

Circulating Levels of M30 and M65 Molecules in Transitional Cell Carcinoma of the Bladder and Their Relation to Tumor Progression

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Abstract

Background: Various markers are suggested for diagnosis and monitoring of transitional cell carcinoma of the bladder (TCC), including cytokeratins (CKs).

Objectives: In the present study, the circulating CK18 (M65) and its caspase-cleaved form, cCK18 (M30), have been investigated in a group of patients with TCC.

Patients and Methods: Serum samples were obtained from 60 patients before surgical resection, among which the samples of 26 patients after resection were also included. We measured the levels of soluble M30 and M65 molecules by enzyme-linked immunosorbent assay. The relation between these markers and patients' clinical characteristics was evaluated.

Results: M30 and M65 in total patient sera were 148 ± 16 U/L and 318 ± 34 U/L, respectively. A correlation existed between pre-operative M30 and M65 levels ($P < 0.0001$, Spearman $r = 0.51$). M65, but not M30, showed a significant relation to tumor stage and grade. The M65 quantity in patients with T3/T4 tumor stages (350 ± 42 U/L) was higher than that of patients with T1/T2 stages (293 ± 45 U/L; $P < 0.038$). Patients with tumor grades III/IV also showed higher levels of M65 compared to patients with tumor grades I/II ($P < 0.04$). The M30:M65 ratio in all patients was 0.54 ± 0.04 . There was a lower M30:M65 ratio in patients with T3/T4 stage tumors and those with tumor grades III/IV ($P < 0.02$). The M30 (133 ± 19 U/L) and M65 levels (240 ± 21 U/L) after surgery did not significantly differ compared to their pre-operative values. However, a correlation between the pre- and post-operative M30:M65 ratio in patients ≥ 70 years was seen ($P = 0.009$).

Conclusions: These data suggested a relationship of both M65 and the M30:M65 ratio to tumor progression which might imply their importance in TCC monitoring.

Keywords: Bladder Cancer, Cytokeratin 18, M30, M65

1. Background

Bladder cancer is a common disease worldwide. The incidence of this cancer varies throughout the world with the highest rates in developed communities (1). Approximately 90% of bladder cancers are transitional cell carcinomas (TCC). The gold standard for detection and surveillance of bladder cancers are cystoscopy and urine cytology (2). Despite its benefit, cytology has low sensitivity, particularly for detection of low grade cancers and cystoscopy is a costly, invasive procedure. Hence, there is a need to develop other simple, non-invasive diagnostic methods that can be used to prolong the intervals between cystoscopies and for disease monitoring (3). An ideal bladder cancer screening and monitoring test should be non-invasive, easy to perform, and have high sensitivity and specificity. In recent years various markers have been sug-

gested for diagnosis and monitoring of bladder cancer. These include bladder tumor antigen (BTA), nuclear matrix protein 22 (NMP22), survivin, telomerase, and cytokeratins (CKs) (2, 4).

CKs are a family of more than 20 intermediate filament proteins expressed in cells of epithelial origin as well as endothelial cells. CKs are divided into two groups. CKs 1-8 are the type II group that comprise neutral to basic proteins whereas CKs 9 - 20 are a type I group that include acidic proteins (5). Epithelial tumors largely maintain the features of specific CK expression of their normal epithelial origin; therefore, these molecules have been extensively used as immunohistochemical markers in diagnostic tumor pathology (6).

CK8, 18 and 19 are most commonly used in clinics. These three markers are expressed by most types of carcinomas, including breast, prostate, lung, colon and ovarian (6, 7).

Their main use as immunohistochemical markers is to monitor treatment, evaluate response to therapy, and provide early prognostic information on tumor progression and metastasis formation (8). Circulating CKs result from release of intact proteins from rapidly proliferating tumor or dead cells (9). Several monoclonal anti-CKs antibodies are available that recognize CK8, CK18 and CK19 (10). The presence of CK18 fragments in cancerous epithelial cells has been reported (5, 11).

CK18 is cleaved by caspases during apoptosis. In order to detect this fragment, termed M30, a specific monoclonal antibody is available, as well as another one, M65 that recognizes total soluble CK18 fragments. By using these monoclonal antibodies it is possible to determine different circulating forms of CK18 in plasma or serum (12, 13). The M30 antibody detects CK18 fragments that contain a neo-epitope at positions 387 - 396 generated by the action of caspases 3, 7 and 9 which are activated during the early stages of apoptosis. This fragment is referred to as caspase-cleaved CK18 (ccCK18). The M65 antibody also detects cleaved fragments, but cannot distinguish between the full-length protein and its fragments (14). Thus, the M65 enzyme-linked immunosorbent assay (ELISA) theoretically measures both caspase cleavage (apoptosis) and cellular release of intact CK18 (necrosis).

The potential diagnostic and prognostic significance of circulating M30 and M65 has been investigated in patients with non-small cell lung cancer (NSCLC) and in colon cancer (15, 16). Their levels correlated with disease stage and recurrence. In other malignancies such as lung cancer (17) and pancreatic cancer (18), high pretreatment CK18 levels indicated a larger tumor burden and less favorable prognosis. A number of clinical trials on breast (19, 20), prostate (21), small cell lung (22) and testicular (23) cancers used both M30 and M65 as biomarkers of cell death from a variety of different chemotherapeutic agents.

2. Objectives

It has been claimed that the M30 assay has both predictive value of drug response (24) and prognostic value for survival (17, 25). M30 and M65 have been used as markers of host tissue toxicity in a number of different clinical conditions such as acute myocardial infarction (26), chronic liver disease (27) and hepatitis C (28). However, there is scant information on the prognostic application of these markers in bladder cancer. The current study aims to characterize baseline levels and post-surgical changes of circulating serum M30 and M65 levels in patients with TCC, and to examine their correlation with various clinical features.

3. Patients and Methods

3.1. Patients

This study enrolled 60 TCC patients (49 male and 11 female) aged 35 - 85 years who referred to Nemazi hospital, Shiraz, Iran. Disease diagnosis was confirmed by histopathologic examination and cystoscopy results. Tumor staging was determined according to the tumor-node-metastases (TNM) classification and grading was confirmed by a pathologist. Available data were obtained from patients' hospital files. All protocols were approved by the ethics committee at Shiraz University of Medical Sciences and informed consent was obtained from the patients.

3.2. Patients' Samples

Blood samples were collected before and 7 - 10 days after operation and the obtained sera were stored at -70°C until analyzed.

3.3. ELISA Assays

The level of ccCK18 and total CK18 in sera were determined using the M30- Apoptosense® ELISA and M65-ELISA assay kits (PEVIVA, Sweden), respectively, according to the protocols described by the manufacturer. Briefly, 25 μL of standard solutions, low and high controls, and samples were added to wells pre-coated with mouse monoclonal CK18 antibody M5 as catcher antibody. Then, 75 μL of the diluted horseradish peroxidase (HRP)-conjugated monoclonal antibody M30 as detector was added. The samples were then incubated for 4 hours at room temperature with constant shaking, after which excess unbound conjugate was removed by five washing steps. Color development was then achieved by the addition of 200 μL of TMB (3, 3', 5, 5'-Tetramethyl benzidine) solution, and incubation for 20 minutes in the dark. The reaction was stopped by the addition of 50 μL of stop solution and the absorbance measured in ELISA reader (Anthos 2020, Australia) at 450 nm. Total level of ccCK18 in the samples was measured through plotting a standard curve of known concentrations of the antigen against absorbance. With respect to M65 ELISA, monoclonal M5 and M6 antibodies directed against two different epitopes of CK18 in both its intact and caspase-cleaved forms was used. Twenty five μL of standard solution, controls and samples were added to wells pre-coated with mouse monoclonal CK18 antibody M6, followed by addition of diluted HRP-conjugated monoclonal antibody M5 as detector. The procedure was continued as described for M30 ELISA method and then CK18 quantity was calculated through plotting the standard curve. Correlation between ccCK18 (M30) and CK18 (M65) levels and clinical characteristics of the patients were evaluated.

3.4. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad software, San Diego, CA, USA). P values of ≤ 0.05 were considered statistically significant. M30 and M65 levels were presented on the basis of mean \pm standard error. Relationship between M30 or M65 levels in pre- and post-operative sera were analyzed by paired t-test two-tailed with Wilcoxon signed rank test. Spearman correlation test was used when determining an association between either M30 or M65 and age, and Mann-Whitney test was used for the relation between M30 or M65 level or M30:M65 ratio with gender or when comparing the markers between two groups. When there were more than two groups, the nonparametric Kruskal-Wallis was performed to test for significant differences among the groups. To compare M30 and M65 pre-operative and post-operative levels, unpaired t-test with Welch's correction was performed.

4. Results

Sera from 60 patients with TCC of the bladder were assessed for M30 and M65 serum levels. Patients' characteristics are shown in [Table 1](#).

4.1. Pre-Operative Serum M30 and M65 Levels

A wide range of M30 values (25 - 724 U/L) was observed in total patients, with a mean of 148 ± 16 U/L (median: 120 U/L; [Figure 1](#)). There was no significant difference between M30 levels in males (148 ± 16 U/L, median: 118 U/L) and females (152 ± 50 U/L, median: 122 U/L). Patients were categorized into three groups according to age: 35 - 49, 50 - 69 and ≥ 70 years. M30 levels showed no correlation with age.

M65 levels also showed a wide range of values (80 - 1375 U/L) with a mean of 318 ± 34 U/L (median: 230 U/L). The M65 level in males was 305 ± 35 U/L compared to females (376 ± 108 U/L; $P = 0.53$). The level of this molecule significantly correlated with age ($P = 0.03$, Spearman $r = 0.27$) and appeared to increase with age.

As shown in [Table 1](#), the majority of patients had stages T1 and T2 (61.7 %) and grades III and IV (55%) tumors. [Figure 2](#) shows the results of M30 and M65 analyses in patients according to tumor stage. As shown, there were no significant differences between M30 levels in patients with different tumor stages. Similarly, the level of M30 showed no significant difference in patients with various tumor grades ([Figure 3](#)). In contrast to M30, M65 levels showed a significant relation to tumor stage and grade. As shown in [Figure 2](#), the quantity of this molecule in patients with T3/T4 stages (350 ± 42 U/L) was higher than patients with T1/T2 stages (293 ± 45 U/L; $P < 0.038$). Patients with tumor grades

Table 1. Demographic and Clinical Characteristics of the Patients (N = 60)

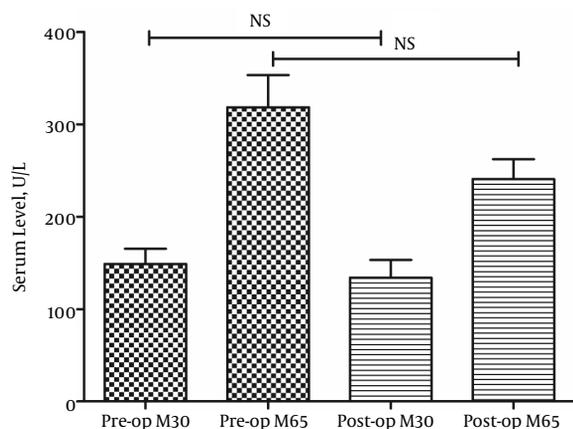
Features	No. (%)
Gender	
Male	49 (81.7)
Female	11 (18.3)
Age, y	
35 - 49	10 (16.7)
50 - 69	29 (48.3)
70 - 85	21 (35)
Stages	
T1	26 (43.4)
T2	11 (18.3)
T3	11 (18.3)
T4	3 (5)
ND	9 (15)
Grades	
I	7 (11.7)
II	15 (25)
III	9 (15)
IV	24 (40)
ND	5 (8.3)

Abbreviation: ND, not determined.

III/IV had higher levels of M65 (383 ± 58 U/L) compared to those with grades I/II tumors (231 ± 26 U/L; $P = 0.04$; [Figure 3](#)).

4.2. Pre-Operative M30:M65 Ratio

We determined the ratio of M30 to M65 for each patient. As shown in [Figure 4](#), this ratio for all patients was 0.54 ± 0.04 . There was no significant difference in ratio between males (0.55 ± 0.05) and females (0.46 ± 0.09). The M30:M65 ratio showed a significant difference between the age groups. This ratio was 0.84 ± 0.20 for patients aged 35 - 49 years, 0.53 ± 0.04 for 50-69 year-old patients and 0.41 ± 0.04 for patients ≥ 70 years of age ($P < 0.02$). There was a significant difference in M30:M65 ratio among patients with different tumor stages. This ratio was 0.61 ± 0.04 in patients with stage T1, which decreased to 0.26 ± 0.008 in patients with stage T4 disease ($P < 0.002$). As shown in [Figure 5](#), the M30:M65 ratio was lower in patients with T3/T4 stages (0.38 ± 0.04) compared to those with T1/T2 stages (0.59 ± 0.06 ; $P < 0.002$). Similarly, we observed a lower M30:M65 ratio in patients with grades III/IV (0.48 ± 0.07) compared to grades I/II tumors (0.64 ± 0.04 ; $P < 0.01$; [Figure 5](#)).

Figure 1. Serum Levels of Pre-Operative (op) and Post-Operative M30 and M65 Molecules in TCC Patients

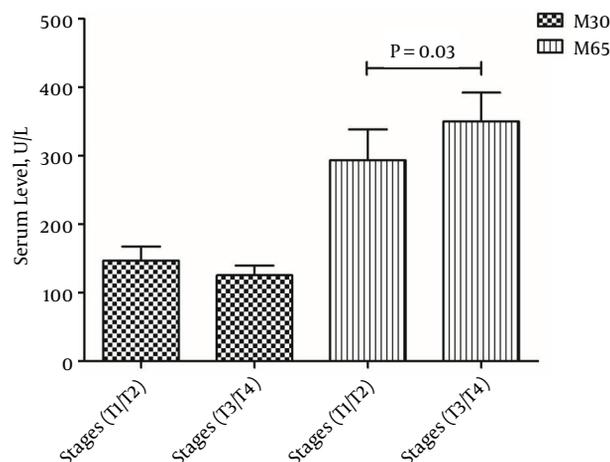
The levels of the soluble M30 and M65 molecules were determined in patients sera pre- (n = 60) and post-operation (n = 26) by ELISA method. Differences between pre- and post-operative M30 and pre- and post-operative M65 were not significant (NS). Data are presented as mean ± standard error.

4.3. Post-Operative Serum M30 and M65 Levels

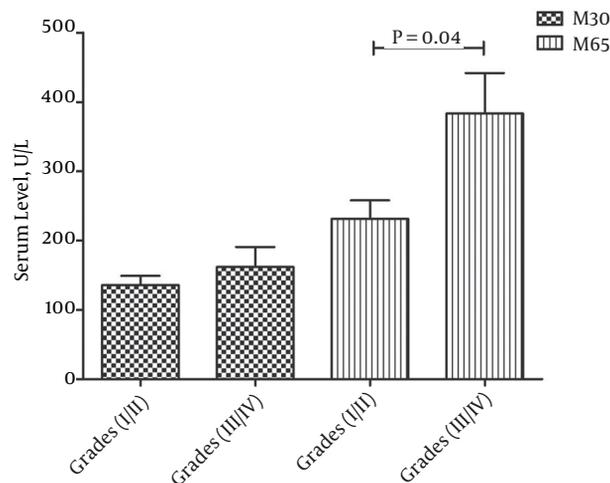
Samples of 26 patients after surgery were available for measuring M30 and M65 levels. As shown in Figure 1, the M30 level in patients after surgery was 133 ± 19 U/L which showed no significant difference with its pre-operative value (148 ± 30 U/L) in this group of patients. The corresponding value for post-operative M65 levels was 240 ± 21 U/L compared to its pre-operative level (319 ± 63 U/L). There was a correlation between pre-operative M30 and M65 ($P < 0.0001$, Spearman $r = 0.51$ Figure 6A) and post-operative M30 and M65 values ($P < 0.02$, Spearman $r = 0.45$; Figure 6B). However, we found no significant difference in M30 or M65 levels before and after surgery. The M30:M65 ratios before and after surgery showed a significant correlation in older patients (≥ 70 years; $P = 0.009$).

5. Discussion

In the present study we have examined sera from TCC patients for levels of circulating M30 and M65. During apoptosis CK18 is cleaved by caspases and subsequently

Figure 2. Serum Levels of Pre-Operative M30 and M65 Molecules in TCC Patients in Relation to Tumor Stage

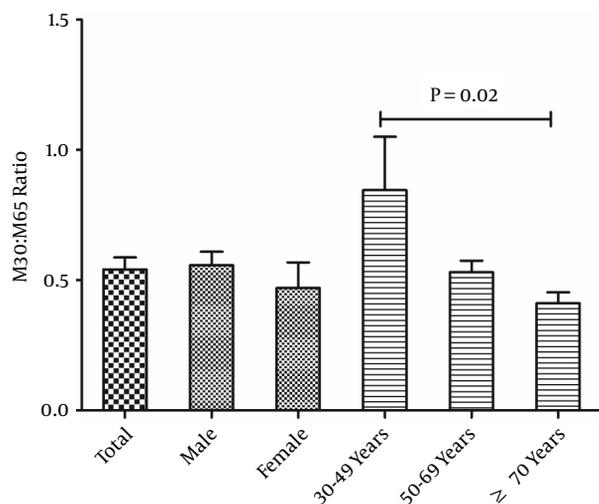
The levels of the soluble M30 and M65 molecules were determined by ELISA method. The M65 but not M30 levels was significantly higher in group of patients with higher tumor stages ($P = 0.038$). Data are presented as mean ± standard error.

Figure 3. Serum Levels of Pre-Operative M30 and M65 Molecules in TCC Patients in Relation to Tumor Grade

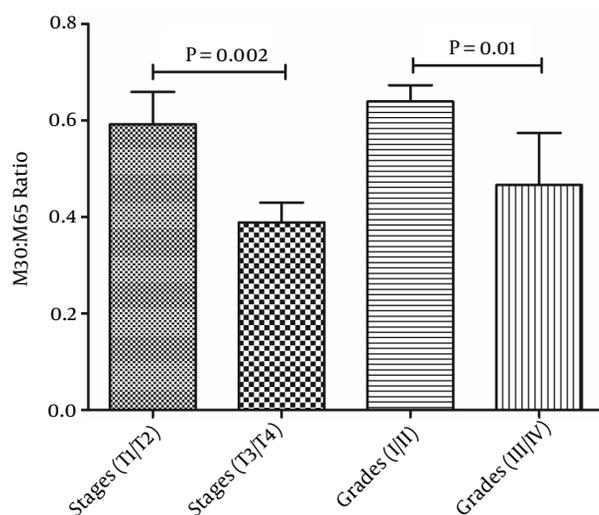
The levels of the soluble M30 and M65 molecules were determined by ELISA method. The M65 but not M30 levels was significantly higher in group of patients with higher tumor grades ($P = 0.04$). Data are presented as mean ± standard error.

released into the extracellular environment and blood (9, 29). These fragments can be detected by ELISA using the M30 monoclonal antibody which recognizes the CK18Asp396 neo-epitope. Therefore, M30 can be postulated to be a selective apoptotic marker.

The M65 assay is based on two antibodies, M5 and M6, directed against two different epitopes of CK18. All CK18

Figure 4. Pre-Operative M30:M65 Ratio in TCC Patients in Relation to Sex and Age

Serum levels of M30 and M65 molecules were determined by ELISA method. Data presented the mean M30:M65 ratio \pm standard error in all patients according to sex and age. A significant higher M30:M65 ratio in younger group of patients (30 - 49 years) vs. older ones (\geq 70 years) was observed ($P = 0.02$).

Figure 5. Pre-Operative M30:M65 Ratio in TCC Patients in Relation to Different Tumor Stages and Grades

Serum levels of M30 and M65 molecules were determined by ELISA method. Data presented the mean M30:M65 ratio \pm standard error in patients with different stages and grades. A significant lower M30:M65 ratio in patients with higher stages ($P = 0.002$) and grades of TCC ($P = 0.01$) was observed.

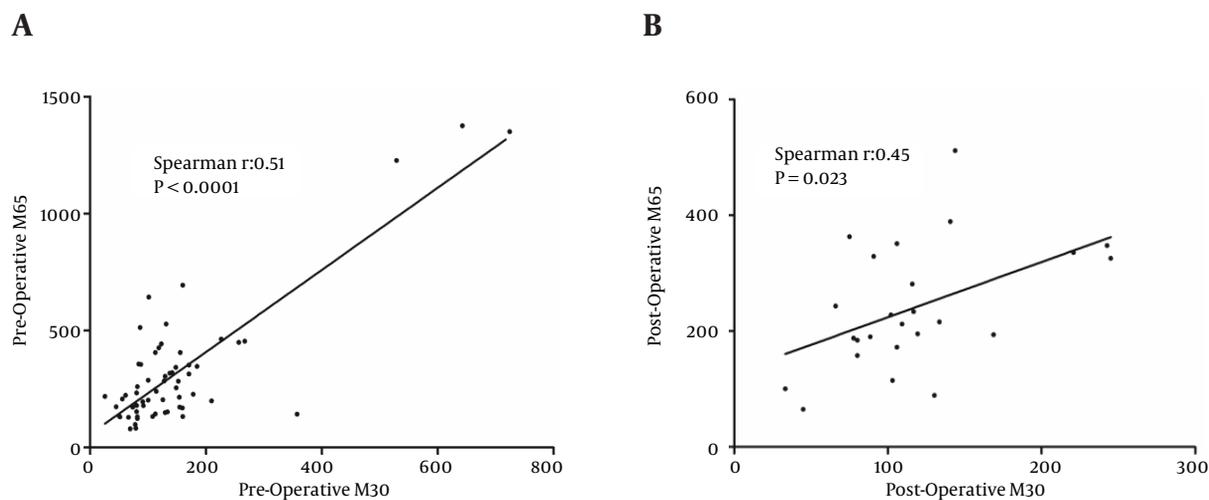
fragments that contain epitopes in the 300 to 390 amino acid region of the protein are recognized. In addition to apoptosis, M65 is thought to measure intact CK18 released during cell necrosis (30-32). Therefore, CK18 lev-

els can be considered as a surrogate marker of cell death activity in tumors and non-tumor conditions. Determining its level in patients can be useful for diagnosis of tumor recurrence, prognosis and monitoring. Additionally, in some experiments circulating CK18 has been used to assess the efficiency of different anticancer drugs during chemotherapy (14). Olofsson et al. suggested that the CK18 marker could be useful for early prediction of the response to chemotherapy in breast cancer and a useful biomarker for clinical trials (33). Circulating CK18 was considered as a biomarker of chemotherapy-induced cell death in testicular cancer (23). Post-surgical plasma CK18 levels showed a correlation with tumor recurrence and presence of residual disease in colorectal cancer (34).

The first report of using CK18 as a diagnostic value in TCC patients was in 2002 by Ramazan Sekeroglu et al. (35). These researchers used a solid-phase two-site chemiluminescence assay to measure CK18 levels. The results suggested that serum CK18 could not be a diagnostic or screening tool in early stages of bladder cancer, but was helpful in diagnosis of higher tumor stages. Song et al. studied TCC of the bladder and benign bladder diseases. They determined that a significant relationship between urinary NMP 22, a tumor marker of bladder cancer, and CK18 levels existed which suggested that NMP22 and CK18 were useful markers for diagnosis and monitoring of TCC. Levels of urinary CK18 significantly differed according to pathological grade and stage of patients' tumors (36).

In the phase I study of intravesical adenoviral transduction of human bladder cells with human interferon- α (Ad-IFN- α) treatment in patients with bladder cancer (37), significant apoptosis and necrosis in the patients' tumors was observed. This study was the first to suggest that analysis of urinary M30 and M65 levels might be used as a potential surrogate biomarker for tumor cell death and prognosis after treatment of non-muscle invasive bladder cancer with any therapeutic agent.

However, to the best of our knowledge no studies examined both M30 and M65 levels in serum of TCC patients. In the current study, we evaluated M30 and M65 serum levels in a group of Iranian patients with TCC. We sought to have an insight regarding the relationship of these markers to patient characteristics and prognostic factors such as tumor stage and grade. Moreover, we measured the changes in the quantity of these markers in a number of our patients after surgery to determine their value for disease monitoring. The results of the study showed a significant correlation between M30 and M65 levels in patients prior to surgery. The levels of M65, but not M30, were significantly related to stage and grade of patients' tumors which emphasized the importance of cell necrosis in TCC biology. Higher levels of M65 in patients with

Figure 6. Correlation Between M30 and M65 Levels in Sera of TCC Patients

A significant correlation between pre-operative M30 and M65 levels ($P < 0.0001$, Spearman $r = 0.51$) and post-operative values ($P = 0.023$, Spearman $r = 0.45$) was observed.

greater stages and grades might suggest the relation of this biomarker with tumor progression. These results were consistent with results obtained by Ramazan Sekeroglu et al. who reported that serum CK18 could be helpful in the diagnosis of higher stage tumors (35). In previous studies, serum levels of M30 and/or M65 significantly correlated with tumor stage in breast (24) and colorectal cancers (38). Unlike these studies, Ozturk et al. observed no difference in serum M30 and M65 levels between patients with stages III and IV of locally advanced head and neck tumors (39). Ausch et al. showed that in colorectal cancer patients, despite the tendency for M65 to decrease with increasing tumor grade, differences between the groups did not reach statistical significance as with M30 (34).

We measured post-operative M30 and M65 levels to determine a possible relation of these marker levels with tumor burden. However M30 and M65 serum concentrations failed to show any decrease following tumor removal. This finding did not agree with the study by Koelink et al. (38) on colorectal cancer patients that showed good correlation with M30 and M65 levels in the plasma of patients before and after surgical resection. This inconsistency might be due to the type of tumor. In addition, we measured M30 and M65 levels only once after surgery; possibly, by measuring these markers at different time intervals and in a higher number of patients, different results would be obtained.

Various studies conducted on different tumors compared the extent of apoptosis to total cell death by calculating the M30:M65 ratio. This ratio might be an impor-

tant factor to select an appropriate treatment strategy for patients. The M30:M65 ratio decreased in endometrial cancer stages III and IV when compared with stage II, which indicated less apoptosis and/or more necrosis during tumor progression (14). In colorectal cancer (38) the M30:M65 ratio tended to decrease with tumor progression. Our results showed a relationship between this ratio and age, tumor stage and grade. The M30:M65 ratio was higher in younger patients compared to older patients. This finding was in line with the positive correlation obtained in this study between M65 levels and age which might suggest a predominance of apoptosis in younger patients versus necrosis in older patients. The pre-operative M30:M65 ratio has shown a tendency to decrease with increase in tumor stage and tumor grade. In this regard, because M30 is considered an indicator of apoptosis, it can be assumed that in TCC patients with more aggressive tumors the rate of apoptosis may be lower than those with less aggressive tumors and cell death is mostly due to necrosis in these types of tumors. We did not find a significant difference between the pre- and post-operative M30:M65 ratio in total patients ($P = 0.08$). However, a correlation between the pre- and post-operative ratio in patients ≥ 70 years was observed.

In conclusion, the serum levels of M65, but not M30, showed a significant correlation with stage and grade of patients' tumors. This suggested a relationship of this marker to tumor progression in TCC. The pre-operative M30:M65 ratio has shown a tendency to decrease with increase in tumor stage and tumor grade. The significantly decreased ratio after surgery in the older group of patients

may imply the importance of this ratio for tumor monitoring in this group of patients. Further studies on a larger number of patients along with follow-up of the patients for tumor recurrence and presence of residual disease will determine the exact value of these markers for TCC monitoring.

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Footnotes

Author Contribution: Study concept and design: Zahra Amirghofran and Abdolaziz Khezri; analysis and interpretation of data and drafting of the manuscript: Zahra Malek-Hosseini; critical revision of the manuscript for important intellectual content: Zahra Amirghofran.

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