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# Preoperative serum placenta growth factor level as a new marker for stage II or III colorectal cancer patients

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***Background/aims:*** *We first reported in this study that serum placenta growth factor and carcinoembryonic antigen in combination were useful markers for selecting early-stage colorectal cancer patients. The aim of the present study was to determine whether serum placenta growth factor could provide carcinoembryonic antigen-independent prognostic information on patients undergoing curative surgery.* ***Methods:*** *Serum and tissue samples were collected from 158 patients with colorectal cancer and from 50 controls. Serum and tissue levels of placenta growth factor were measured by enzyme-linked immunosorbent assay. The serum placenta growth factor levels in colorectal cancer patients were compared with those in healthy controls, and we retrospectively assessed the association between serum placenta growth factor levels and clinicopathological findings and survival.* ***Results:*** *Expression of placenta growth factor was significantly higher in colorectal cancer tissues compared with non-tumor tissues. The mean serum placenta growth factor level in patients was significantly higher than that in controls and significantly higher in patients with large tumor, lymph-node involvement and distant metastasis.* ***Conclusions:*** *Elevated serum placenta growth factor levels are significantly associated with colorectal cancer development, lymph or distant invasive phenotypes and survival, especially in stage II or III patients.*

**Key words**: Colorectal cancer, placenta growth factor, carcinoembryonic antigen, stage II, stage III

# Evre II ve III kolorektal kanserli hastalar için yeni bir prognostik belirteç olarak preoperative serum plasenta büyüme faktör seviyesi

***Amaç:*** *Serum plasenta büyüme faktörü ve karsino embriyonik antijen düzeylerinin kombinasyonunun erken evre kolorektal kanserli hastaların saptanmasında yararlı belirteçler olduğunu gösteren bir çalışmayı sunuyoruz. Çalışmamızın amacı serum plasenta büyüme faktörü düzeyinin küratif amaçlı cerrahiye gidecek hastalarda karsino embriyonik antijen'den bağımsız prognostik bir bilgi verip vermediğini saptamaktır.* ***Yöntem:*** *Kolorektal kanserli 158 hastanın ve 50 kontrolün serum ve doku örnekleri toplandı. Serum ve doku plasenta büyüme faktörü düzeyleri enzim bağlı immunosorbent ölçüm yöntemi ile ölçüldü. Plasenta büyüme faktörü düzeyleri retrospektif olarak sağlıklı bireylerle, klinikopatolojik bulgular ve yaşam süreleri ile karşılaştırıldı.* ***Bulgular:*** *Plasenta büyüme faktörü expresyonu tümör olmayan dokulara göre kolerektal kanserli dokularda belirgin olarak yüksekti. Ortalama serum plasenta büyüme faktörü düzeyleri; kolerekral kanserli hastalarda kontrollere göre belirgin olarak yüksekti. Ayrıca büyük tümörlü, lenf nodu tutulumlu ve uzak metastazlı olgularda da plasenta büyüme faktörü düzeyleri yüksek bulundu.* ***Sonuç:*** *Artmış serum plasenta büyüme faktörü ve doku düzeyleri, kolorektal kanserin gelişiminde lenf, uzak metastaz ve yaşam süreleri ile ilişkili bir belirteçdir. Özellikle evre II ya da evre III hastalarda da önemli olduğunu düşünmekteyiz.*

**Anahtar kelimeler:** Kolorektal kanser, plasenta büyüme faktör, CEA, Evre II, Evre III

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## INTRODUCTION

Colorectal cancer is the third most common cancer and the fourth most frequent cause of cancer deaths worldwide (1). After potential curative surgery, ~30% of the patients will eventually develop metastases, often in spite of adjuvant therapies, such as chemotherapy and radio-chemotherapy (2).

The main factors that determine the prognosis in colorectal cancer include lymph node involvement, size of tumor and local diffusion of disease (3). However, these factors do not fully predict individual clinical outcomes especially among patients with stage II and stage III disease (1). Although adjuvant chemotherapy provides significant survival benefit in stage III patients, it is controversial whether this treatment has any effect on patients with stage II colon cancer, 20-30% of whom eventually experience tumor relapses (4). Adjuvant chemotherapy was shown to increase the survival of certain populations of stage II patients (5); moreover, almost 60% of stage III patients were not relapsing, even if adjuvant chemotherapy was not received (6). Therefore, identification of high-risk patients among stage II and III colorectal cancer patients would be of great benefit in selecting appropriate candidates for standard or intense adjuvant therapy.

Carcinoembryonic antigen (CEA) is a complex glycoprotein that is up-regulated in ~90% of advanced colorectal cancers and contributes to the malignant characteristics of tumors (7). However, it is not useful in detecting asymptomatic cancer, because the sensitivity of CEA determination for early colorectal cancer is as low as 30-40% (8). Moreover, CEA is not significantly associated with survival among patients with stage I and II lesions, and CEA testing is relatively insensitive to tumors with local or peritoneal involvement (9).

Placenta growth factor (PlGF), a dimeric glycoprotein with 53% homology to vascular endothelial growth factor (VEGF) (10,11), which binds to VEGF receptor-1 (Flt-1), but not to VEGF receptor-2 (Flk-1), may function by modulating VEGF activity (12).

Recently, several reports showed that in gastric cancer, breast cancer, renal cell cancer, and non-small cell lung cancer in humans, PlGF was overexpressed and displayed prognostic value (13-16).

In one study, they showed that the PlGF mRNA expression was regulated in colorectal cancer tissue (17). The extent of up-regulation correlated with disease progression and patient survival. Therefore, the results suggest that expression levels of PlGF in colorectal cancer may be used as a prognostic marker for patients with colorectal cancer (18).

In this study, we further assessed a possible role for pre-operative serum PlGF as a potent predictor of prognosis in colorectal cancer patients undergoing surgery with curative intent. The aim of the present study was to determine whether serum PlGF could provide CEA-independent prognostic information in patients undergoing surgery with curative intent and whether a new biomarker would be of more benefit to specific subgroups of patients, namely stage II and stage III patients, than the existing systems and serum tumor markers such as CEA.

## MATERIALS AND METHODS

### Patients

One hundred fifty-eight patients who underwent resection of colorectal carcinoma at our institution between September 2000 and September 2009 were enrolled in this retrospective study. The patients included 99 men and 59 women with a mean age of 60 (range: 30-78) years. The locations of the tumors and distant metastases were determined by barium enemas, colonoscopies, computerized tomography (CT), and magnetic resonance imaging (MRI). The primary lesion was located in the rectum in 55 patients, sigmoid colon in 61, ascending colon in 22, transverse colon in 11, and descending colon in 9. Thirty-two patients were diagnosed as having synchronous liver metastasis and eight patients were diagnosed with both liver metastasis and peritoneal dissemination. Tumor resection was performed in all patients and simultaneous partial hepatectomy for liver metastases was performed in 20 patients. No pre-operative mortalities were observed among these patients. Eighteen patients had a poorly differentiated adenocarcinoma, whereas in 140 patients, the adenocarcinoma was well or moderately differentiated. All patients were classified according to the Union for International Cancer Control (UICC) stage classifications using resected specimens. There were 24 patients with stage I disease (15.1%), 59 with stage II disease (37.3%), and 57 with stage III disease (36%). Eighteen patients with distant metastases were classified as having stage IV disease (11.3%). Stage III and IV patients received fluorouracil-based chemotherapy, whereas in sta-

ge I and II patients, no adjuvant therapy was applied postoperatively. Patients were observed at three-month intervals for 24 months after the completion of surgery, then every six months for three years, and then yearly. A history was taken and physical examination was performed at each visit, and chest X-ray, colonoscopy and CT were performed once per year. The median follow-up time was 65 months (mean: 50.2 ± 19.7). Of 158 patients studied, 70 patients died due to primary or recurrent disease. The clinicopathological parameters studied for prognostic value were tumor size, T classification, vessel involvement, lymphatic invasion, lymph node metastases, distant metastases, and serum concentration of CEA.

### Serum and Tissue Protein Assays

Peripheral venous blood samples were obtained from all 158 patients before surgery. Serum samples obtained from 50 normal healthy age-matched volunteers were used as controls. The absence of disease was assessed by clinical history, physical examination and routine laboratory tests, including liver and renal function tests. Serum samples were allowed to clot and serum was stored at -80°C until use. The levels of PlGF in cancer tissue and adjacent normal tissue were analyzed in 89 of the 158 enrolled patients. These specimens were homogenized and tissue extracts were obtained. Before collection of serum and tissue extracts from patients and healthy controls, their informed consent was obtained for the use of the samples in future experiments. The concentrations of PlGF were quantified with use of a Quantikine® human PlGF immunoassay (R&D Systems, Inc., Minneapolis, MN). Sera were incubated overnight at 4°C on microtiter plates coated with a murine monoclonal antibody against human PlGF. Unbound proteins were washed off, and an enzyme-linked polyclonal antibody specific for PlGF was added to 'sandwich' the PlGF immobilized during the first incubation. A substrate solution for horseradish peroxidase was added, and color was developed in proportion to the amount of antibody-bound PlGF. The absorbance of the color was read at 450 nm. Concentrations of PlGF were expressed as picograms per milligram. Protein concentration was measured by the BCA protein assay (Pierce, Rockford, IL). The lower limit of detection for serum PlGF concentration was 0.01 pg/ml. The tissue concentrations were expressed as pg/ml/protein.

CEA concentrations were determined by enzyme immunoassay (ELISA).

### Statistical Analysis

Data are presented as means ± standard deviation (SD). Comparisons were performed using the nonparametric Mann-Whitney *U* test for continuous variables and the chi-square test for categorical data. Correlations were analyzed by Spearman's coefficient analysis. Analyses of receiver operating characteristics (ROC) were performed to calculate the cut-off values. The survival probabilities were calculated using the product limit method of Kaplan-Meier methods, considering treatment-related deaths and deaths caused by colorectal cancer. Differences between two groups were determined using the log-rank test. The influence of each significant predictor identified by the log rank test was assessed by multivariate analysis using Cox's proportional hazards model. Two-sided *p*-values of $<0.05$ were considered statistically significant.

## RESULTS

### Association between Serum PlGF Levels and the Clinicopathological Characteristics of Colorectal Cancer

Serum PlGF levels were analyzed in 158 colorectal cancer patients and 50 normal controls. There were no age or gender differences between colorectal cancer patient and control groups. The serum concentration of PlGF in patients ranged from 20.2-105.5 pg/ml. The mean serum PlGF concentration in patients was significantly higher than that in normal volunteers ($p<0.0001$).

Table 1 shows the relationship between serum PlGF levels and clinicopathological variables in all patients. Table 2 shows the relationship between serum CEA levels and clinicopathological variables in all patients. Serum PlGF was associated with factors reflecting disease progression, such as tumor size >41 mm ($p=0.0001$), lymph node involvement ($p=0.001$) and the presence of distant metastases ($p<0.0001$). In addition, serum PlGF levels increased significantly in accordance with the progression of UICC stage classification ($p<0.0001$). To examine the predictive value of serum PlGF for different clinicopathological characteristics, we conducted chi-square and Mann-Whitney *U* tests. We defined elevated serum PlGF levels according to the best predictive values calculated on ROC analyses for tumor size >41 mm (25 pg/ml), lymph node metastasis (47.4 pg/ml), and distant metastasis (47.9 pg/ml), and used the criteria of 47.9 pg/ml for analyses of other parameters. Elevated serum

**Table 1.** Relationships between PlGF level and clinicopathological factors in 158 patients with colorectal cancer

| Variable | n | PlGF (pg/ml) | p |
|---|---|---|---|
| Gender | | | |
| Male | 95 | 75.9 ± 29.2 | |
| Female | 63 | 78.3 ± 20.6 | 0.49† |
| Age (y) | | | |
| <65 | 80 | 91.4 ± 30.1 | |
| ≥65 | 78 | 89.5 ± 25.7 | 0.21† |
| Tumor size (mm) | | | |
| <41 | 79 | 33.8 ± 10.2 | |
| ≥41 | 79 | 95.7 ± 23.1 | 0.0001† |
| Lymph node metastasis | | | |
| N0 | 88 | 67.0 ± 24.5 | |
| N1-3 | 70 | 98.3 ± 21.6 | 0.0001† |
| Distant metastasis | | | |
| M0 | 122 | 56.4 ± 21.8 | |
| M1 | 36 | 89.7 ± 19.3 | 0.0001† |
| UICC classification | | | |
| I | 21 | 31.0 ± 12.7 | |
| II | 61 | 57.8 ± 20.5 | |
| III | 48 | 83.1 ± 23.9 | |
| IV | 28 | 99.5 ± 15.2 | 0.0001* |

†Mann-Whitney *U* test. *Kruskal-Wallis analysis.

**Table 2.** Relationships between CEA level and clinicopathological factors in 158 patients with colorectal cancer

| Variable | n | CEA (ng/ml) | p |
|---|---|---|---|
| Gender | | | |
| Male | 95 | 3.7 ± 2.1 | |
| Female | 63 | 2.9 ± 1.0 | >0.05 |
| Age (y) | | | |
| <65 | 80 | 2.9 ± 1.1 | |
| ≥65 | 78 | 2.8 ± 1.0 | >0.05 |
| Tumor size (mm) | | | |
| <41 | 79 | 3.0 ± 1.0 | |
| ≥41 | 79 | 2.9 ± 1.0 | >0.05 |
| Lymph node metastasis | | | |
| N0 | 88 | 2.4 ± 1.3 | |
| N1-3 | 70 | 3.0 ± 1.5 | <0.05 |
| Distant metastasis | | | |
| M0 | 122 | 2.3 ± 1.2 | |
| M1 | 36 | 3.1 ± 1.4 | 0.001 |
| UICC classification | | | |
| I | 21 | 2.1 ± 1.4 | |
| II | 61 | 2.3 ± 1.6 | |
| III | 48 | 3.0 ± 1.6 | |
| IV | 28 | 3.2 ± 1.7 | 0.001 |

PlGF level was associated with advanced stage (stage III, IV; p=0.0001), tumor size >41 mm (p<0.0001) and metastasis (p<0.0001).

## Associations between Serum PlGF and Survival in All Patients with Special Reference to Serum CEA

In our colorectal cancer patient population, we defined elevated serum PlGF and CEA levels according to the best predictive values calculated in ROC analyses, which found the best pair of values for highest sensitivity and highest specificity using a peak of each cut-off point. Patients with elevated serum PlGF and CEA levels had significantly poorer prognosis than patients whose levels were below the cut-off value (log-rank test, PlGF: p<0.0001, CEA: p<0.0001, respectively). On the basis of Cox univariate proportional hazards analysis, advanced UICC stage (III, IV; p<0.0001), tumor size (>41 mm; p=0.0008), lymph node metastasis (p<0.00019), distant metastasis (p<0.0001), elevated serum CEA levels (p<0.0001), and elevated serum PlGF levels (p<0.0001) were significant prognostic factors for poor overall survival. By multivariate analysis, distant metastasis (p<0.0001) and elevated serum PlGF level (p<0.0001) were only the independent risk factors for predicting poor prognosis (Table 3).

**Table 3.** Univariate and multivariate analysis for predictor of survival in colorectal cancer

| Variables | Univariate (all patients) | | |
|---|---|---|---|
| | HR | 95%CI | p-value |
| UICC classification (III, IV vs. I, II) | 8 | 3.12-12.8 | 0.0001 |
| Tumor size (≥41 vs. <41) | 3.8 | 2.5-5.12 | 0.001 |
| Lymph node metastasis (yes vs. no) | 4.12 | 3.0-6.15 | 0.0001 |
| Distant metastasis (yes vs. no) | 15.2 | 9.81-19.7 | 0.0001 |
| PlGF (≥47.9 vs. <47.9) | 3.9 | 2.1-5.5 | 0.0001 |
| CEA (≥3.5 vs. <3.5) | 4.5 | 2.51-6.9 | 0.0001 |
| **Variables** | **Multivariate (all patients)** | | |
| | HR | 95%CI | p value |
| UICC classification (III, IV vs. I, II) | 0.77 | 0.23-2.13 | 0.75 |
| Tumor size (≥41 vs. <41) | 1.44 | 0.81-2.80 | 0.49 |
| Lymph node metastasis (yes vs. no) | 2.09 | 1.01-4.96 | 0.17 |
| Distant metastasis (yes vs. no) | 16.73 | 11.15-27.5 | 0.0001 |
| PlGF (≥47.9 vs. <47.9) | 3.0 | 1.53-6.21 | 0.0001 |
| CEA (≥3.5 vs. <3.5) | 2.24 | 1.39-4.38 | 0.41 |

Figure 1 is a scattergram showing PlGF protein expression in cancer tissues and normal mucosa.

## Associations between Serum PlGF and Survival in Potentially Curative Patients with Special Reference to Serum CEA

In the stage II and III, stage II or stage III colorectal cancer population, we defined elevated serum PlGF and CEA levels according to the best pair of

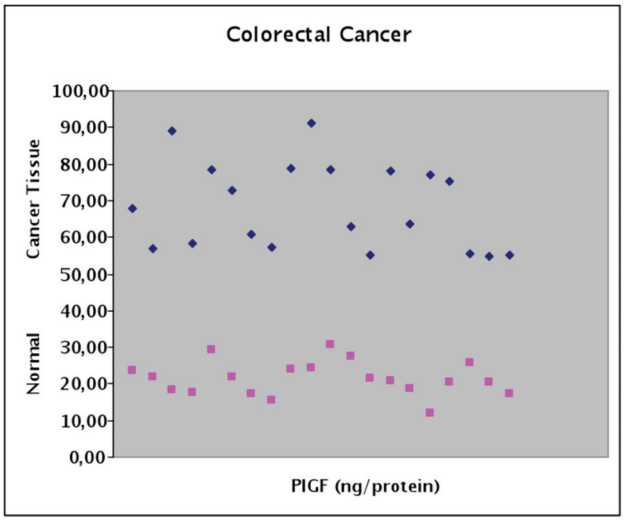


**Figure 1.** Scattergram of PlGF protein expression in cancer tissues and normal mucosa.

values for highest sensitivity and highest specificity using a peak of each cut-off point.

The survival curves in stage II and stage III patients were subdivided on the basis of serum PlGF (>39.5 pg/ml) and CEA (>3.5 ng/ml) levels. Elevated serum PlGF and CEA levels were associated with poor prognosis in patients with stage II and III classification ($p<0.0001$, $p=0.03$, respectively). On the basis of Cox univariate proportional hazards analysis, tumor size (>41 mm) ($p<0.01$), lymph node metastasis ($p=0.04$), elevated serum CEA levels ($p=0.03$), and elevated serum PlGF levels ($p<0.0001$) were significant prognostic factors for poor overall survival. By multivariate analysis, elevated serum PlGF level ($p=0.01$) was the only independent risk factor predicting poor prognosis instead of lymph node metastasis.

Furthermore, elevated serum PlGF level was associated with poor survival (stage II, stage III: $p=0.003$, $p=0.005$, respectively), and was the only independent prognostic factor in stage II or stage III patients (stage II, stage III: $p=0.001$, $p=0.03$, respectively).

## DISCUSSION

In this study, we reported that preoperative serum PlGF levels and expression levels in tissue in colorectal cancer patients is an independent, powerful predictor of their prognosis. Moreover, especially in stage III colorectal cancer patients, serum PlGF was found to be an indicator for survival. However, further studies with large series could help us to confirm the results.

These results suggest that PlGF, which functions in angiogenesis and carcinogenesis in colorectal cancer, might be used as a serum marker for cancer screen.

In the present study, we showed that an increase in the preoperative circulating PlGF levels was significantly correlated with factors associated with tumor size and nodal and distant metastasis, which are well-known conventional prognostic factors. Furthermore, elevated serum PlGF levels were found to be of prognostic value, whereas the prognostic values of CEA levels and UICC classification were highly impacted by other clinical factors. A previous study showed that the serum CEA levels were associated with prognosis in the stage III or IV patients, but not in the stage I or II patients (19).

In first our study, these findings showed that there is no correlation between serum PlGF levels and CEA levels in colorectal cancer patients. These findings support our hypothesis that PlGF and CEA are independently regulated.

The UICC staging system provides the most reliable information on prognosis, and is certainly useful for discriminating patients with early-stage disease from those with every advanced disease. However, its prediction of prognosis in patients with intermediate levels of tumor invasion is less accurate. Therefore, identification of sensitive prognostic markers in this subgroup would allow the use of postoperative adjuvant therapy in a subset of patients with a worse prognosis, with a resultant improvement in survival. Our study showed that the preoperative serum concentration of PlGF was the only pre-therapeutic prognosis factor in stage II and III colorectal cancer patients. This ability to identify stage II patients with poor prognosis who need clinical chemoprevention of recurrence could lead to an improvement in cancer survival. By contrast, from a clinical point of view, classification of patients with stage III tumors is also important, because intensive adjuvant chemotherapy and/or treatment with oxaliplatin could improve their survival rates (20). Interestingly, serum PlGF was the strongest prognostic factor in stage II and III patients, instead of lymph node metastasis, for which patients are routinely offered postoperative adjuvant chemotherapy.

In conclusion, preoperative serum PlGF may be a novel prognostic marker in colorectal cancer patients, especially in stage II and/or III patients, outweighing the negative prognostic values of UICC stage and CEA levels in these patients.

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