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RESEARCH ARTICLES

Serum M30 and M65 values in patients with advanced stage non-small-cell lung cancer compared with controls

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Abstract
Background M30 and M65 are derivatives of cytokeratin 18 and released from the epithelial cell during cell death. These markers can be used to evaluate prognosis and chemotherapy response in several tumours. We evaluated serum M30 and M65 values in patients with advanced non-small-cell lung cancer (NSCLC) compared with those in a healthy group.
Material and methods Thirty-two patients with advanced NSCLC and thirty-two healthy people were included in the study. Serum M30 and M65 values were measured by quantitative ELISA method. The best cut-off value for serum M65 was calculated by ROC analysis and then univariate analysis was performed to determine the importance of M65 value in predicting progression-free survival (PFS).
Results There were no differences between mean serum M30 values between patients and controls (445.44±536.17 vs. 340.56±345.07, p=1). The mean serum M65 values were found to be significantly higher in patients than in healthy controls (1421.30±1662.59 vs. 648.85±341.17, p<0.001). The best cut-off value for serum M65 predicting PFS was 1311.64 U/l (AUC 0.58, sensitivity and specificity were 45.5% and 85.7% respectively). The patients with serum M65 values ≥1311.64 U/l had worse PFS than patients with serum M65 values <1311.64 U/l, p=0.01). There was no correlation between serum M30 value and PFS in the patient group (p=0.4).
Conclusions Our results indicated that serum M65 values elevated in advanced NSCLC compared to a healthy control group and elevated serum M65 level can predict PFS in patients.

Keywords Non-small-cell lung cancer · M30 · M65 · Progression-free survival · Cut-off · Advanced stage

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Introduction

Cytokeratin 18 (CK-18) is a cytoskeletal protein of the epithelium and released to circulation during apoptotic or non-apoptotic cell death of epithelial cells [1, 2]. Intact or caspase-cleaved forms of CK-18 are usually accepted as markers of cell death in the malignant transformation [1, 3]. In the circulation CK-18 fragments can be detected by M30 and M65 antibodies using enzyme-linked immunoabsorbent assays (ELISAs) [4]. M30 is the monoclonal antibody that recognises the caspase-cleaved CK-18 fragments so it is the selective biomarker of epithelial apoptosis [4, 5]. Monoclonal antibody M65 recognises all CK-18 fragments that contain full-length epitopes of the protein released from both necrotic and apoptotic cells [4]. In cancer patients, plasma M30 and M65 antigens were stable for 2 years when stored at –80°C [6].

Both M30 and M65 assays have been reported as having important prognostic and predictive markers in several malignancies [1, 7–10]. They were reported to be increased in pancreatic cancer [2], advanced gastric cancer [1], head and neck tumour [10], lung cancer [7] and breast cancer [11]. In addition, M65 levels were associated with poor prognosis in pancreas cancer [2] and testicular cancer [12], and also there was a correlation between M30 levels and survival in gastric cancer [1] and testicular cancer [12].

Lung cancer is the leading cause of cancer-related death in the world. It has a poor prognosis because it is generally diagnosed in an advanced stage [13]. The five-year overall survival (OS) rate for non-small-cell lung cancer (NSCLC) is approximately 15% [14]. There have been several studies evaluating prognostic factors in lung cancer. Identification of the biomarkers used to evaluate prognosis might improve outcomes for patients with lung cancer. Ulukaya et al. [7] first reported that M30 levels were higher in lung cancer patients compared with benign lung disease and healthy patients. Their study might show the role of serum M30 levels in lung cancer, however there are no data related to M65 levels and its impact on the progression-free survival (PFS) in lung cancer patients. In the current study, we evaluated the clinical significance of serum M30 and M65 levels in advanced NSCLC.

Materials and methods

Study group

A total of 64 cases participated in the study. Thirty-two of them were diagnosed and treated due to advanced NSCLC at the Medical Oncology Department of Dr. Lutfi Kirdar Kartal Education and Research Hospital. The others were healthy people without any known malignancy who were selected from healthy relatives of our patients, after informed written consent was obtained. The eligibility criteria for the control group was absence of previous malignancy or systemic disease. NSCLC was staged clinically according to TNM classification, 7th version [15]. Recently diagnosed NSCLCs with clinically advanced stage with inoperable disease (stage IIIA, IIIB or IV) were selected for the patient group, and patients with liver or renal dysfunction were excluded from the study. The clinical information for the patient group, such as age at diagnosis, performance status, tumour stage, histopathological type and age, and gender of control group, were obtained from patients' charts after informed written consent was obtained from each subject included in the study. All patients received cisplatinum-based chemotherapy and also concomitant radiotherapy for stage III patients.

Collection of serum samples

Samples of 5 ml of peripheral blood were collected from 32 healthy people and 32 patients with NSCLC in dry tubes and centrifuged at 1000 g for 10 min to obtain sera within half an hour of blood sampling. Sera from patients were collected prior to the chemotherapy and stored at –20°C for 4 months, until the evaluation. All samples were analysed simultaneously. Serum M30 and M65 levels were measured in both groups and mean values were compared.

M30 and M65 measurement

The concentrations of M30 and M65 levels were measured using ELISA kit, M30-Apoptosense and M65-ELISA assays (Peviva AB, Sweden). Samples were diluted to a ratio of 1:1 by using Standard A solution which included 0 U/l M30 or M65. This ELISA uses monoclonal antibody as catcher for recognising 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 vs. measured absorbances, the M30 and M65 levels were expressed as U/l. All determinations were done according to manufacturers' instructions. Units of M30 and M65 ELISA were defined using a synthetic peptide (1 U=1.24 pmol). The measuring ranges for M30 and M65 levels were 0–1000 U/l and 0–2000 U/l respectively.

The serum M30 and M65 levels of patients with NSCLC were compared with those of a healthy control group. The characteristics of the patient group included serum M30 and M65 levels were analysed with regard to their OS and PFS respectively.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software. Because the distribution of study parameters was non-normal, non-parametric tests were used. The comparison of median M30 and M65 levels between control and patient groups was analysed by the Mann-Whitney *U*-test. In order to evaluate the importance of M65 levels for PFS, a receiver operating characteristics (ROC) analysis was performed. 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 or 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. Factors analysed by univariate analysis were also examined with multivariate analysis by using a Cox proportional hazards model for predicting risk factors for relapse. Multivariate *p* values were used to characterise the independence of

Table 1 The patient and control group characteristics

	Control group	Patient group
No.	32	32
Median age (years), range	56 (38–79)	59 (44–72)
Gender		
Female (%)	7 (21.9)	2 (6.2)
Male (%)	25 (78.1)	30 (93.8)
Mean M30 level±SD (U/l)	340.56±345.07	445±44
Mean M65 level±SD (U/L)	648.85±341.17	1421.30±1662.59

Table 2 The mean M30 and M65 levels according to patient characteristics

	M30±SD (U/l)	M65±SD (U/l)
Gender		
Female	773.37±890.06	2594±3090.73
Male	423.58±521.61	1343.07±1588.76
Age (years)		
<55	339.95±225.14	1163.48±673.16
>55.1	486.72±616.70	1522.19±1921.57
Stage		
IIIA	234.20±57.96	750.12±17.96
IIIB	388.81±307.23	981.86±459.40
IV	500.46±657.55	1746.37±2088.95
Histopathology		
SCC	372.44±318.09	1205.22±908.70
Adenocarcinoma	773.75±995.79	2408.78±3178.77
NSCLC nos	277.81±97.78	903.15±260.84

SCC, squamous cell carcinoma; nos, not otherwise specified

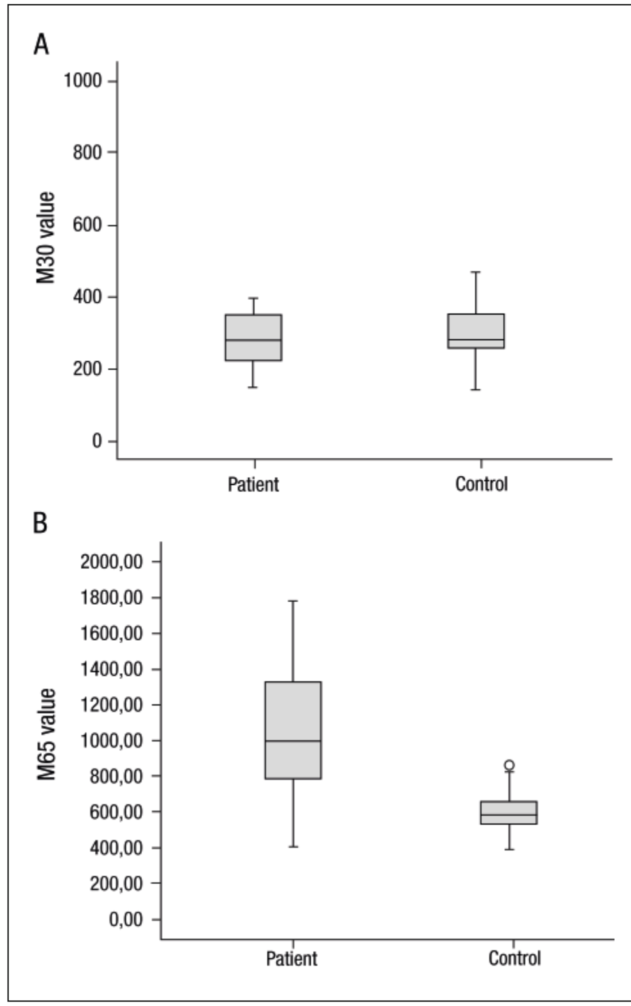

Fig. 1A M30 values of patient and control groups. **B** M65 values of patients and controls

these factors. The 95% confidence interval (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

Patient characteristics

A total of 64 cases were included in the study: 32 of them were patients with advanced NSCLC and the other 32 cases were a healthy control group. The patient and control group characteristics are summarised in Table 1. The median ages of the two groups were similar with 56 vs. 59 years for the control and patient groups, respectively. Over 93% of the patient group was male and 78% of the control group was male. All of the patients were advanced stage NSCLC; 2 of them were staged as IIIA, 11 of them were stage IIIB and the rest were stage IV. Histopathological subtypes of the tumours were adenocarcinoma (21.8%) and squamous cell carcinoma (62.5%), while histopathological subtypes could not be determined in 15.6% of the patients.

Serum M30/M65 levels in the study group

The serum M30 and M65 levels of the patient group according to clinical characteristics are listed in Table 2. Figures 1A and 1B show serum M30 and M65 levels of the control and patient groups, respectively. There were no significant differences between serum M30 and M65 levels and gender, age, stage and histopathological type of the patients. While the mean serum M30 levels of the patient (445.44 U/l) and control groups (340.56 U/l) were not significantly different, the mean serum M65 levels were significantly higher in the patient group (1421.30 U/l) compared to the control group (648.85 U/l) (p<0.001) (Table 3).

Progression-free survival

Median PFS of the patients was 27 months (1-year PFS rate 65%) during the median 7 months (range 1–56 months) of the follow-up period. Median OS time could not be

Table 3 Comparison of mean value of M30 and M65 between controls and patients

	Control	Patients	*p*
Mean M30 value (U/l)	340.56±345.07	445.44±536.17	1
Mean M65 value (U/l)	648.85±341.17	1421.30±1662.59	<0.001

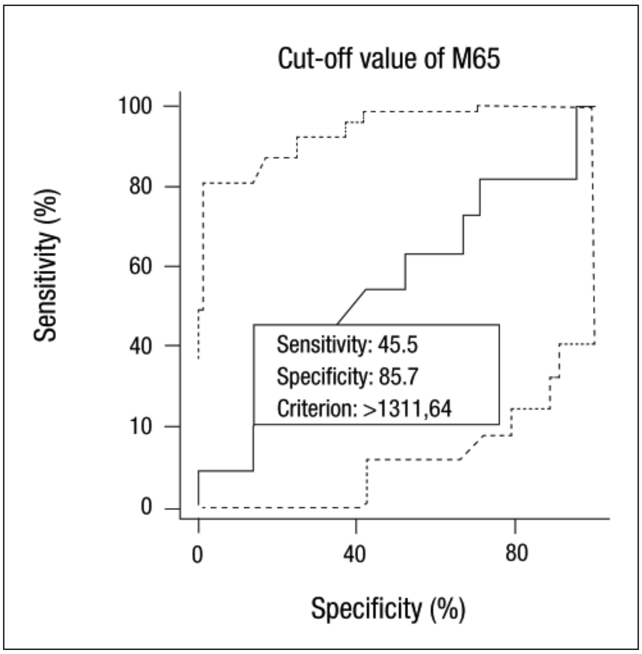

Fig. 2 The cut-off value of serum M65 level (AUC: 0.58)

reached, but the 1-year OS rate was 87%. There were no significant correlation between tumour characteristics such as histopathological type (*p*=0.6), clinical stage (*p*=0.5), patient's age (*p*=0.5), gender (*p*=0.6) and PFS.

Prognostic importance of serum M30 and M65 levels for PFS

The prognostic significance of the M65 levels for PFS was evaluated with ROC analysis. The cut-off value of M65 predictive for PFS was 1311.64 U/l, with 45.5% sensitivity and 85.7% specificity (AUC=0.56, *p*=0.58), which were predictive of PFS, accurately (Fig. 2). Moreover, patients with serum M65 levels greater than the cut-off value

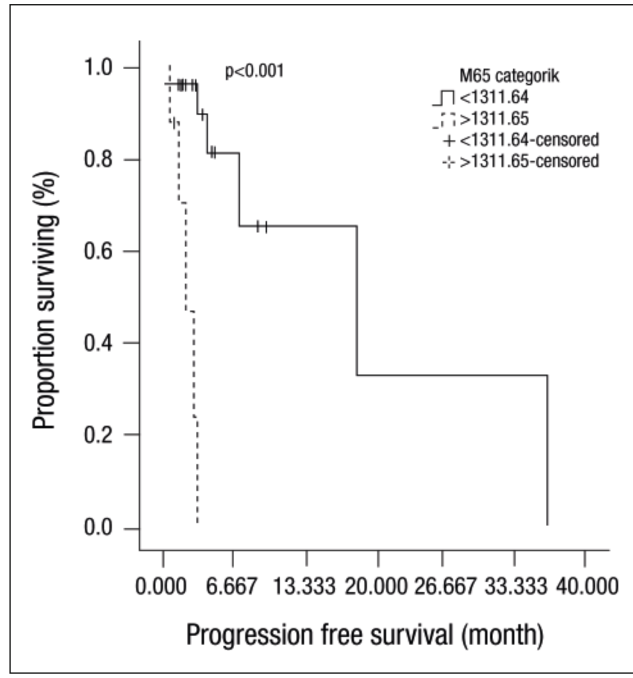

Fig. 3 TPFS curve of patients according to M65 cut-off value (1311.64 U/l)

(>1311.65 U/l) had worse PFS (3.3 months) than those with M65 levels lower than 1311.64 U/l (27.5 months) (*p*<0.001) (Fig. 3). Table 4 shows the results of univariate analysis. Serum M65 level in the patient group was also found to be an important prognostic factor for PFS in the multivariate analysis (*p*=0.002). The results of the multivariate analysis are shown in Table 5.

Discussion

CK-18 is an intermediate filament of the cytoskeleton in epithelial cells. Following caspase-dependent apoptosis of the epithelial cells, cleaved cytokeratin fragments (M30) release into the extracellular space following breakdown of the cell membrane and M30 ELISA assay detects caspase cleaved CK-18 [4]. M65 antigen represents the total CK-18 fragments released both during tissue necrosis and caspase-cleaved fragments. Kramer et al. [4] showed that extracellular CK-18 fragments could be detected by M65 ELISA during necrosis of breast epithelial cells. Both of these

Table 4 The comparison of PFS according to cut-off value of M65 levels by univariate analysis

	Number (%)	Median PFS (months)	95% CI	*p*	Median OS (months)	95% CI	*p*
M65 level (U/l)							
<1311.64	24 (75)	27.53±12.71	2.62–52.44				
>1311.65	8 (25)	3.30±0.83	1.65–4.94	<0.001	na	na	<0.001

na, not applicable

Table 5 The results of multivariate analysis of PFS in the patient group

Characteristics	Wald	*p*	HR	95% CI
Gender	0.00	0.9	7332.5	0–NA
Age	1.63	0.2	0.22	0.02–2.22
Stage	0.00	0.9	1.009	0.29–3.41
Histopathology	2.86	0.09	0.13	0.01–1.37
M30 level	2.43	0.1	0.1	0.006–1.78
M65 level	9.25	0.002	0.008	0–0.17

NA, not applicable; HR, hazard ratio; CI, confidence interval

assays were reported to be markers predicting response to chemotherapy and survival [1, 2, 7, 8, 12].

We found that the mean serum M65 level of patients with advanced NSCLC was higher than that of healthy controls. De Haas et al. [12] reported that serum M30 levels were an important prognostic factor, like LDH, AFP and ß-hcG in testicular cancer. In addition it was shown that M30 levels were correlated with grade, stage and Ki-67 index of endometrial cancer [16]. In our study, there was no correlation among clinicopathological features like age, gender, performance status, stage and either serum M30 or M65 levels of the patient group. We included only 32 eligible advanced-stage NSCLC patients and our sample size was limited, so this might have influenced the results.

Ueno et al. [11] showed higher M30 levels in breast cancer patients compared to healthy subjects. However they could not find any relation between M30 level and prognosis. Dive et al. [2] reported higher median M65 levels in metastatic pancreas cancer than in locally advanced or resectable pancreas cancer. In their study they classified pancreas cancer as metastatic, locally advanced and resectable, however they did not compare M65 levels between healthy control and pancreas cancer patients. In addition they found that M65 levels were associated with poor OS in the univariate analysis, but they could not confirm this by multivariate analysis. Ulukaya et al. [7] analysed only M30 levels among patients with NSCLC or benign lung disease and a healthy group and they reported that M30 levels were higher in the NSCLC group ($p<0.001$). Moreover, they showed an association of poorer survival of patients with increasing M30 levels. Hou et al. documented that M30, M65 and circulating tumour cells had higher levels in small cell lung cancer and they were found to be important prognostic factors for survival of patients with small cell lung cancer [17]. We evaluated M30 and M65 levels of NSCLC patients relative to healthy people to see if they had any utility as clinical potential biomarkers for PFS of advanced-stage NSCLC patients. Our study is noteworthy because we showed firstly that serum M65 level was an important biomarker in predicting PFS of NSCLC patients. Because our median follow-up time was short (7 months), we could not achieve OS time, therefore we did not analyse important factors that were important for OS.

There has been limited data on M65 levels in cancer patients. Ausch et al. [18] reported M65 as a valuable marker to identify patients with high incidence of systemic disease with colon cancer and elevated serum M65 levels in early and advanced stages of colon cancer. Ozturk et al. [10] evaluated M30 and M65 levels of patients with locally advanced head and neck cancer, but despite increasing M30 levels in patients compared to healthy people, they did not show an increase in M65 level in the patient group ($p=0.07$) and they did not analyse the importance of these assays in survival or PFS of patients with head and neck cancer. Yaman et al. [1] reported that the serum M30 level was correlated with survival in advanced gastric cancer patients and they found that the best cut-off value for prediction of death was 83.8 U/l for the M30 antigen. However, serum M65 level was not related to survival in their study.

Koelink et al. [19] reported that plasma M30 and total CK-18 levels in colorectal cancer patients were related with disease stage, tumour diameter and disease-free survival (DFS). Although they indicated that M30 and M65 levels were significantly correlated with DFS of patients by univariate analysis ($p=0.003$ and $p=0.02$ for M30 and M65, respectively), they did not find serum M65 levels to be an important factor in predicting DFS by multivariate analysis ($p=0.055$). The authors also did not use ROC analysis to define the best cut-off value of M30 and M65 levels in predicting DFS. In our study serum M65 level was found to be an important biomarker for predicting PFS of advanced NSCLC patients. We found the best cut-off value for serum M65 level to be 1311.64 U/l. Patients with serum M65 levels greater than 1311.64 had worse PFS (3.3 months) than those with M65 levels lower than 1311.64 (27.5 months) ($p<0.001$).

Herein, we found that serum M65 levels were higher in patients with advanced NSCLC and they were related with poor PFS. It is known that necrosis is a major process for hypoxic tumours and necrosis does not need ATP for processing so it is different from apoptosis. Hypoxia prevents apoptosis so it leads to resistance to treatment [20]. Our results might indicate the reasons for a low response rate to chemotherapy of advanced NSCLC. New studies, comparing M65 levels of early-stage and advanced-stage lung cancer patients, are needed to predict treatment response of lung cancer.

Long-term stability of both M30 and M65 antigen is limited. Although M65 antigen was reported as stored for 2 years at –80°C without any degradation in cancer patients [6], M30 antigen value was reported to increase after extended storage [21]. In the literature, serum M30 levels of lung cancer patients were evaluated and these assays were compared among NSCLC patients, patients with benign lung disease and a healthy control group [7]. In this study, all serums were stored at –20°C until the analysis. We also stored serum of our study group at –20°C until the evaluation.

In conclusion, we demonstrated firstly the higher serum M65 levels in the NSCLC patients compared with healthy subjects. Moreover, this assay was also an important factor in predicting PFS of the patients. In the future M65 or M30 might be evaluated as prognostic factors in the OS of lung cancer patients in studies with larger sample sizes and having longer follow-up times.

Conflict of interest The authors declare that they have no conflict of interest relating to the publication of this manuscript.

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