure 2B** (Table 3). When diagnostic sensitivity according to tumor stage was compared, MIF was more useful than CEA for the detection of early stage cancer **Figure 2C** (Table 3). The diagnostic sensitivity and specificity of MIF were 71.4% and 90.6% in stage I, 50% and 90.6% in stage II, 38.5% and 90.6% in stage III, and 37.0% and 90.6% in stage IV, respectively. In contrast, the diagnostic sensitivity and specificity of CEA were 4.8% and 100% in stage I, 26.2% and 100% in stage II, 30.8% and 100.0% in stage III, and 51.9% and 100.0% in stage IV, respectively.

The combination of these 2 markers showed greater sensitivity and specificity **Figure 2D**. Combined, sensitivity was 61.2% and specificity was 92.5% (Table 3).

Discussion

In this study, we identified MIF as a candidate diagnostic biomarker for colorectal carcinomas. We identified a large number of genes that are up-regulated in colon cancer and have coding sequences for secreted proteins. Among these

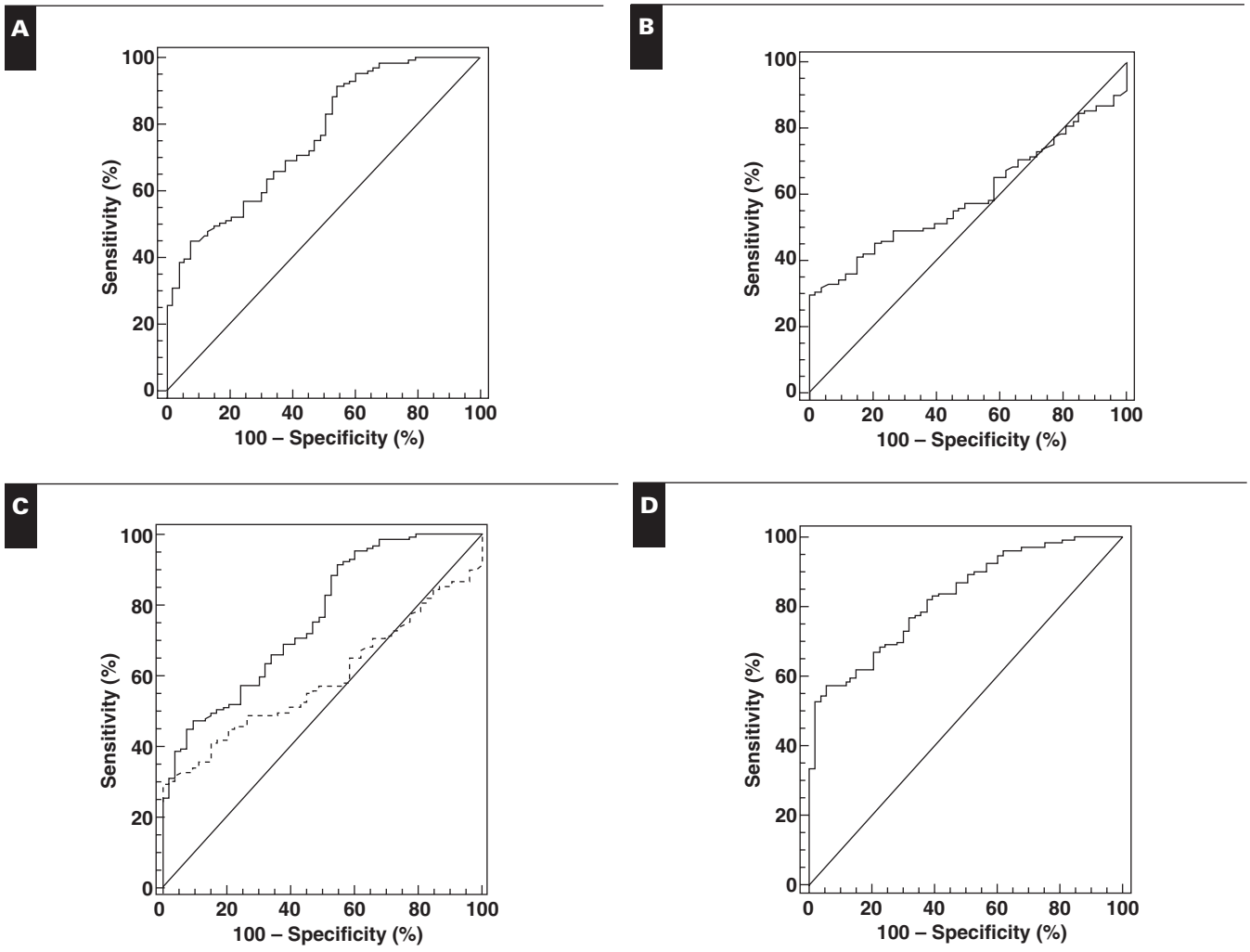

Figure 2 Comparison of carcinoembryonic antigen (CEA) and macrophage migration inhibitory factor (MIF) as diagnostic markers. The receiver operating characteristic (ROC) curve was used to determine the sensitivity, specificity, and SE of each marker. The SE, area under the curve (AUC), and significance level are summarized in Table 3. The ROC curve of each marker represents 90.6% specificity and 47.3% sensitivity for MIF according to a criterion of >34.8 ng/mL (**A**), 100% specificity and 29.5% sensitivity for CEA according to a criterion of >6.3 ng/mL (6.3 μg/L; **B**), and comparison of MIF (solid line) and CEA (dotted line) using ROC methods (**C**). AUC of MIF, 0.761; AUC of CEA, 0.585. **D**, Combination of MIF and CEA; specificity, 92.5%; sensitivity, 61.2%.

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Table 3
Summary of Receiver Operating Characteristic Curve Methods for MIF and CEA

	Specificity (%)	Sensitivity (%)	SE	Area Under the Curve	Significance Level *P* (Area = 0.5)
MIF	90.6	47.3	0.036	0.761	.0001
CEA	100.0	29.5	0.045	0.585	.0418
MIF and CEA	92.5	61.2	0.03	0.827	.0001

CEA, carcinoembryonic antigen; MIF, macrophage migration inhibitory factor.

proteins, we found that serum levels of MIF are frequently up-regulated in patients with colon cancer compared with levels in control subjects. These data suggest that MIF may be useful as a diagnostic marker.

By DNA array analysis, we identified 65 genes that were up-regulated in all 40 carcinomas compared with matched normal-appearing mucosal samples. Many of these dysregulated genes were also reported to be up-regulated at the transcript level in previous reports.[3-10] These genes belonged to the functional categories of nucleic acid metabolism (18 genes, 29.5%), development (12 genes, 19.7%), cell cycle (10 genes, 16.4%), cell proliferation (10 genes, 16.4%), and organelle organization and biogenesis (7 genes, 11.5%) according to the functional bioinformatics database.[22] Nine genes (about 13% of those detected) were identified as genes encoding secreted proteins in the secreted protein database. In this study, we selected *MIF, CXCL10,* and inhibin A for further serum evaluation.

MIF is a secretory cytokine and is known to contribute to the development and promotion of malignant tumors.[23] Overexpression of MIF has been reported in several types of cancer. MIF has been shown to promote malignant cell transformation, enhance neovascularization, and inhibit tumor cell–specific cytolytic responses. Overexpression of MIF was reported to be related to poor outcome and early metastasis.[23] In the gastrointestinal tract, MIF is generally expressed and increased in sporadic colorectal adenomas and carcinomas.[24] In this study, we showed overexpression of MIF in early-stage colon tumors and elevated serum MIF levels. These findings suggest that MIF may be a good candidate as a diagnostic marker for colon cancer. However, we also found a relatively broad range of MIF serum levels in healthy people without cancer. This breadth of the range might result from the fact that MIF expression is increased in inflammatory disorders, including inflammatory bowel disease, pancreatitis, and hepatitis.[25] At present, we have no data concerning the serum MIF level in inflammatory diseases, but several previous studies showed that the serum MIF level is elevated in some inflammatory diseases.[26-29] We believe that future studies should include and evaluate the serum MIF level from patients with inflammatory diseases as another control group to support our hypothesis. Moreover, it also should be noted that our small-scale study is not sufficient for clinical application of MIF as a diagnostic marker. Future study should involve testing in an unrelated data set to validate this marker with large numbers of samples.

Two other markers selected for this study, inhibin A and CXCL10, were increased in colon cancer tissue samples, but their serum levels were not significantly increased compared with levels in control samples. CXCL10 is one of the chemokine, small cytokine–like secreted proteins[30] and is reported to be overexpressed in several cancers. Recently, interactions between chemokines and chemokine receptors have been found to be important in the development and progression of cancer,[31,32] and overexpression of CXCL10 and its receptor, CXCR3, have been reported in breast cancer cells.[33] Recently, in colorectal cancer, overexpression of CXCL10 and CXCR3 has been reported.[34] Inhibin and activin are dimeric polypeptides. Inhibin A is a dimmer of inhibin βA and inhibin α. Inhibin and activin contribute to cell growth and development through binding to transmembrane receptors with serine/threonine kinase activity. Overexpression of activin A in stage IV colorectal carcinoma has been reported.[35] Although we have demonstrated overexpression of CXCL10 and inhibin A in colon cancer tissue samples, we could not demonstrate a statistically significant increase in serum levels in patients with colon cancer. These findings indicate that CXCL10 and inhibin A cannot be used as diagnostic markers in the serum. However, the possibility of prognostic or predictive markers of CXCL10 and inhibin A in cancer tissues remains and warrants further study. In addition, because the other 6 markers also have potential as colorectal cancer diagnostic biomarkers, evaluation of these markers might also be useful for biomarker discovery.

We compared MIF levels with CEA levels and found that the combination of these 2 markers can be used to increase the sensitivity of colorectal carcinoma diagnosis. CEA is a glycoprotein involved in cell adhesion that is normally produced during fetal development, but production stops before birth, and CEA is not usually present in the blood of healthy adults. However, it was been found that serum from people with colorectal, gastric, pancreatic, lung, and breast carcinoma has higher levels of CEA than that from healthy people. The previously reported sensitivity of

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CEA is about 20% to 40%, and specificity is about 70% to 100%. In accordance with previous reports, the serum CEA level is related to tumor progression, and, thus, evaluating serum CEA has limited value in detecting early-stage colorectal cancer. We, therefore, suggest that the serum MIF level has diagnostic value in colon cancer detection alone and in combination with CEA.

We identified MIF as a candidate diagnostic marker for colorectal carcinoma. Moreover, the combination of MIF and CEA may be valuable in the early detection of colorectal carcinomas.

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