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Original Article

The Importance of Fecal and Plasma CEA, COX-2, MMP-7, and TIMP-1 in the Diagnosis of Colorectal Cancer

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Abstract

Purpose: Colorectal cancer (CRC) is one of the most common and death related cancers in the world. Therefore, the early diagnosis of CRC remains with a great importance to prevent its further progression and increase survival rates. Colonoscopy and pathological examinations which are invasive and painful procedures, are needed to make a definitive diagnosis of CRC. The carcinoembryonic antigen (CEA) is particularly used for postoperative follow-up of CRC patients. The imbalance between matrix metalloproteinases (MMPs) and their tissue inhibitors of metalloproteinases (TIMPs) leads to degradation of extracellular matrix which is the most important step in invasion and metastasis. It was also observed that cyclooxygenase-2 (COX-2) has crucial roles in the development and progression of colorectal cancer. The purpose of our study is to detect fecal and plasma MMP-7, COX-2, TIMP-1, and CEA protein levels in patients with colorectal cancer, colorectal polyps, and healthy individuals, and assess their association with each other and with clinicopathological variables. We also aimed to evaluate plasma and fecal MMP-7, COX-2, TIMP-1, and CEA protein levels as potential diagnostic markers in colorectal carcinoma.

Methods: Plasma and fecal samples were taken from patients with fifteen colorectal cancers, twenty-six colorectal polyps, and thirty-three healthy volunteers. Protein extraction was carried out from fecal samples. Plasma and fecal MMP-7, TIMP-1, and COX-2 protein levels were determined by ELISA whereas plasma and fecal CEA protein levels were detected with CEIA.

Results: Plasma and fecal CEA levels were significantly higher in CRC than the control. In addition, plasma TIMP-1 and plasma CEA levels were significantly elevated in cancer according to polyp group. We also detected decreased plasma MMP-7 levels in polyps compared to control group. Positive correlations were observed among plasma COX-2 and TIMP-1 levels (r=0.571), fecal COX-2 and CEA levels (r=0.764) in CRC. However, no association was found between biochemical parameters and clinicopathological variables. ROC analysis for discriminating CRC from healthy controls showed that area under curves (AUC) for fecal and plasma CEA levels were 0.763 and 0.692, respectively. Plasma CEA (AUC=0.735), plasma TIMP-1 (AUC=0.706), and their combination (AUC=0.760) exhibited significant diagnostic performances to differentiate CRC from polyp. In discrimination colorectal polyps from healthy tissues, MMP-7 showed the highest AUC value (0.667).

Conclusion: Here we suggested that plasma and fecal CEA protein levels may be potential predictive noninvasive markers for diagnosis of colorectal adenocarcinoma. In addition, plasma CEA and TIMP-1 are also valuable biomarker candidates in differentiating colorectal cancer from colorectal polyps.

Keywords: Colorectal cancer, colorectal polyps, tissue inhibitors of metalloproteinases-1, matrix metalloproteinase-7, cyclooxygenase-2, carcinoembryonic antigen

INTRODUCTION

The prevalence of colorectal cancer (CRC) is higher than that of all other malignancies. It has been reported that colorectal cancer is the third most frequently seen cancer type in males, after prostate and lung cancer. In females, colorectal cancer is the third most frequently seen cancer type, after breast and lung cancer (1). Within cancer-related deaths, colorectal carcinoma is the second most frequent cause of death after lung cancer (2). The majority of the colorectal cancer cases (98%) are adenocarcinomas, which may develop from the adenomatous polyps of the colon.

Colonoscopy and biopsy methods are used to make the definitive diagnosis of colorectal cancer. Most studies use the carcinoembryonic antigen (CEA) as a tumor marker, as the level of CEA increases in parallel with the increasing stages of the disease. However, CEA has a low sensitivity, specificity, and positive value in the early stages of colorectal cancer (3), and therefore, it is not suitable for extended mass screening. Currently, there is no widely used biochemical marker for colorectal cancer. Therefore, there is a critical need for biochemical markers in the early diagnosis and prevention of recurrence and metastasis in postoperative patients with colorectal cancer.

The extracellular matrix (ECM) is a complex structure that surrounds and supports the cells. ECM and basal membrane destruction by matrix metalloproteinases (MMPs), the zinc dependent endopeptidases, are important steps for tumor invasion and metastasis. Therefore, MMPs play a crucial role in several physiological and pathological processes such as wound healing, tumor invasion, and metastasis. Excessive matrix metalloproteinase-7 (MMP-7) expression has been reported in various premalignant and malignant tumors of the gastrointestinal system, especially in cancers of the esophagus (4), gastric (5), colon (6), and pancreas (7). During the process of transformation from normal colonic mucosa to adenomatous mucosa, there is a rapid increase in MMP-7 expression. In addition, patients with familial adenomatous polyposis (FAP) also have an excessive expression of MMP-7 in the polyps, which was correlated with size and dysplasia (8). The activity of MMPs is regulated by some specific tissue inhibitors, tissue inhibitors of metalloproteinases (TIMPs), which bind to the active site of MMPs. TIMP-1 expression levels were higher in subjects with colorectal cancer than in healthy subjects. Furthermore, expression levels of TIMP-1 were high in the stromal and epithelial cells of both adenoma and adenocarcinoma. In this study, the intensity of staining increased from hyperplastic polyps to tubulovillous adenoma and adenocarcinoma (9).

Cyclooxygenase-2 (COX-2) is an inducible enzyme by several cytokines and growth factors, and has a pivotal role in tumorigenesis such as cell proliferation, invasion, and metastasis (10). It has been repeatedly observed that overexpression of COX-2 was found 50% in adenoma, and 85% in adenocarcinoma in colorectal cancer. In addition, this expression increases from the aberrant crypt phase to the metastatic carcinoma phase, and is related to poor prognosis in CRC (11).

The purpose of our study is to determine fecal and plasma TIMP-1, COX-2, MMP-7, and CEA protein levels in colorectal cancer, colorectal polyp, and healthy individuals. In addition, fecal and plasma TIMP-1, COX-2, MMP-7 and CEA protein levels were compared with each other, and with clinicopathological variables of colorectal carcinoma and colorectal polyps. Furthermore, we evaluated the diagnostic value of fecal and plasma TIMP-1, COX-2, MMP-7, and CEA protein levels in colorectal cancer.

METHODS

Patients and Tissue Samples

In this study, blood and fecal samples were taken during colonoscopy from patients with colorectal polyps and/or colorectal cancer. The protocol of this study was approved by the Dokuz Eylul University Non-invasive Clinical Research Ethics Committee and informed consent forms were signed by each participant. Patients were excluded from the study if they had coagulopathy, renal and/or liver failure, inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, or if they had used non-steroidal anti-inflammatory drugs in the past 3 days. Smoking was not included in the exclusion criteria due to the low number of cases. The final definitive diagnosis was established by histopathological examination, and a total of 26 patients with colon polyps, 15 patients with colorectal cancer, and 33 healthy individuals were included in the study. The clinicopathological variables of patients with colorectal cancer and colorectal polyps are summarized in Tables 1 and 2, respectively. The blood samples were immediately centrifuged at 3000 rpm for 10 min at room temperature, the plasma supernatant was collected, and the plasma and fecal samples were stored at -40°C until further analysis.

Protein Extraction from Fecal Samples

1.5 ml extraction buffer (0.1 M Tris pH 8.0, 0.1 M citric acid, 1 M urea, 0.01 M $CaCl_2$ with protease inhibitor cocktail) was added to 150 mg fecal sample, and homogenized in a Tissuelyser II

Table 1. Clinicopathological variables of patients with colorectal cancer

Parameter	Number	Percentage (%)
Gender		
Male	6	40
Female	9	60
Age (years)		
≤65	11	73.4
>65	4	26.6
Tumor Type		
Adenocarcinoma	14	93.4
Signet ring cell carcinoma	1	6.6
Tumor Location		
Colon	7	46.6
Rectum	8	53.4
Tumor Size		
<5 cm	6	60
≥5 cm	4	40
Distant Metastasis		
Presence	6	46
Absence	7	54
T Staging		
Early Stage (Tis-T1-T2)	3	30
Late Stage (T3-T4)	7	70
N Staging		
N0	5	50
N1	3	30
N2	2	20
Perineural Invasion		
Presence	1	10
Absence	9	90
Lymphatic Invasion		
Presence	7	70
Absence	3	30
Venous Invasion		
Presence	1	10
Absence	9	90

Table 2. Clinicopathological variables of patients with colorectal polyps

Parameter	Number	Percentage (%)
Gender		
Male	14	53.8
Female	12	46.2
Age (years)		
≤65	21	80.7
>65	2	19.3
Polyp Size		
≤6 mm	19	82.6
>6 mm	4	17.4
Polyp Type		
Hyperplastic	3	13.2
Tubular	16	69.5
Tubulovillous	4	17.3

homogenizer (25 Hz) (Qiagen Valencia, CA, USA) for 2 minutes at +4°C. Next, the samples were centrifuged at 1200 g for 10 minutes at +4°C. The supernatants were transferred to 5 µm filters and centrifuged at 5000 g for 10 minutes at +4°C. The total protein concentrations in the eluates were determined via a bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA), and the samples were stored at -80°C until further analysis.

ELISA

In plasma and fecal eluates, MMP-7, TIMP-1 (R&D Systems, Minneapolis, MN, USA), and COX-2 (Calbiochem, San Diego, CA, USA) protein levels were determined with commercial ELISA kits according to the manufacturers' instructions.

CEIA

The chemiluminescent enzyme immunometric assay (CEIA) was used to measure CEA protein levels in plasma and fecal samples with an Immulite 2000 analyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

STATISTICAL ANALYSIS

All data were evaluated with SPSS 15.0 Software (SPSS Inc., Chicago, IL, USA) and GraphPad Prisim 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The Mann Whitney U test was used for pairwise comparisons and subgroup analysis. Spearman's Correlation test was used to assess correlations. The independent groups were compared with Kruskal-Wallis variation analyses. The binary logistic regression and receiver operating characteristic (ROC) curve analyses were performed to evaluate the diagnostic performance of fecal/plasma TIMP-1, MMP-7, COX-2, and CEA protein levels. A value of $p<0.05$ was accepted as statistically significant.

RESULTS

1. Fecal and plasma MMP-7, TIMP-1, COX-2 and CEA protein levels in colorectal cancer, and colorectal polyps

When we evaluate our results, we did not detect COX-2 and MMP-7 protein levels in our fecal samples probably due to their low secretion levels to the colon. The plasma CEA protein levels were significantly elevated in the cancer group than in the polyp (p=0.012) and control (p=0.008) groups. But there was no significant difference in plasma CEA protein levels between polyp and control groups (p=0.604) (Figure 1a). Besides, fecal CEA protein levels of cancer group were significantly higher than those of the control group (p=0.033) (Figure 1b). In addition, a significant increase of plasma TIMP-1 protein levels was detected

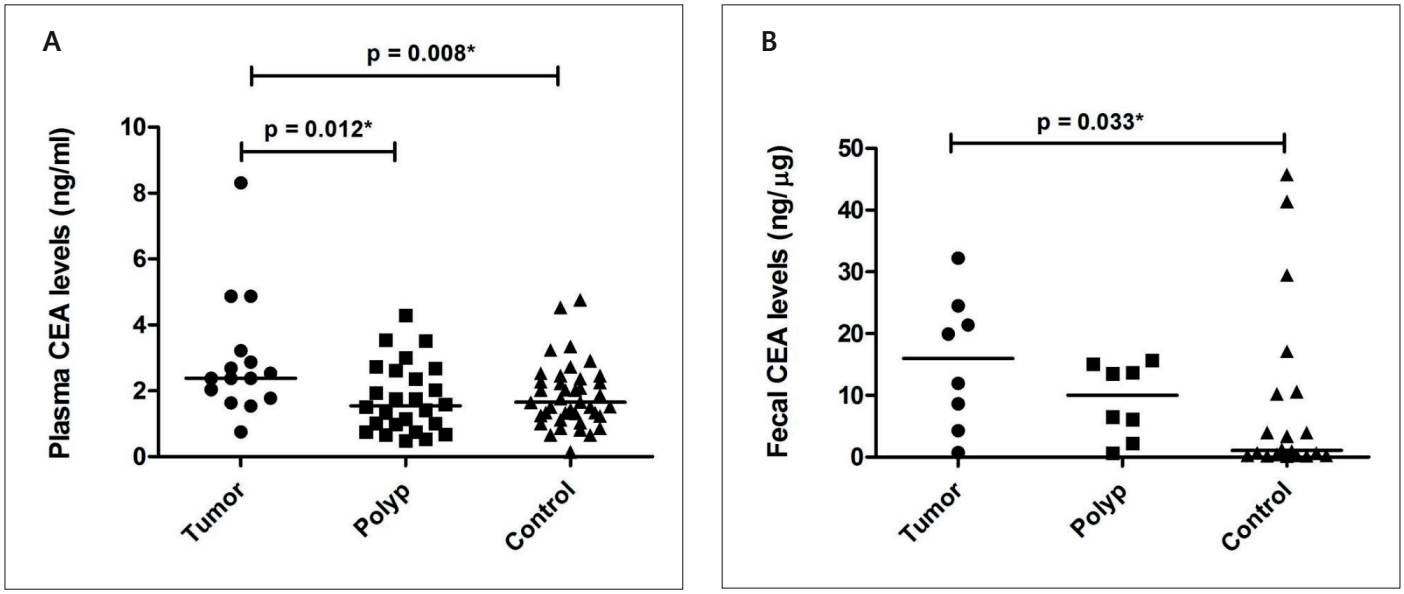

Figure 1. Plasma **(A)** and fecal **(B)** CEA protein levels in cancer, polyp, and control groups. Horizontal lines represent the median values, *p≤0.05 is statistically significant.

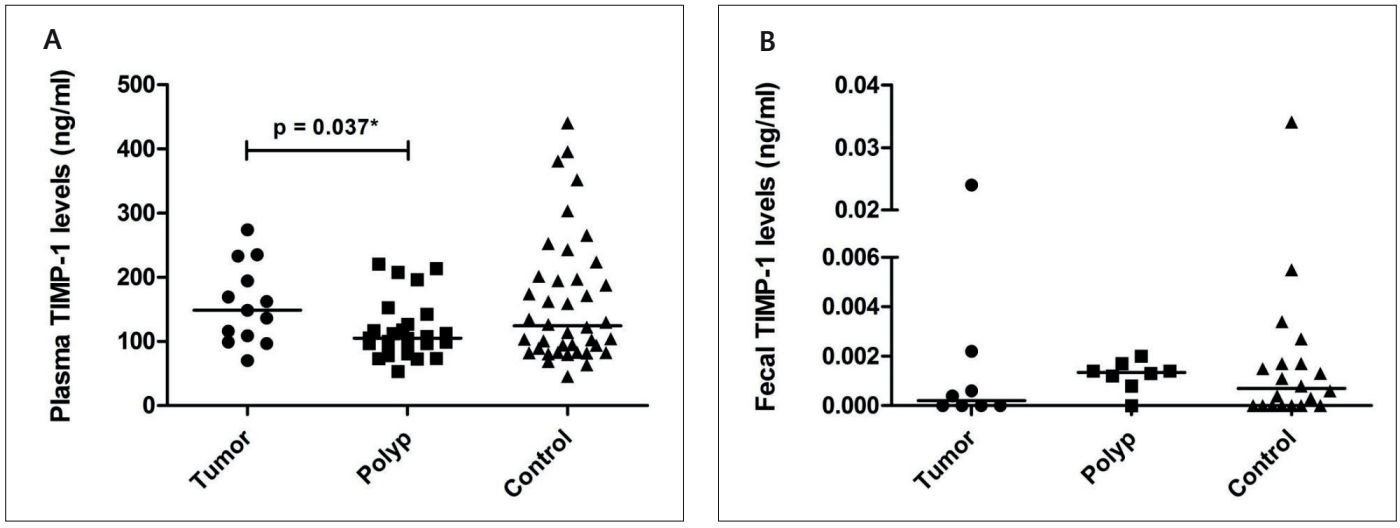

Figure 2. Plasma TIMP-1 **(B)** and fecal TIMP-1 **(C)** protein levels in cancer, polyp, and control groups. Horizontal lines represent the median values, *p≤0.05 is statistically significant.

in cancer group compared to polyp group (p=0.037) (Figure 2a), whereas fecal TIMP-1 protein levels were not statistically different between the groups (Figure 2b). Plasma MMP-7 protein levels were decreased in polyp group according to control (p=0.019) (Figure 3a). However, no significant differences were found in the plasma COX-2 protein levels between cancer, polyp, and control groups (Figure 3b).

The relationship between the clinicopathological parameters and the plasma and fecal levels of TIMP-1, MMP-7, COX-2, and CEA in the polyp and cancer groups was analyzed with Spearman's Correlation test. No significant correlations were observed between biochemical parameters and clinicopathological parameters of both polyp and cancer groups (p>0.05). When we assessed the association of biochemical parameters with each other, we found positive correlation between plasma COX-2 and TIMP-1 levels as well as between fecal COX-2 and CEA levels in colorectal cancer (Table 3).

Table 3. The correlation between biochemical parameters in colorectal cancer

Biochemical Parameters	Correlation coefficient (r)	p value
Plasma COX-2 vs. Plasma TIMP-1	0.571	0.041*
Fecal COX-2 vs. Fecal CEA	0.764	0.027*

*p≤0.05 is statistically significant.

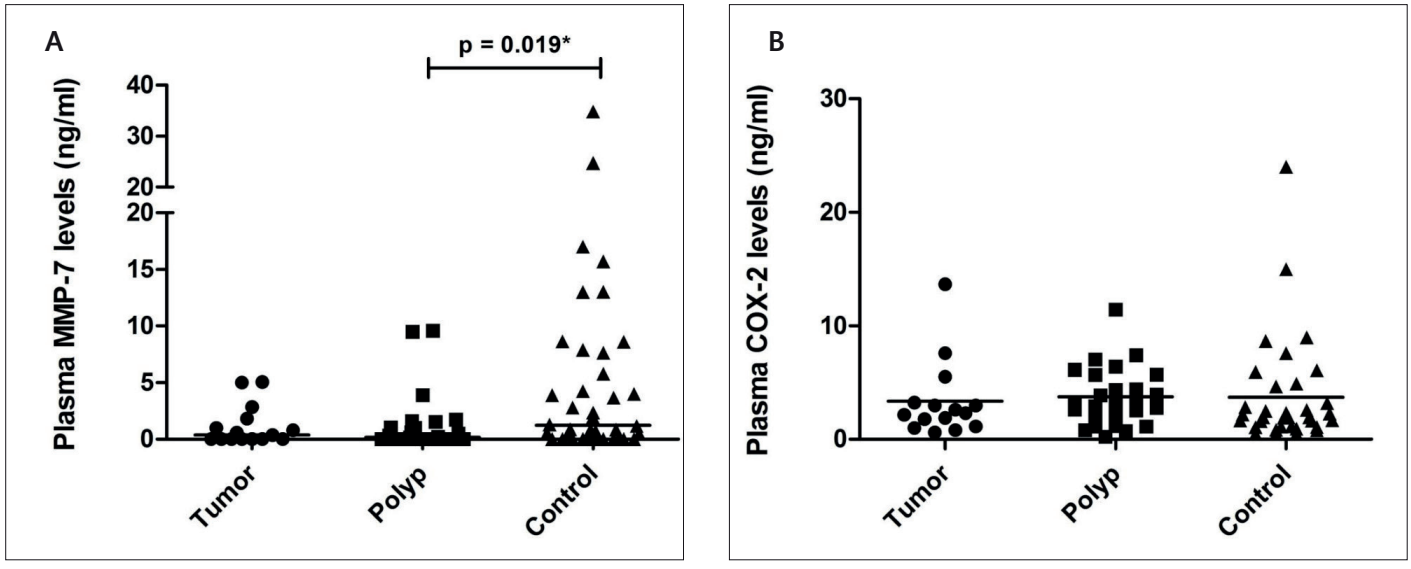

Figure 3. Plasma MMP-7 **(A)** and plasma COX-2 **(B)** protein levels of cancer, polyp, and control groups. Horizontal lines represent the median values, *p≤0.05 is statistically significant.

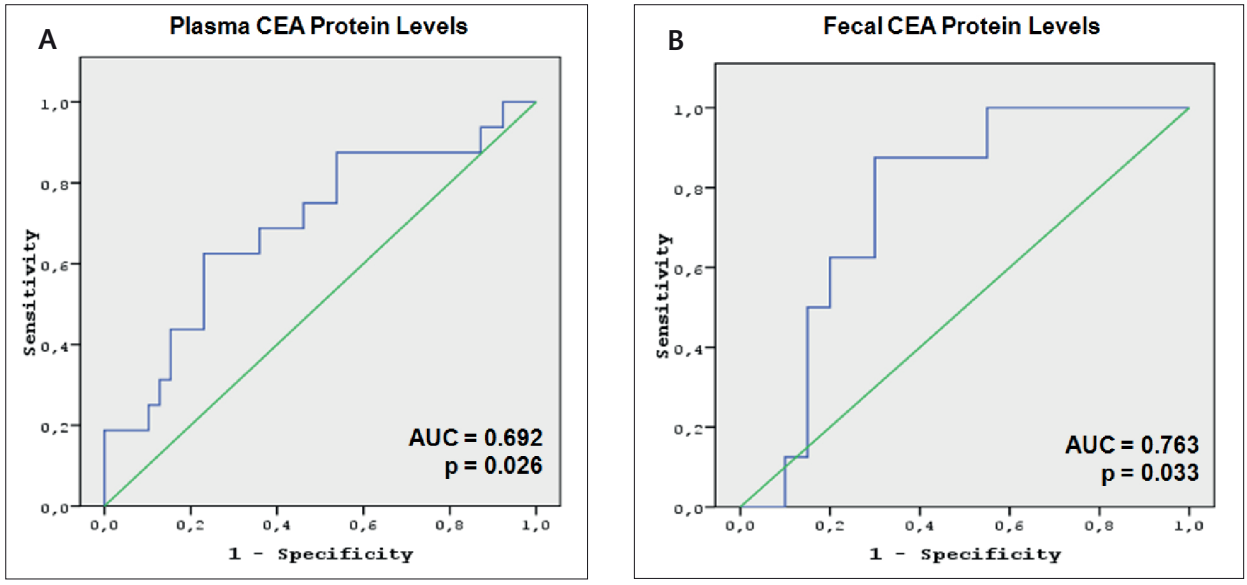

Figure 4. ROC curve and corresponding AUC statistics for plasma CEA (A) and fecal CEA (B) protein levels in differentiating cancer from control groups, *p≤0.05 is statistically significant.

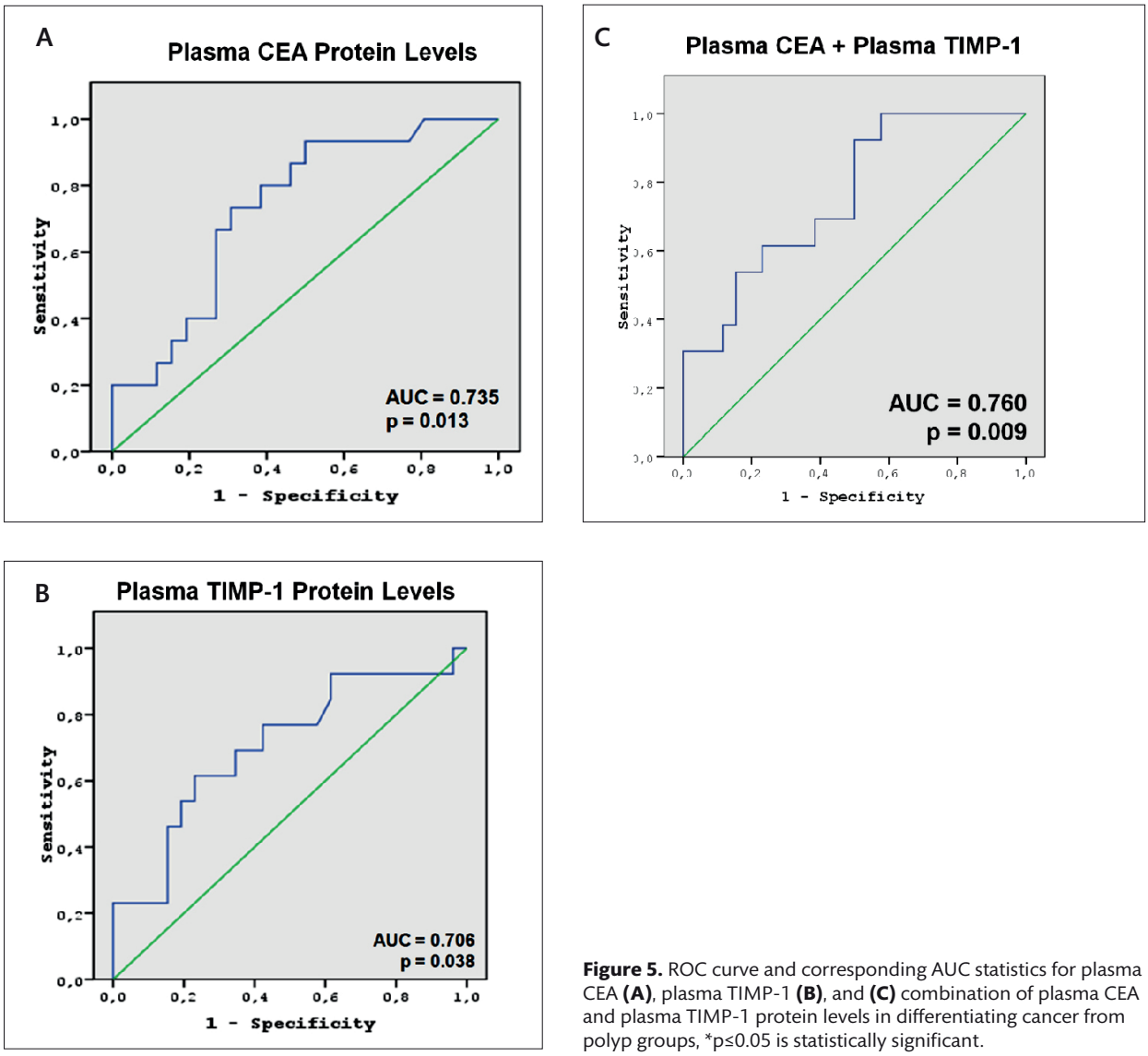

Figure 5. ROC curve and corresponding AUC statistics for plasma CEA **(A)**, plasma TIMP-1 **(B)**, and **(C)** combination of plasma CEA and plasma TIMP-1 protein levels in differentiating cancer from polyp groups, *p≤0.05 is statistically significant.

2. Diagnostic performance of fecal and plasma MMP-7, TIMP-1, COX-2 and CEA protein levels in differentiating colorectal cancer and colorectal polyps

When the diagnostic significance evaluated, plasma CEA protein levels with cut-off value of 2.375 ng/ml had a sensitivity of 62.5% and specificity of 76.9% (AUC=0.692) (Figure 4a), and fecal CEA protein levels with cut-off value of 4.11 ng/µg (AUC=0.763) had a sensitivity of 87.5% and specificity of 70% to differentiate colorectal cancer from healthy controls (Figure 4b). At a cut-off value of 2.03 ng/ml, plasma CEA protein levels had a sensitivity of 73.3% and specificity of 69.2% (AUC=0.735) and at a cut-off value of 108.14 ng/ml, plasma TIMP-1 protein levels had a sensitivity of 76.9% and specificity of 57.7% (AUC=0.706) in discriminating colorectal cancer from polyps. The combination of plasma CEA and plasma TIMP-1 had an AUC=0.760, which was more effective compared to plasma CEA or plasma TIMP-1 alone. The sensitivity and specificity of the combination were 92.3% and 50.0%, respectively (Figure 5). With an optimal cut-off value of 0.67 ng/ml of plasma MMP-7 protein levels to distinguish polyp from healthy control, sensitivity and specificity were 69.2% and 62.5%, respectively (AUC=0.667) (Figure 6). However, we did not find any diagnostic value of plasma COX-2 and fecal TIMP-1 protein levels for colorectal polyps and colorectal cancer.

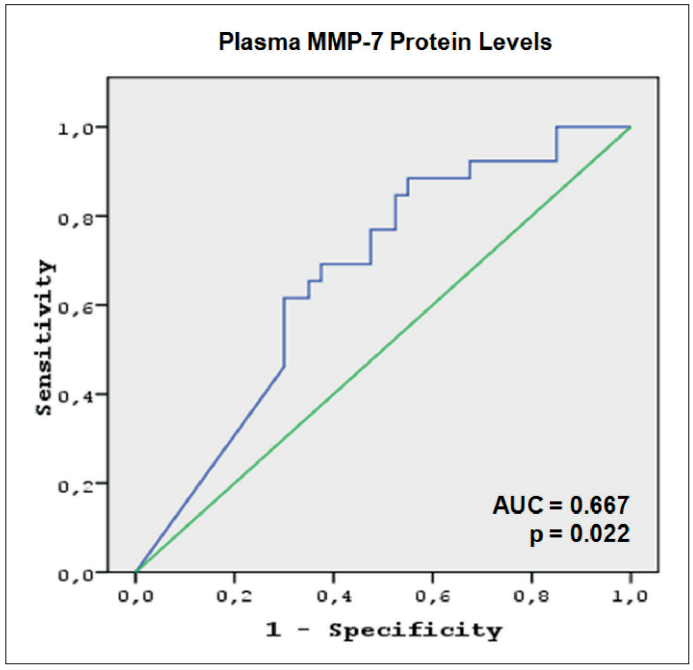

Figure 6. ROC curve and corresponding AUC statistics for plasma MMP-7 protein levels in differentiating polyp from control groups, *p≤0.05 is statistically significant.

DISCUSSION

The development of colorectal cancer is a long, complex, and multiple-staged process that involves genetic and phenotypic diversity. Proteolytic degradation of the extracellular matrix by MMPs plays a significant role in development and progression of gastrointestinal malignancies. Prior studies indicated that MMP-7 expression and activity levels were elevated in the onset and early stages of tumors. It is also suggested that MMP-7 involves in the growth, invasion, and metastasis of CRC (12, 13). Maurel et al. found that serum MMP-7 protein levels were higher in patients with CRC metastasis compared to patients without metastasis and the control group. In addition, serum MMP-7 protein levels were not significantly different between patients without CRC metastasis and control group (14). Consistently, we found no differences in plasma MMP-7 protein levels between cancer and control as well as cancer and polyp groups. In contrast to the findings of Maurel et al., plasma MMP-7 levels were lower in our CRC and polyp groups compared to the controls.

It is known that TIMP-1 inhibits MMPs to regulate proteolytic activity, but it also has a stimulating effect on tumor growth and malignant transformation (15). The studies showed that an increase in plasma TIMP-1 level was a significant diagnostic factor for determining survival (16–18). According to these studies, late stage patients with poor differentiation had higher TIMP-1 levels (16). Holten-Anderson et al. found no significant difference between polyp and control plasma TIMP-1 protein levels, while TIMP-1 levels in cancer patients were significantly higher than those of the polyp and control groups (17). In our study, we also recorded a significant increase in plasma TIMP-1 protein levels in cancer group according to polyp group.

A large number of observations emphasized that COX-2 expression levels increased in colorectal carcinomas when compared to normal tissue samples. It was reported that COX-2 expression was 90% in adenocarcinomas, and 60% in adenomas (19). Wasilewicz et al. showed that the expression of COX-2 in colon polyps is associated with polyp length (20). In addition, Han et al. found higher COX-2 expression in patients with colorectal cancer who also had polyps, but consistent with our results, there was no correlation between the clinicopathological variables and COX-2 levels (21). Here we measured, for the first time, plasma COX-2 protein levels in control, polyp, and cancer groups; however, no significant differences were found.

CEA is used to determine the independent prognostic factors in patients without metastasis who have undergone surgery (22), and it is also frequently used for follow-up of recurrences after surgery (23). Since currently available follow-up marker for CRC is CEA, we also evaluated CEA in our study. We found that plasma CEA protein levels were significantly higher in the cancer group than the polyp and control groups. Fecal CEA protein levels were also significantly elevated in the cancer group when compared to control group which was consistent with the previous reports (24–26). As far as we know that this is the first study which compares the fecal CEA levels of polyp-cancer and polyp-normal groups. However, there were no significant differences between the fecal CEA levels of healthy individuals and patients with colorectal polyp.

To obtain a definitive diagnosis, CRC patients often undergo colonoscopy, which is an invasive and expensive method that may lead to disturbing complications. The fecal occult blood test (FOBT) is a simple and non-invasive test that has been shown to

decrease mortality rates associated with CRC (27). However, it was reported that a positive FOBT result was observed in less than 10% of the patients with CRC (28). Thus, new non-invasive tests with good diagnostic performance are needed. There are only limited number of studies aimed at developing new fecal and plasma markers for diagnosis of CRC (29–31). Takai et al. added fecal MMP-7 mRNA levels to COX-2, and termed this combination the "fecal RNA test". The sensitivity of their fecal RNA test for CRC was 90% (with 95% confidence interval) (29). According to our results, in the differentiation among patients with cancer and healthy individuals, the AUCs for fecal and plasma CEA were 0.763 and 0.692, respectively. Plasma CEA yielded an AUC of 0.735, and plasma TIMP-1 yielded an AUC of 0.706 when differentiating CRC from colorectal polyps. More importantly, binary logistic regression and combined ROC analyses revealed that combination of plasma CEA and TIMP-1 had an elevated AUC of 0.760 with 92.3% sensitivity and 50.0% specificity. Plasma MMP-7 levels also had a significant AUC of 0.667 which is important to separate patients with polyps from healthy controls. Mroczko et al. suggested that the serum TIMP-1 and CEA levels are useful biomarkers in the diagnosis of colorectal carcinoma (32). Karl et al. also quantified fecal TIMP-1 and CEA protein levels in colorectal cancer and evaluated the individual and combined sensitivity of 6 markers, including TIMP-1 and CEA (30). They showed that the sensitivity of fecal TIMP-1 was 72%; however, its combination with S100A12 and hemoglobin-haptoglobin had 95% specificity and 88% sensitivity.

In conclusion, these results clearly indicated that fecal and plasma CEA levels are valid candidates as biochemical markers for the diagnosis of colorectal carcinogenesis. Furthermore, combination of plasma TIMP-1 and plasma CEA might be promising markers to distinguish colorectal cancer from colorectal polyps. In order to increase the diagnostic value of plasma and fecal markers, large-scale clinical studies are needed.

Ethics Committee Approval: The protocol of this study was approved by Dokuz Eylul University Non-invasive Clinical Researchs Etics Committee

Informed Consent: Informed consent forms were signed by each participant.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - GO, RK; Design - GO, RK; Supervision - GO, OT; Resource - GO; Materials - GO, OT, MY; Data Collection and/ or Processing - DK, RK, GB, MY; Analysis and/or Interpretation - DK, RK, GB, MY; Literature Search - RK, DK; Writing - RK, DK, GB, GO, OT, MY; Critical Reviews - GO, OT

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Conflict of Interest: The authors declare that there are no conflicts of interest.

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