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ORIGINAL ARTICLE

Is there any impact of plasma M30 and M65 levels on progression-free survival of patients with advanced gastric cancer?

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Abstract
Purpose M30 and M65 are different circulating fragments of cytokeratin 18. They release during apoptotic cell death, so it is believed that they reflect cell death of epithelial tumors. The aim of this study was to determine the prognostic value of plasma M30 and M65 levels in predicting of survival for patients with advanced gastric cancer compare with healthy controls.
Methods Thirty-four patients with advanced gastric cancer and thirty-two healthy controls were included. Plasma M30 and M65 values were measured by quantitative ELISA method.
Results The median age of patients and control groups was 60 and 56 years, respectively. No difference was detected between patient and control groups with respect to plasma median M30 values (390.4 vs. 270.7 U/l, respectively, $P = 0.10$). The median plasma M65 values of patients were significantly higher than those of control group (1232.1 vs. 580.1 U/l, $P < 0.001$). The best cut-off values for plasma M30 and M65 for predicting progression-free survival (PFS) were 277.7 and 1434.9 U/l in ROC analysis. The patients whose plasma M30 values were higher than 277.7 U/l had worse PFS than patients with plasma M30 value <277.7 U/l (8.9 vs. 11.2, respectively, $P = 0.01$). The median PFS of patients whose M65 levels lower than or equal to 1434.9 U/l was better than that of patients whose M65 levels were >1434.9 U/l (12.4 vs. 10.4, respectively, $P = 0.04$). But plasma M30 and M65 level in patient group were not found to be an important prognostic factor for PFS in the multivariate analysis.
Conclusions These results showed that plasma M65 values were significantly elevated in patients with advanced gastric cancer compared to healthy people. Moreover, both increased plasma M30 and M65 levels can predict PFS in patients with gastric cancer.

Keywords Plasma M30 level · Plasma M65 levels · Gastric cancer · Survival

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Introduction

Cytokeratin 18 (CK-18) is a member of the intermediate filament family of cytoskeletal protein and is widely found in epithelial and endothelial cells lining of the respiratory and gastrointestinal tract [1, 2]. It is released into circulation during apoptotic cell death, and it is believed that it is a surrogate of drug-induced cancer cell death [3]. M30 and M65 are caspase cleaved and intact forms of CK-18, respectively, and they were detected in the circulation by using enzyme-linked immunosorbent assays (ELISAs) [4]. M30 antibody recognizes a neo-epitope of CK-18 formed, therefore it is a more selective biomarker of apoptosis [5, 6]. However, monoclonal antibody M65 measures all CK-18 fragments that contain full-length epitopes of the protein, which are released during both necrotic and apoptotic

cell death [4, 7]. Prognostic significance of both M30 and M65 assays has been evaluated, and it has been shown that they may have an important prognostic and predictive markers in several malignancies [8–14]. It was reported that plasma M65 levels were associated with poor prognostic factors in pancreas cancer [15] and testicular cancer [16], and plasma M30 levels were related with survival in gastric cancer [8] and testicular cancer [16].

Gastric cancer is the second leading cause of cancer death worldwide. Although the incidence rate has declined, the prognosis of patients with gastric cancer has not improved much [17, 18]. A correct definition of poor prognostic factors may help to guide more aggressive treatment protocols. Recently, new prognostic indicators have been documented by advances in molecular and histochemical studies [19, 20]. CK-18 expression has been previously detected in tissue blocks of some patients with gastric cancer [21]. In a study performed by Xu et al. [22], it showed that patients with gastric cancer had overexpression of CK-18 compared with benign gastric pathologies such as gastric ulcers. Recently, Yaman et al., firstly evaluated prognostic significance of serum M30 and M65 levels in patients with gastric cancer. They showed that both serum M30 and M65 levels were significantly increased in patients compared to control group and only serum M30 levels and clinical stage were an independent prognostic indicator for survival [8]. In the present study, we aimed to determine the prognostic significance of the plasma M30 and M65 levels in predicting of progression-free survival (PFS) for patients with advanced gastric cancer compare to healthy controls.

Materials and methods

This case–control study included a total of 34 patients with histologically confirmed gastric cancer who were chemotherapy-naive and followed-up at Dr. Lutfi Kirdar Kartal Education and Research Hospital, Department of Medical Oncology. All patients were staged as locally advanced or metastatic according to the AJCC/UICC TNM staging classification for gastric cancer [23]. Patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status of $\leq$2, and laboratory requirements for the inclusion were having normal levels of bilirubin, hepatic enzymes, and renal functions. Hematologic requirements were a white blood cell count >3,000 mm^3, absolute neutrophil count (ANC) >1,000 mm^3, and platelets >100,000/mm^3. Moreover, thirty-two age- and sex-matched healthy people without any known malignancy who were selected between healthy relatives of patients were constituted as the control group. The Local Ethics Committee of our hospital approved the study, and the informed written consents were obtained from each patients and healthy controls.

Patients who had received chemotherapy in the prior 6 months and with prior malignancies except for basal cell carcinoma or cervical carcinoma in situ were excluded from the study. The clinical informations of the patient group such as age at diagnosis, performance status, tumor stage, histopathological type and age, gender of the control group were obtained from patients' charts.

Collection of serum samples

Five millimeters of peripheral blood samples were collected from 32 healthy people and 34 patients with advanced gastric cancer into the dry tubes, and samples were centrifuged at 1,000*g* for 10 min to obtain serum within the half an hour after blood sampling. Patients' samples were collected prior to the chemotherapy, and all plasma samples were stored at −20°C until the evaluation. Plasma M30 and M65 antibody levels were measured in both groups, and mean values were compared after all samples were analyzed simultaneously.

M30 and M65 measurement

An ELISA kit, M30-Apoptosense, and M65-ELISA (Peviva AB, Sweden) were used to measure M30 and M65 levels. Samples were diluted in the ratio of 1:1 by using Standard A solution that included 0 U/l M30 or M65. This ELISA uses monoclonal antibody as catcher for recognizing CK-18 and horseradish peroxidase conjugated M30 as detector. Excess unbound conjugate was removed, and then TMB substrate was added. Finally, the absorbance was measured in a microplate reader at 450 nm. By plotting a standard curve, from known concentrations versus measured absorbances, the M30 and M65 levels were expressed as U/l. All determinations were done according to manufacturer's instructions. Units of M30 and M65 ELISA were defined using a synthetic peptide (1U = 1.24 pmol). The measuring range for M30 and M65 levels were 0–1,000 U/l and 0–2,000 U/l, respectively.

Plasma M30 and M65 levels of patients with gastric cancer were compared with those of control group. Patient characteristics included plasma M30 and M65 levels were analyzed according to PFS and overall survival, respectively.

Statistical analysis

Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) software. Descriptives of the parameters are quoted as median. Because the distribution of study parameters was non-normal distribution, non-parametric tests were used. The significance of the

differences among the medians was determined by the Mann–Whitney *U* test and Kruskal–Wallis test. Median plasma M30 and M65 levels between patient and control groups were also compared using Mann–Whitney *U* test. Pearson's correlation analysis was used for the correlation between plasma M30 and M65 levels in patients. Survival analysis and curves were established according to the Kaplan–Meier method and compared by the log-rank test. PFS was defined as the time from diagnosis to the last follow-up and the time until relapse as being the time since diagnosis to the first evidence of relapse. In addition, OS was described as the time from diagnosis to the date of the patient's death or last known contact. Multivariate analysis to assess the significance of plasma M30 and M65 levels and the other clinicopathological features as prognostic factors was performed by the Cox regression analysis after univariate analysis was carried out. Multivariate *P* values were used to characterize the independence of these factors. The receiver operating characteristics (ROC) analysis was performed in order to evaluate the significance of M30 and M65 levels for PFS. The 95% confidence (CI) was used to quantify the relationship between survival time and each independent factor. All *P* values were two-sided in tests, and *P* values less than or equal to 0.05 were considered to be statistically significant.

Results

Thirty-four patients with advanced gastric cancer and 32 healthy subjects as control group were analyzed. Twenty-one patients (61.8%) were men and 13 (38.2%) were women, with a median age of 60 years (range; 26–82 years). The majority of patients ($n = 30$, 88.2%) were metastatic disease, and the most frequent site of metastases (53.6%) was liver. In addition, the moderately differentiated tumors were the most common type of differentiation (52.9%). Histopathological subtypes of the patients were adenocarcinoma in 76.5% of patients, while there were only four signet-ring cell carcinoma and four mixed type. Patient characteristics are listed in Table 1. In the control group, the median age was 56 years (range; 38–79) and 23 healthy subjects were men, while only nine people were women.

The plasma M30 levels in patients were higher than those of control groups (median; 390.4 vs. 270.7 U/l), but this difference was not significant ($P = 0.10$). However, plasma M65 levels were found to be significantly elevated in patients compared to the control group ($P < 0.001$). Table 2 shows the comparison of plasma M30 and M65 levels among two groups. The relationship between plasma M30 or M65 levels and age, gender, the presence of surgery, histopathological subtypes, tumor differentiation were not found ($P > 0.05$); however, we detected a significant correlation between tumor localization and both plasma M30 and M65 levels. Thus, both plasma M30 and M65 levels were significantly greater in cases with tumor located in the lower stomach compared to the upper, middle, or diffuse tumor involvement ($P = 0.01$ and 0.01,

Table 1 Patient characteristics

Characteristics	*n* (%)
All	34
Age (years)	
Range	26–82
Median	60
Gender	
Male	21 (61.8)
Female	13 (38.2)
Tumor site	
Upper	5 (14.7)
Middle	14 (41.2)
Lower	13 (38.3)
Diffuse	2 (5.9)
Surgery	
Present	11 (32.4)
Absent	23 (67.6)
Clinical stage	
Locally advanced	4 (11.8)
Metastatic	30 (88.2)
Histopathology	
Adenocarcinoma	26 (76.5)
Signet-ring cell carcinoma	4 (11.8)
Mixed	4 (11.8)
Tumor differentiation	
Well-differentiated	2 (6)
Moderately differentiated	18 (52.9)
Poorly differentiated	14 (41.1)
Site of metastasis	
Liver	16 (53.6)
Peritoneum	8 (26.6)
Lungs	1 (3.3)
Bone	1 (3.3)
Ovary	2 (6.6)
Paraaortic lymph nodes	2 (6.6)

Table 2 The serum M30 and M65 levels in patients and control groups

Serum levels	Patients $n = 34$	Control $n = 32$	*P* value
Median M30 (U/l)	390.4	270.7	0.10
Range	140–3129.1	137.7–2182.2	
Median M65 (U/l)	1232.1	580.1	<0.001
Range	241.1–11439.9	393.8–2409	

Table 3 The correlation between plasma M30 and M65 levels and clinicopathological characteristics of the patients group

Variables	*n* (%)	M30 [median (range, U/l)]	*P* value	M65 [median (range, U/l)]	*P* value
Age (years)			0.10		0.10
≤50	11 (32.4)	328.7 (140–1146.5)		899.7 (241–8682)	
>50	23 (67.6)	391.6 (176.2–3129)		1470.9 (610–11440)	
Gender			0.20		0.13
Male	21 (61.8)	417.5 (140–3129)		1434.9 (522–11440)	
Female	13 (38.2)	265.1 (164.4–1146.5)		876.8 (241–8682)	
Tumor site			0.01		0.01
Upper	5 (14.7)	202.5 (164.4–1607)		696.5 (679.2–2052.2)	
Middle	14 (41.2)	258.8 (140–1146.5)		816 (522–8682)	
Lower	13 (38.3)	757.2 (265–3129)		2417.6 (951.7–11440)	
Diffuse	2 (5.9)	251.6 (174.5–328.7)		570.4 (241–890)	
Surgery			0.65		0.43
Present	11 (32.4)	391 (164.4–1345)		1386.2 (610–8682)	
Absent	23 (67.6)	265.1 (140.3129)		1013.7 (241–11440)	
Clinical stage			0.04		0.23
Locally advanced	4 (11.8)	396.4 (140–3129)		1453 (241–11440)	
Metastatic	30 (88.2)	196.6 (172–265)		839.7 (685.5–1014.7)	
Histopathology			0.28		0.19
Adenocarcinoma	26 (76.5)	390.4 (140–3129)		1232 (241–11440)	
Signet-ring cell carcinoma	4 (11.8)	605.4 (277.7–1345)		2322 (951.7–3467.8)	
Mixed	4 (11.8)	232 (195.7–502.6)		707.5 (688.6–1435)	
Tumor differentiation			0.33		0.15
Well-differentiated	2 (6)	600.3 (172–1345)		2686 (685.5–3588.4)	
Moderately differentiated	18 (52.9)	389.8 (140–3129)		1077.8 (241–11440)	
Poorly differentiated	14 (41.1)	328.7 (164.4–761)		1013.7 (610–2798)	

respectively). The correlation between plasma median M30 and M65 levels and the clinicopathological findings are shown in Table 3.

In patients with metastatic disease, plasma median M30 levels were significantly higher than those of the patients with locally advanced disease (396.4 vs. 196.6 U/l, $P = 0.04$). Although patients with metastatic disease had higher plasma M65 levels compared with the patients with locally advanced disease, this difference was not statistically significant (median; 1453 vs. 839.7 U/l, $P = 0.23$). On the other hand, plasma M30 and M65 levels were positively correlated with each other in patients with gastric cancer ($P < 0.001$, $r = 0.881$).

At the median follow-up of 12.8 months (range; 3–24 months), 1-year PFS rate and the median PFS time were 24.2% and 8.9 months, respectively, while 1-year OS rate and the median OS time were 67% and 17.3 months, respectively. The univariate analysis for PFS showed that only the presence of surgery was an important prognostic factor ($P = 0.03$). In other word, the median PFS time in patients who had undergone surgery was better than that of the patients without surgery (10.5 vs. 7.7 months). There were no significant correlations between clinicopathological factors, such as the presence of metastasis, histopathological type, clinical stage, tumor differentiation, tumor localization, patient's age, gender, and PFS ($P > 0.05$). Similarly, the relationship between clinicopathological factors and OS was not detected in the univariate analysis ($P > 0.05$). The results of univariate analysis for PFS are summarized in Table 4.

The prognostic significance of the M30 and M65 levels for PFS was evaluated with ROC analysis. The ROC analysis indicated a cut-off value of 277.7 U/l for M30 with 84.6% sensitivity (95% CI: 54.6–98.1) and 61.9% specificity (95% CI: 34.8–81.9) (AUC = 0.73, $P = 0.014$), which were predictive of PFS, accurately. In addition, a cut-off value of 1434.9 U/l for M65 with 69.2% sensitivity (95% CI: 38.6–90.9) and 71.4% specificity (95% CI: 47.8–88.7) (AUC: 0.707, $P = 0.036$) was shown. Patients with serum M30 level greater than cut-off value (>277.7 U/l) had worse PFS (8.9 months) than M30 levels lower than or equal to 277.7 U/l (11.2 months) ($P = 0.01$) (Fig. 1). Furthermore, the median PFS time of patients whose M65 levels lower than or equal to 1434.9 U/l was better than

Table 4 Univariate analysis of patients with advanced gastric tumor for progression-free survival (PFS) according to clinicopathological factors

Factors	6-month PFS rate (%)	Log-rank χ^2 value	*P* values
Gender		0.07	0.93
Male	84		
Female	69.2		
Age (years)		0.01	0.97
≤50	72.7		
>50	73.9		
Tumor site		4.39	0.22
Upper	72.4		
Middle	76.7		
Lower	57.4		
Diffuse	NA		
Surgery		4.38	0.03
Present	100		
Absent	66.1		
Tumor differentiation		0.28	0.86
Well-differentiated	83.3		
Moderately differentiated	71.8		
Poorly differentiated	83.9		
Clinical stage		1.81	0.17
Locally advanced	NA		
Metastatic	75.4		
Histopathology		3.63	0.16
Adenocarcinoma	70.5		
Signet-ring cell carcinoma	NA		
Mixed	NA		

NA not applicable

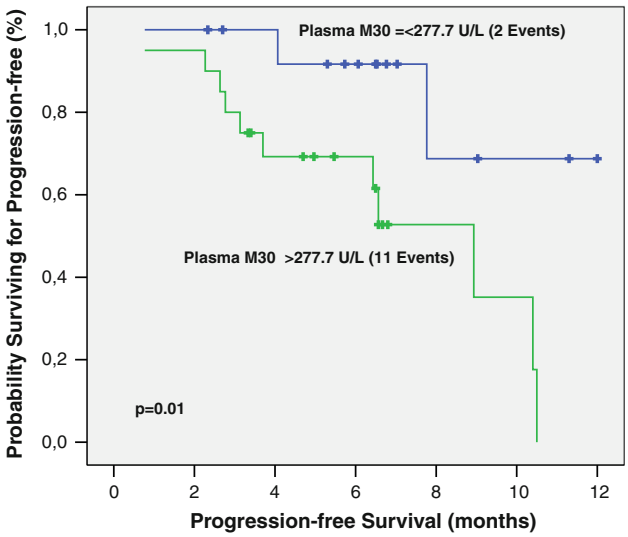

Fig. 1 Progression-free survival curves of the patients whose M30 levels were greater than 277.7 U/l (median; 8.9 months) were significantly worse than that of the cases whose M30 levels <277.7 U/l (median; 11.2 months)

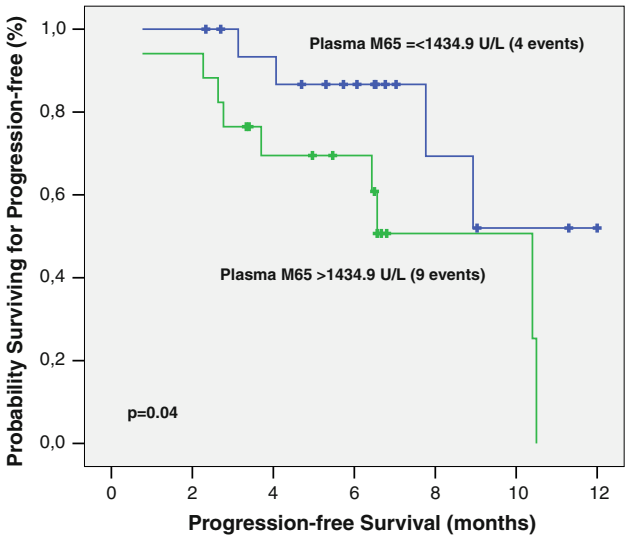

Fig. 2 Progression-free survival curves are shown in patients with M65 levels <1434.9 or >1434.9 U/l in patients with advanced gastric cancer

that of patients whose M65 levels were >1434.9 U/l (10.4 vs. 8.1 months, respectively, $P = 0.04$, Fig. 2). Table 5 shows the results of univariate analysis according to a cut-off value of M30 and M65. On the other hand, plasma M30 and M65 levels in patient group were not found to be an important prognostic factor for PFS in the multivariate analysis ($P = 0.06$ and 0.37, respectively), as were the other clinicopathological factors. The results of the multivariate analysis are shown in the Table 6.

Discussion

In the current study, we found that plasma M65 levels of patients with gastric cancer were significantly higher than those of healthy control, but not M30. The patients with metastatic disease had significantly elevated plasma M30 levels compared to levels in cases with locally advanced disease, reflecting higher tumor load. In addition, we detected a significant correlation between tumor localization and both plasma M30 and M65 levels. Our results also indicated that patients with both elevated plasma M30 and M65 levels had worse PFS compared to patients with low levels.

M30 and M65 are relatively new markers that detect different circulating forms of the epithelial cell structural protein CK-18. Plasma M30 levels indicate the caspase-cleavage form of CK-18 in the serum, and M65 detects a common epitope present in full-length protein as well as in the caspase-cleaved fragment, thus it is an indicator of CK-18 released by tissue necrosis in addition to apoptosis

Table 5 The PFS and OS of the patients with gastric cancer according to cut-off value of M30 and M65 levels by univariate analysis

Cut-off point	*n* (%)	Median PFS (month)	95% CI	*P*	Median OS (month)	95% CI	*P*
M30 level (U/l)							
≤277.7	14 (41.2)	11.2	7.4–14.4	0.01	NA	NA	0.02
>277.7	20 (58.8)	8.9	5.8–12		NA	NA	
M65 level (U/l)							
≤1434.9	17 (50)	10.4	6.1–14.6	0.04	NA	NA	0.01
>1434.9	17 (50)	8.1	6.9–9.7		NA	NA	

PFS progression-free survival, *OS* overall survival, *CI* confidence interval, *NA* not applicable

Table 6 Multivariate analysis of the prognostic factors in patients with gastric cancer for progression-free survival

Factors	Wald	*P*	HR	95% CI
Gender (male vs. female)	0.79	0.37	2.93	0.27–13.3
Age (≤50 vs. >50)	1.18	0.27	0.26	0.02–2.92
Tumor site	2.84	0.09	0.42	0.15–1.14
Surgery (present vs. absent)	0.44	0.50	0.34	0.01–7.69
Clinical stage	0.001	0.98	0.01	0.001–1.12
Histopathology	3.86	0.06	0.07	0.01–0.99
Tumor differentiation	0.002	0.96	0.97	0.26–3.60
Cut-off value of M30 (≤277.7 vs. >277.7 U/l)	1.63	0.06	11.7	0.26–23.5
Cut-off value of M65 (≤1434.9 vs. >14394.9 U/l)	1.17	0.37	0.21	0.01–3.45

HR hazards ratio, *CI* confidence interval

[3, 4]. It has been reported that these markers, especially M30 levels may be predict response to chemotherapy and survival as a marker [9, 11, 12].

In a study performed by De Haas et al. [16], the authors showed that serum M30 level was an important prognostic factor like LDH, AFP, β-hcG in testicular cancer. Moreover, it was reported that M30 levels were correlated with grade, stage, Ki-67 index of the endometrium cancer [24]. We found a correlation between tumor localization and M30 and M65 levels. In addition, the association of M30 levels with clinical stage was detected in our study.

Ueno et al. [10] showed higher M30 levels in patients with breast cancer compared to healthy subjects. However, they could not find any relation between M30 levels and prognosis. Dive et al. [15] reported that the median M65 levels in patients with metastatic pancreas cancer were higher than those of cases with locally advanced or resectable pancreas cancer. However, they did not compare M65 levels both in healthy control and patients with pancreas cancer. In addition, they found that M65 levels were associated with poor OS in the univariate analysis, but they could not confirm it by multivariate analysis. Ulukaya et al. [11] evaluated only M30 levels among patients with NSCLC or benign lung disease and healthy group, and they indicated that M30 levels were higher in the NSCLC group ($P < 0.001$). Moreover, they found the poorer survival of patients with increasing M30 levels. Hou et al. [25] documented that M30, M65, and circulating tumor cells were higher in small cell lung cancer. The authors found M30 and M65 to be the important prognostic factors for survival of patients.

To date, the significance of M30 and M65 levels in advanced gastric cancer has been investigated in only one study performed by Yaman et al. [8]. They found that both serum M30 and M65 levels were significantly increased in patients with advanced gastric cancer compared to control group. In addition, the authors showed that patients with metastatic disease had significantly higher M30 levels compared to patients with locally advanced disease. When these assays were evaluated with respect to survival, the authors found the median survival time in patients with high M30 antigen was worse than that of patients with lower plasma M30 levels, but there was no effect of plasma M65 level on survival. In their study, serum M30 levels and clinical stage were found to be an important prognostic factor for OS. In this current study, we found that plasma M65 levels were significantly increased in patients compared to healthy control, but did not M30. However, our results showed that patients with elevated M30 and M65 levels had significantly shorter median PFS interval compared to patients with lower M30 and M65 levels. Moreover, we detected that plasma M30 and M65 levels were associated with poor PFS in the univariate analysis, but we could not confirm these data by multivariate analysis.

The small sample size and short follow-up time of our study could be considered as significant limitation and might have influenced these results. But, although prospective studies including large sample size are needed, our results contribute to the literature, because we showed firstly that increased serum M30 and M65 levels were related to PFS in patients with advanced gastric cancer.

In patients with cancer, M65 levels have been investigated by limited studies data. Ausch et al. [26] indicated that serum M65 levels were an important marker to identify patients with high incidence of systemic disease with colon cancer**.** Ozturk et al. [13] evaluated M30 and M65 levels of patients with locally advanced head and neck cancer, and they showed that serum M30 levels were significantly found to be higher than healthy subjects, while increased M65 levels were not significant compared to control groups. Our results were compatible with their study. However, they did not analyze the impact of these markers on OS or PFS of patients. In contrast to Ozturk's study, Yaman et al. reported that serum M30 level was correlated with OS in patients with advanced gastric cancer, but not M65 level [8]. On the other hand, we detected that the best cut-off value for prediction of progression for M30 and M65 was 277.7 and 1434.9 U/l, and high levels of both M30 and M65 were associated with poor PFS. To our knowledge, this is the first study in which these assays were evaluated for PFS of patients with advanced gastric cancer. But prognostic significance of both M30 and M65 could not be verified by multivariate analysis. It may be attributed to small sample size of our study.

In this research, both plasma M30 and M65 levels were significantly greater in cases with tumor localized in the lower of stomach compared to tumor in the upper, middle stomach, or diffuse involvement ($P = 0.01$ and 0.01, respectively). It may indicate that tumor in the lower stomach associated with higher M30 and M65 has worse prognosis than other localization so treatment may change according to site of the tumor. But it should be confirmed with larger sample size study. In patients with metastatic disease, plasma M30 levels were significantly higher than those of the patients with locally advanced disease ($P = 0.04$), reflecting higher tumor burden. Nevertheless, while plasma M65 levels were higher in metastatic disease compared with locally advanced disease, this difference was not statistically significant ($P = 0.23$). Our results were thus compatible with the study of Yaman et al. [8] with respect to correlation between high M30 levels and the presence of metastasis.

In conclusion, our results show that serum M65 levels in patients with advanced gastric cancer were higher compared with healthy subjects. In addition, we found firstly that both the elevated plasma M30 and M65 levels were related with PFS of patients, but in the multivariate analysis, the significance of these markers as prognostic factor could not be confirmed in predicting PFS. Future prospective studies including large sample size will need to address the possible impact of M30 and M65 levels on response to treatment of patients, and these markers might be evaluated as a prognostic factor in OS of patients with gastric cancer.

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