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Clinically Applicable Serum Biomarkers Among 14 Candidates Associated With Recurrence of Stage II and III Colorectal Cancer

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Abstract. *Background/Aim: We evaluated the predictive value of candidate serum biomarkers for recurrence in stage II and III colorectal cancer (CRC) after curative surgery. Patients and Methods: A total of 33 and 120 patients with CRC with or without recurrence at 5 years after curative surgery were included in the training set and the validation set, respectively. Possible serum biomarkers were examined for associations with CRC recurrence using receiver operating characteristics (ROC) curve analysis. Results: In the training set, the expression levels of the 14 biomarkers were compared according to recurrence. Among them, five biomarkers that had significantly different expression levels were validated in 60 patients with recurrence at 5 years after curative surgery and 60 patients without. Multivariate analysis showed that natural log-transformed values of carcinoembryonic antigen (CEA), cyclin-dependent kinase regulatory subunit 2 (CKS2), 2'-5'-oligoadenylate synthetase 2 (OAS2), and autophagy-related gene 5 (ATG5) in preoperative serum were significantly related to recurrence. ROC analysis showed that these biomarkers were able to discriminate patients with recurrence from those without (area under the curve=0.828, 95% confidence interval=0.755-0.990). Conclusion: Preoperative serum levels of CEA, CKS2, OAS2 and ATG5 were independent risk factors for recurrence. A combination of serum CEA, CKS2, OAS2 and ATG5 predicted tumor recurrence well in patients with stage II and III CRC.*

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of worldwide cancer-related death (1). In the Republic of Korea, CRC is the second most common cancer and was the third leading cause of cancer-related death during 2016 (2). Approximately 30-50% of patients with CRC develop disease recurrence despite curative resection (3). Among the clinicopathologicaI risk factors, T4 or N2 disease, the presence of lymphovascular or perineural invasion, and poorly differentiated histology are considered as significant risk factors for recurrence. A number of promising serum- and plasma-based molecular markers for CRC recurrence have been identified over the past several years. However, despite the large number of published studies on blood-based biomarkers, there are few robust biomarkers that predict recurrence or the response to treatment.

For decades, carcinoembryonic antigen (CEA) has been used as an unparalleled serum biomarker for the diagnosis and recurrence of CRC (4-6). Generally, a high preoperative serum concentration of CEA is associated with worse oncological outcome (6). However, some studies have reported that there were no significant relationships between CEA concentration and oncological outcomes (7, 8).

By reviewing published studies and our previous work, we selected candidate serum biomarkers for CRC, namely autophagy-related gene 5 (ATG5), ATG10, small proline-rich repeated protein 3 (SPRR3), aldehyde dehydrogenase 1A1

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(ALDH1A1), insulin-like growth factor-binding protein 1 (IGFBP1), gelsolin (GSN), 2'-5'-oligoadenylate synthetase 2 (OAS2), triggering receptor expressed on myeloid cells-1 (TREM1), signaling lymphocyte activation marker family member 7 (SLAMF7), transcription factor activating enhancer-binding protein 2e (TFAP2E), and lysyl oxidase (LOX) (9-18). We also added two potential molecules, metastasis associated in colon cancer-1 (MACC1) and cyclin-dependent kinase regulatory subunit 2 (CKS2), which were repeatedly shown to be correlated with metastasis and prognosis of CRC, as well as the well-known biomarker plasma CEA (19). In the present study, we assessed these proteins in preoperative blood samples and correlated the results with the recurrence and metastasis of CRC. Moreover, we evaluated a combination of possibly potent serum markers for its value for predicting the recurrence of CRC.

Patients and Methods

Training set. Thirty-three patients with serum stored at the Bio-Resource Center of Asan Medical Center (Seoul, Republic of Korea) were randomly chosen and divided into the recurrence group (N=14) and no-recurrence group (N=19) to identify candidate proteins for use as biomarkers. All patients provided written informed consent regarding the use of their fresh or frozen serum samples for this purpose.

Verification set. The candidate biomarkers identified from the training set were further verified in 120 patients with stage II or III CRC who underwent curative surgery at Asan Medical Center between January 2011 and May 2013, and underwent regular follow-up for 5 years. We retrospectively analyzed the medical records of these patients, who were divided equally according to the presence of recurrence within 5 years of surgery. The preoperative serum samples of the selected patients were obtained from the Bio-Resource Center of Asan Medical Center. The study protocol for the training and verification sets was approved by the Institutional Review Board of Asan Medical Center (IRB no.: 2014-0150), and the study was performed in accordance with the Declaration of Helsinki.

Determination of biomarker expression level. Quantitation of candidate biomarkers was carried out on serum samples at 1:10 dilution, and measured *via* an enzyme-linked immunosorbent assay kit (ELISA; MyBioSource, San Diego, CA, USA) according to the manufacturer's instruction. Absorbance was measured on a microplate reader with absorbance at 450 nm (Tecan, Melbourne, Australia). The limits of detection and quantification were ALDH1A1: 2.88 and 2.94; ATG5: 0.02 and 0.41; ATG10: 0.002 and 0.18; CKS2:0.58 and 0.60; GSN: 8.39 and 8.85; IGFBP1: 0.14 and 0.30; LOX: 0.21 and 0.74; MACC1:0.09 and 0.37; OAS2: 0.03 and 0.5; SLAMF7: 0.005 and 0.291; SPRR3: 0.002 and 0.19; TFAP2E: 0.001 and 0.12; TREM1: 0.003 and 0.173 ng/ml, respectively. The level of serum CEA was measured by using enzyme immunoassay (ELISA-2-CEA kit; CIS Biointernational, Marcoule, France). The normal level of CEA concentration was customarily defined as ≤6 ng/ml.

Evaluation. Before surgery, all patients underwent staging workups that included a colonoscopy, chest radiography, abdominopelvic computed tomography (CT), and measurement of serum CEA. In some patients, positron-emission tomography-CT scan, single contrast-enhanced magnetic resonance imaging of the liver, with/without chest CT scan were combined to further verify the recurrence. All tumors were histologically examined and staged in accordance with the staging of the American Joint Committee on Cancer (eighth edition) (20).

Follow-up. Patients underwent standardized postoperative follow-up including clinical examination, complete blood count, blood chemistry tests, measurement of serum CEA concentrations, and chest radiography every 3 months for the first 2 postoperative years, and every 6 months thereafter. Patients underwent abdominopelvic CT every 6 months, and colonoscopy was performed within 1 year of the operation and then once every 2-3 years. Patients with suspected recurrence underwent specific examinations with CT, magnetic resonance imaging, with/without positron-emission tomography-CT). diagnosis of recurrence was primarily determined histologically using samples from surgical resection or biopsy, and radiological changes otherwise.

Statistical analysis. Categorical variables were compared using chi-squared tests, and continuous variables were compared using independent sample *t*-tests or Mann-Whitney tests. Analysis of associations between recurrence and biomarker levels was determined using a log-transformed formula. Receiver operating characteristic (ROC) curves were plotted for each biomarker for predicting recurrence. The area under the curve was estimated along with its 95% confidence interval. All statistical tests were determined on a two-sided verification, and a where of $p<0.05$ indicated statistical significance. All analyses were carried out using SPSS for Windows version 21.0 (SPSS, Inc., IBM, Armonk, NY, USA) and R version 3.5.1.

Results

Identification of potential biomarkers from the training set. A total of 33 patients with CRC were divided into the recurrence group (N=14) and the no-recurrence group (N=19) (Figure 1) and their preoperative serum samples were analyzed to identify potential candidate biomarkers among ATG5, ATG10, SPRR3, ALDH1A1, IGFBP1, GSN, OAS2, TREM1, SLAMF7, CKS2, TFAP2E, LOX and MACC1. Among these, five biomarkers whose levels were found to significantly differ according to recurrence, namely ATG5, CKS2, IGFBP1, SLMF7 and OAS2, were selected and analyzed in the verification set.

Baseline characteristics of patients in the verification set. The verification set for the five biomarkers included 60 patients with recurrence at 5 years after curative surgery for stage II or III CRC and 60 patients without. The two groups did not differ significantly in baseline characteristics such as age, histological type, tumor stage, lymphovascular invasion and microsatellite instability status. On the other hand, the group with recurrence had a significantly higher serum CEA concentration, a higher prevalence of rectal cancer, and a

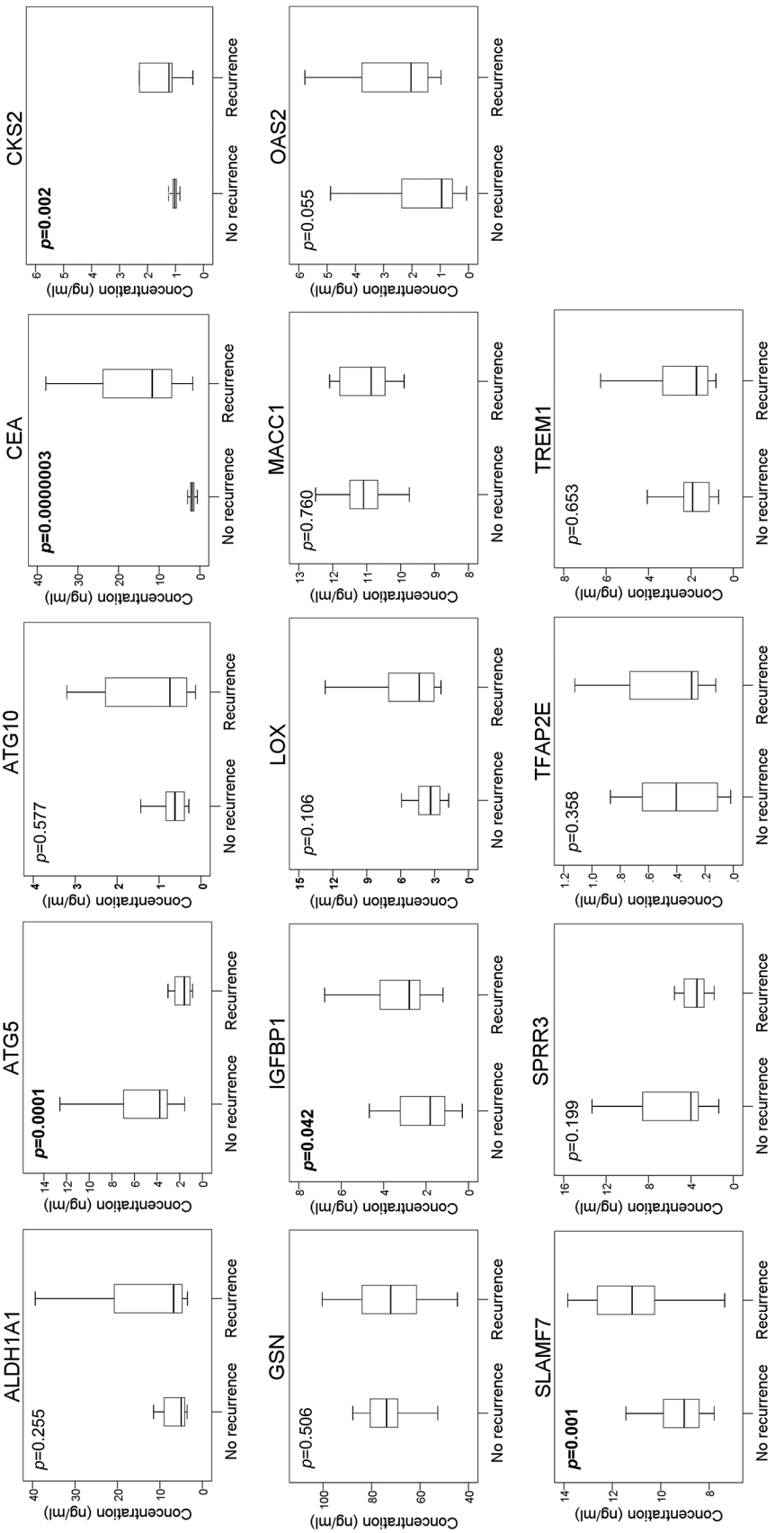

Figure 1. *Expression of candidate biomarkers in patients with (N=14) and without (N=19) recurrence who underwent curative resection for stage II or III colorectal cancer. Box plots of concentration obtained in enzyme-linked immunosorbent assay showing median expression (horizontal lines). Error bars represent the standard error. Data were obtained from three independent experiments performed in duplicate. Statistically significant p-values are shown in bold. ALDH1A1: Aldehyde dehydrogenase 1A1; ATG5: autophagy-related gene 5; CEA: carcinoembryonic antigen; CKS2: cyclin-dependent kinase regulatory subunit 2; GSN: gelsolin; IGFBP1: insulin-like growth factor-binding protein 1; LOX: lysyl oxidase; MACC1: metastasis associated in colon cancer-1; OAS2: 2'-5'-oligoadenylate synthetase 2; SLAMF7: signaling lymphocyte activation marker family member 7; SPRR3: small proline-rich repeated protein 3; TFAP2E: transcription factor activating enhancer-binding protein 2e; TREM1: triggering receptor expressed on myeloid cells-1.*

Table I. *Clinicopathologic characteristics*.

Characteristic	No recurrence (N=60)	Recurrence (N=60)	*p*-Value
Gender, n (%)			
Male	28 (46.7)	41 (68.3)	**0.02**
Female	32 (53.3)	19 (31.7)	
Age, years			
Mean±SD	62±12	61±11	0.59
Serum CEA, ng/ml			
Mean±SD	8.4±12.0	18.4±50.0	0.12
Serum CEA, n (%)			
<6 ng/ml	40 (66.7)	26 (43.3)	**0.01**
≥6 ng/ml	20 (33.3)	34 (56.7)	
Tumor location			
Colon	45 (75.0)	27 (45.0)	**0.001**
Rectum	15 (25.0)	32 (53.3)	
Colon and rectum	0	1 (1.7)	
pStage			
II	37 (61.7)	27 (45.0)	0.07
III	23 (38.3)	33 (55.0)	
Histology, n (%)			
WD/MD	51 (85.0)	55 (91.7)	0.26
PD/Muc/SRC	9 (15.0)	5 (8.3)	
LVI, n (%)			
Absent	43 (71.7)	37 (61.7)	0.25
Present	17 (28.3)	23 (38.3)	
PNI, n (%)			
Absent	50 (83.3)	36 (60.0)	**0.005**
Present	10 (16.7)	24 (40.0)	
MSI status, n (%)			
MSS	46 (76.7)	53 (88.3)	0.40
MSI-Low	3 (5.0)	2 (3.3)	
MSI-High	5 (8.3)	2 (3.3)	
Not assessed	6 (10.0)	3 (5.0)	

CEA: Serum carcinoembryonic antigen; LVI: lymphovascular invasion; MD: moderately differentiated; MSI: microsatellite instability; MSS: microsatellite stable; Muc: mucinous; PD: poorly differentiated; PNI: perineural invasion; SD: standard deviation; SRC: signet-ring cell; WD: well-differentiated. Statistically significant *p*-values are shown in bold.

higher proportion with perineural invasion compared with the non-recurrence group (Table I).

Univariate and multivariate analysis for candidate biomarkers. In univariate analysis, log-transformed values of serum CEA, CKS2, OAS2, ATG5, IGFBP1 and SLAMF7 concentrations were significantly higher in the recurrence group than in the non-recurrence group (Figure 2). On multivariate analysis, CEA, CKS2, OAS2, and ATG5 were shown to be significantly associated with recurrence (Table II), and were included in additional validation with ROC analysis. The results showed that the four molecules potently discriminated patients with recurrence from those without (area under the curve=0.828, 95% confidence interval=0.755-0.990) (Figure 3).

The formula for the ROC curve using the odds ratio was as follows: . Therefore, the final formula was as follows: The threshold was calculated by determining the maximum Youden index (sensitivity+specificity−1) from the ROC curve (21). We found that the maximum Youden index was 2.1787 (sensitivity=0.7833; specificity=0.7333), which indicates that the probability of recurrence increases with a Youden index of 2.1787 or higher. The positive predictive value was 75%, and negative predictive value was 67% using this formula.

Discussion

The current tumor staging system mainly consists of three parameters *i.e.* tumor, lymph nodes, and remote metastases, whereas pathologic al risk factors (*e.g.* lymphovascular and perineural invasion, tumor budding and poorly differentiated cluster, and extranodal extension), clinical risk factors (*e.g.* perforation, obstruction), and serum markers (*e.g.* CEA, cancer antigen 19-9) are graded with lesser importance in determining prognosis and recurrence (20). Among them, only CEA is widely used as a serum biomarker. However, as a traditional biomarker, it shows only modest sensitivity for the detection of CRC (6, 22). Particularly, there is controversy on whether the preoperative values of serum CEA are able to accurately predict recurrence (6). Although a number of promising blood-based biomarkers for the recurrence of CRC have been identified over the past several years, only few potent biomarkers are presently available in clinical settings. Thus, we aimed to identify potent serum biomarkers that can predict the recurrence of stage II and stage III CRC in patients who undergo curative resection, principally among our previously reported biomarkers. As a result, we found that several biomarkers, such as CEA, CKS2, OAS2 and ATG5, can be used to predict the recurrence of curatively resected CRC.

The overexpression of CKS2 is closely related to tumor aggressiveness and prognosis in various malignancies of the stomach, bladder, liver, and colorectum (23-26). Yu *et al.* reported that high CKS2 expression in CRC tissues was related to larger tumor size and higher tumor-node-metastasis (TNM) stage, and that CKS2 facilitated tumor metastasis by regulating the tight junction protein claudin 1. They also reported that CKS2 and TNM stage were independent prognostic factors for poor outcomes in CRC (17). Another study showed that overexpression of the three genes, namely block of proliferation 1 (*BOP1*), *CKS2*, and nuclear factor interleukin-3 (*NFIL3*), in non-metastatic cells was potent enough to induce experimental liver metastasis, while knockdown of the endogenous genes in SW620 cells reduced metastasis. CRC cells expressing BOP1, CKS2, or NFIL3 exhibited the biological characteristics of epithelial-mesenchymal transition *via* the phosphatidylinositol-4,5-

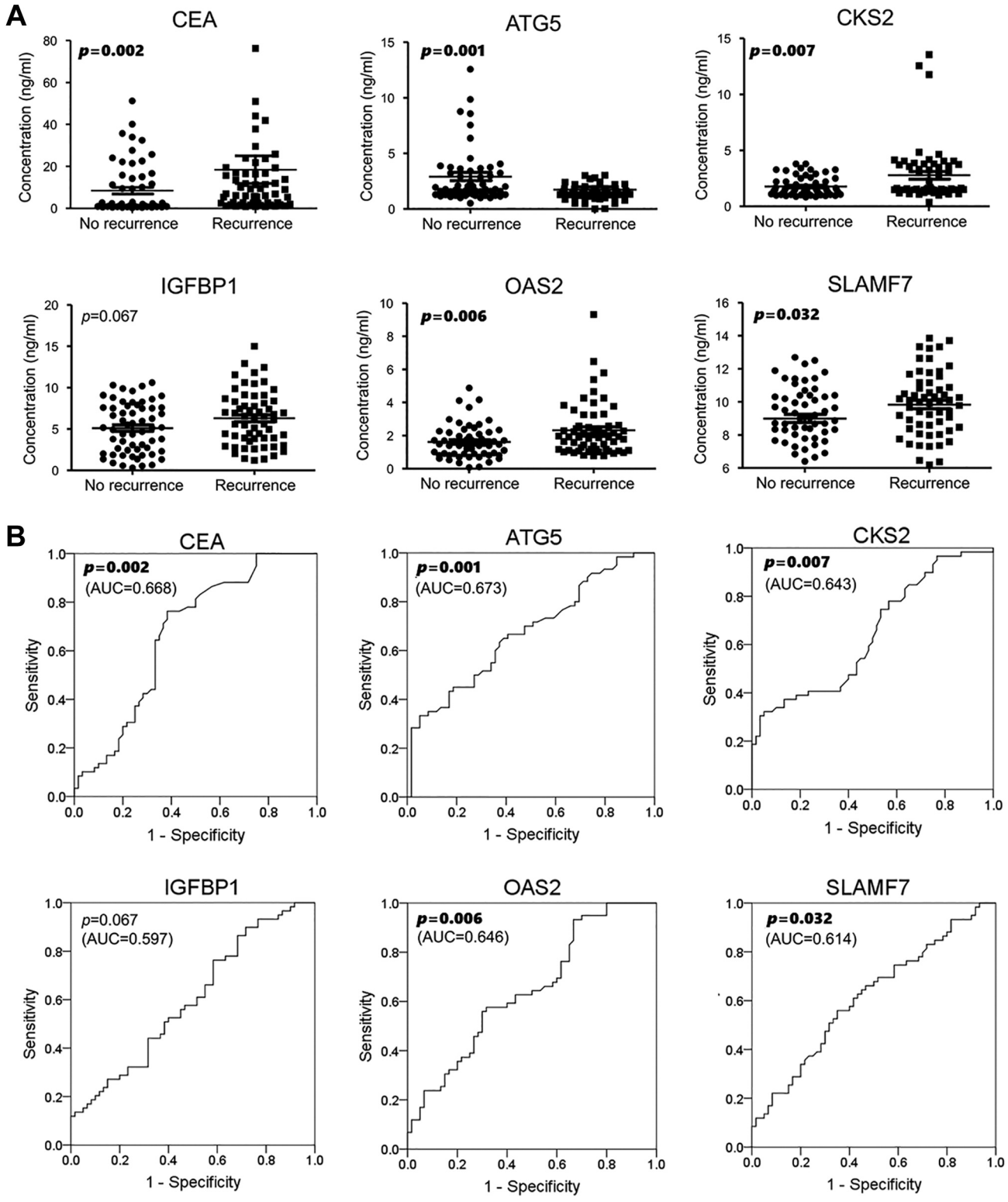

Figure 2. *Levels of protein expression (A) and receiver operating characteristic curves (B) of candidate biomarkers assessed in serum from patients with (N=60) and without (N=60) recurrence who underwent curative resection for stage II or III colorectal cancer. Median expression is shown by horizontal lines. ATG5: Autophagy-related gene 5; AUC: area under the receiver operating characteristics curve; CEA: carcinoembryonic antigen; CKS2: cyclin-dependent kinase regulatory subunit 2; IGFBP1: insulin-like growth factor-binding protein 1; OAS2: 2'-5'-oligoadenylate synthetase 2; SLAMF7: signaling lymphocyte activation marker family member 7.*

Table II. *Univariate and multivariate analysis of candidate biomarkers using natural log-transformed concentrations in serum.*

	Univariate analysis			Multivariate analysis		
Serum marker	OR	95% CI	*p*-Value	OR	95% CI	*p*-Value
CEA	1.555	1.188-2.075	**0.002**	1.496	1.112-2.057	**0.01**
CKS2	2.527	1.281-5.367	**0.011**	5.614	1.821-21.719	**0.006**
OAS2	2.666	1.458-5.314	**0.003**	9.541	3.098-38.295	**<0.001**
ATG5	0.316	0.140-0.614	**0.002**	0.317	0.121-0.695	**0.01**
IGFBP1	1.863	1.123-3.248	**0.02**			
SLAMF7	7.151	1.285-44.849	**0.03**			

ATG 5: Autophagy-related gene 5; CEA: carcinoembryonic antigen; CI: confidence interval; CKS2: cyclin-dependent kinase regulatory subunit 2; IGFBP1: insulin-like growth factor-binding protein 1; OAS2: 2'-5'-oligoadenylate synthetase 2; OR: odds ratio; SLAMF7: signaling lymphocyte activation marker family member 7. Area under the receiver operating characteristics curve=0.828 (95% CI=0.755-0.900). Hosmer-Lemeshow test *p*=0.455. Statistically significant *p*-values are shown in bold.

*bis*phosphate 3-kinase (PI3K) pathway, concurrently acting as direct WNT/β-catenin target genes (27).

Expression of OAS2 was reported in patients with viral infections, chronic infections, and autoimmune diseases (28). For malignant tumors, it was reported that overexpression of OAS2 was associated with oral cancer (29). The OAS family is associated with the regulation of apoptosis, one of the ways that organisms react in response to viral infection in an effort to eliminate virus-infected cells, and a key mechanism for inhibiting tumorigenesis (30). In our previous study, OAS2 in primary CRC tissues was negatively associated with recurrence, although OAS2 was closely related to lymphovascular invasion (13). Noting the changes in the plasma level of OAS in patients with infection or undergoing interferon treatment prompted us to look at the role of OAS in modulating the immune system (31). Whereas OAS2 expression in tissues appears to suppress tumor recurrence, an elevated level of serum OAS2 during recurrence indicates the dynamic immune regulation of tumor microenvironment over time. This finding of the dual reaction of OAS2 needs further investigation.

In general, autophagy is particularly important in cancer. The role of autophagy in tumorigenesis is complex and paradoxical. Autophagy plays a critical role in preventing cancer development; however, once cancer is established, increases in autophagic flux may enable tumor cell survival and growth (32). Therefore, in premalignant lesions, enhancers of autophagy might prevent cancer development. Conversely, in advanced cancer, both the enhancement of autophagy and its inhibition have been suggested as therapeutic strategies (33). ATG5 is known to play a role in gastrointestinal cancer tumorigenesis by altering autophagic and apoptotic cell death (34). In our previous study, *ATG5* was strongly down-regulated in CRC (9), and another study showed that negative expression of ATG5 was associated with poor prognosis for patients with CRC (35). Similarly, an elevated serum ATG5 level was also negatively correlated with recurrence in this study.

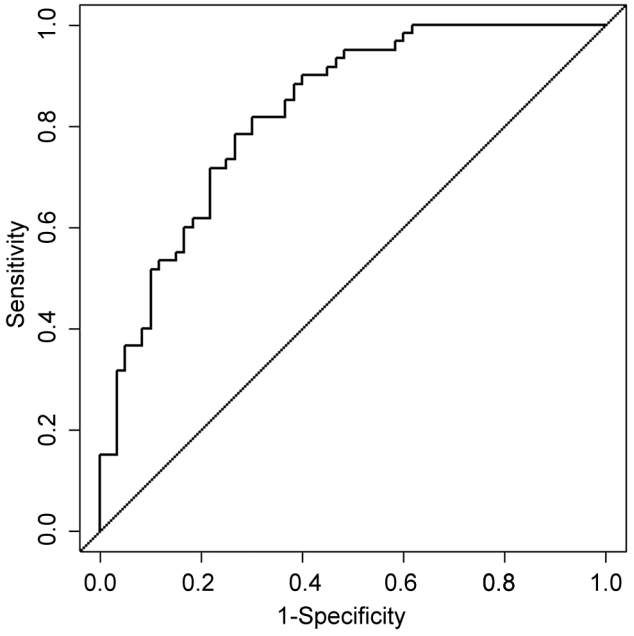

Figure 3. *Receiver operating curve analysis using the combination of preoperative carcinoembryonic antigen (CEA), cyclin-dependent kinase regulatory subunit 2 (CKS2), 2'-5'-oligoadenylate synthetase 2 (OAS2), and autophagy-related gene 5 (ATG5). Analysis of the receiver operating characteristics curve showed an area under the curve value of 0.828 (95% confidence intervaI=0.755-0.900) for discriminating patients with recurrence from those without.*

In our previous study, IGFBP1 and SLAMF7 have been reported as metastatic potentiator and suppressor of natural killer celld, respectively (12, 18). However, these molecules

were previously studied in patients with stage IV, not stage II or III disease. It is presumed that this may have influenced the results. Consequently, in this study, they were not found to be meaningful serum markers that could predict recurrence.

Our study has several limitations that affect the application of the conclusions to clinical settings. Firstly, the size of the validation cohort used in the study was relatively small; using more large-scale data would derive a more accurate formula for predicting recurrence. Secondly, the observed discrepancies in the results between cell-based assays and the current serum study (*e.g.* OAS2 and ATG5) require further biological validation. However, as most studies of biomarkers for cancer recurrence or therapeutic responsiveness ended in one-off reports without further validation studies, we intended to validate the molecules that we had previously identified as being possibly related to the recurrence of CRC in cell-based assays. For practical purposes, we examined the serum levels of these molecules and found that a combination of CEA, CKS2, OAS2 and ATG5 predicted tumor recurrence well in patients with stage II and III CRC who undergo curative resection. The approach adopted in our study seems to be useful for testing whether previously identified biomarker molecules have translational values.

In conclusion, a combination of serum CEA, CKS2, OAS2 and ATG5 predicted tumor recurrence well in patients with stage II and III CRC. Continuing studies, by using panels of these molecules in a sufficient number of patients, are ongoing and may further strengthen our findings.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization: Chan Wook Kim, Ye Jin Ha, Jin Cheon Kim; Methodology: Chan Wook Kim, Ye Jin Ha, Ka Hee Tak, Seon Ae Roh, Seon-Kyu Kim; Resources: Chan Wook Kim, Jin Cheon Kim; Data curation: Chan Wook Kim, Ye Jin Ha, Ka Hee Tak, Seon-Kyu Kim, Seon-Young Kim, Dong-Hyung Cho, Jin Cheon Kim; Investigation: Chan Wook Kim, Ye Jin Ha, Ka Hee Tak, Seon-Kyu Kim, Seon-Young Kim, Jin Cheon Kim; Formal analysis: Chan Wook Kim, Ye Jin Ha, Ka Hee Tak, Seon-Kyu Kim, Seon-Young Kim, Yong Sung Kim, Dong-Hyung Cho, Jin Cheon Kim; Funding acquisition: Chan Wook Kim, Jin Cheon Kim; Project administration: Chan Wook Kim, Jin Cheon Kim; Supervision: Chan Wook Kim, Seon-Young Kim, Yong Sung Kim, Dong-Hyung Cho, Jin Cheon Kim; Manuscript writing: Chan Wook Kim, Ye Jin Ha, Jin Cheon Kim.

All Authors read and approved the article.

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