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Quantitative comparison of immunohistochemical and PCR analysis of midkine expression in breast cancer types and serum midkine level

Hülya ÇETİN SORKUN[1,*], Metin AKBULUT[2], Yaşar ENLİ[3], Emre TEPELİ[4], Sevgi ÖZKAN[5], Ergün ERDEM[6]

[1]Denizli Health Services Vocational School, Pamukkale University, Denizli, Turkey
[2]Department of Pathology, Faculty of Medicine, Pamukkale University, Denizli, Turkey
[3]Department of Biochemistry, Faculty of Medicine, Pamukkale University, Denizli, Turkey
[4]Department of Medical Biology, Faculty of Medicine, Pamukkale University, Denizli, Turkey
[5]School of Health, Pamukkale University, Denizli, Turkey
[6]Department of General Surgery, Faculty of Medicine, Pamukkale University, Denizli, Turkey

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Background/aim: Midkine (MK), a heparin-binding growth factor, has an important role in cancer progression. The aim of this study was to determine MK expression in breast tissue and the preoperative and postoperative serum levels of patients with breast cancer.

Materials and methods: Sixty-one patients with breast cancer participated in our study. The MK serum levels were measured pre- and postoperatively for these patients. We also analyzed breast tissues of the 61 patients immunohistochemically. We examined serum midkine levels in 49 healthy volunteers.

Results: MK expression was observed in 44 (72.1%) of 61 breast cancer patients. In breast cancer patients the serum MK levels (3.68 ± 2.13 ng/mL (mean ± SD)) were significantly higher than in the control group (1.77 ± 0.38 ng/mL) before tumor removal (P = 0.000). After tumor removal, serum MK levels (2.47 ± 1.00 ng/mL) were significantly (P = 0.000) decreased according to preoperative levels. Increased serum levels of MK were related with tumor stages when clinical parameters were analyzed.

Conclusion: We found that increased serum MK levels and protein expressions were associated with the carcinogenesis of breast cancer. MK levels decreased after tumor removal. According to our findings, MK might be a useful tumor marker for patients with breast cancer.

Key words: Midkine, breast cancer, serum, immunohistochemistry, real-time quantitative PCR, ELISA

1. Introduction

Growth factors are proteins with low molecular weight that establish intercellular communication at both short and long distances. These proteins move in specific cells through their specific cell surface receptors and regulate cell differentiation, migration, survival, and proliferation (1).

It has been reported that there is a rise in more than one growth factor in malignant tumors and such factors play a role in tumor development, growth, invasion, angiogenesis, and metastasis. As one of the growth factors, midkine (MK) has been investigated recently in studies focusing on elucidating the process of carcinogenesis in various cancer types (2–5). The product of the human MK gene is a protein that is localized on the 11p11.2 chromosome; it consists of 143 amino acids, which are rich in histidines and cysteine, and weighs approximately 13 kDa (6–8). MK has been reported to be a retinoic acid-sensitive gene product and a heparin-binding growth factor in embryonic carcinoma cells (6,9). It has been argued that MK increases mitotic activity and causes some cells to turn malignant (10,11). It is said to be expressed at a minimum level in normal tissues but at a high level in many malignant and inflammatory diseases (12). MK expression has been reported to increase often and regularly in human cancer types and that it can serve as a tumor marker and a molecular target for cancer treatment (13). The serum MK level is said to increase considerably in the early stages of many cancer types (14).

Today, cancer is one of the major factors leading to human deaths. Lung, breast, prostate, and colorectal cancers constitute nearly half of all cancer deaths and new cancer cases. It is stated that there has been a decrease at a rate of 0.4% in the incidence of cancer every year from 2001 to 2010. This decrease in cancer incidence was found to occur in males while rates remained fixed in females.

* Correspondence: hcsorkun@pau.edu.tr

In fact, the incidence of breast cancer in women is said to increase (15). With its different histological manifestations, biological performance, clinical findings, and responses to treatment, breast cancer is a disease with malignancy having many pathological subtypes (16,17). Deaths due to breast cancer generally occur due to the growth of distant metastases that cannot be detected with the existing treatments (18). Approximately 69% of women who are diagnosed with advanced-stage breast cancer die within 5 years (19).

Breast cancer is seen in one of every four women. Our aim was to explore the presence of MK expression in cancerous breast tissues, which is thought to be a marker capable of detecting cancer at an early stage, through immunohistochemical and cDNA techniques and its serum levels.

2. Materials and methods

Included in the study were 110 individuals who presented to the General Surgery Breast Outpatient Clinic of the Faculty of Medicine of Pamukkale University between August 2013 and August 2014 and who volunteered. Care was taken to ensure that the subjects did not have any other disease (liver disorders, rheumatoid arthritis, diabetes, hypertension, etc.). Our patient group consisted of 61 subjects who were diagnosed with breast cancer. As study material, we used the subjects' tissue samples that were fixed in 10% formaldehyde solution and then embedded in paraffin blocks. Serum samples were obtained from blood samples that were taken before the patients diagnosed with breast cancer were operated on and from samples that were taken for routine examinations 15–30 days after the operation. The control group consisted of 49 female patients who underwent a breast operation for noncancerous reasons and had nontumoral breast tissues, and their preoperative serum samples were also obtained. The pathology reports of the patients containing data on their ages, menopause statuses, biopsy methods, and presence of any other disease as well as their information from the breast outpatient clinic were reviewed and such reports were used to determine the subject groups according to their tumor grades and stages.

2.1. Immunohistochemistry

Each case's samples with maximum microvessel density and minimum hemorrhage and necrosis were selected and were fixed in 10% formalin, and sections of 3–5 μm from paraffin-embedded tissue were placed on adhesive polylysine-coated slides. Sections were incubated in blocking solution to eliminate nonspecific immunoreactivity and then the anti-MK monoclonal antibody (mid green (C terminus) rabbit monoclonal antibody, Medical Biogen) was reacted with it. After removal of excess antibody by washing with PBS, the samples marked with horseradish peroxidase goat antimouse or antirabbit antibodies were incubated for 60 min. DAB substrate reaction can be monitored by the system and opposite sections were treated with a hematoxylin staining procedure (20,21). Samples were considered negative if less than 5% of the cells stained for MK. Weak positive was defined as 5%–25% staining, positive as 25%–50% staining, and strongly positive as more than 50% of cells stained positively in cytoplasm (22).

Each section was evaluated by two pathologists without patient identification information and clinical diagnosis was performed under a conventional light microscope.

2.2. RNA extraction and cDNA synthesis

Total RNA from formalin-fixed, paraffin-embedded (FFPE) sections of 4–5 μm was isolated using the RNeasy FFPE kit (QIAGEN, Germany) according to the manufacturer's instructions. Total RNA concentrations were measured with a biophotometer (Eppendorf, Germany) and 1 μg of RNA was used as a template for the synthesis of complementary DNA (cDNA) using the QuantiTect Reverse Transcription Kit (QIAGEN). Reverse transcription was carried out 42 °C for 15 min followed by incubation at 95 °C for 3 min. The cDNAs were stored at –20 °C until they were used as a template in real-time PCR.

2.2.1. Real-time quantitative PCR

Quantitation of the MK gene and an internal reference gene (β-actin) at mRNA level was done using a fluorescence-based real-time detection method (LightCycler 2.0, Roche, Germany). Final reaction volume for the analysis of MK expression at mRNA level was 20 μL: 2 μL of each primer (final concentration: 0.5 μM), 2 μL of probe (final concentration: 0.2 μM), 4 μL of 5X LightCycler TaqMan Master Mix, 5 μL of DNA or cDNA sample, and 5 μL of PCR-grade water. The cycling conditions were 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 1 s. All runs included one negative DNA or cDNA control consisting of DNase- and RNase-free water. MK expression at mRNA level was relatively quantified using β-actin in each tumor and the final results were obtained with LightCycler software (version 3).

2.3. Serum MK measurement

Serums were separated by centrifuging the blood samples for approximately 30 min after they were taken into plain gelled tubes. The serums were then stored at –20 °C immediately after they were separated until the time they would be studied. When the measurements were to be carried out, the frozen serums were melted at room temperature and 100 μL of these serums were used for measurements. After performing the procedure steps as instructed by the ELISA kit (Cusabio, China), readings were taken at 450 nm to measure MK levels in ng/mL.

2.4. Statistical analysis

Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation and categorical variables as numbers and percentages. Continuous variables were compared with the independent sample t-test or Mann–Whitney U test with one-way ANOVA. $P < 0.05$ was considered statistically significant for all tests.

3. Results

When the mean age of the 61 subjects in the patient group, which was 53.38 ± 13.74 years, was compared to that of the control group, which was 44.00 ± 12.89 years, it was statistically highly significant ($P = 0.000$). Two of our subjects diagnosed with cancer were males (3.3%).

Positive MK expression was seen in the breast tissues of 44 (72.1%) of the 61 cancer patients who took part in our study. As for immunohistochemical staining statuses of breast tissues, the breast tissue samples of 17 patients did not show any staining (Figure 1A); 17 showed weak positive (Figure 1B), 11 positive (Figure 2A) and 16 strongly positive staining (Figure 2B) (Table 1). When the MK expression in breast tissues was evaluated with respect to immunohistochemical staining status, no significant difference was found in terms of tumor diameter, lymph node metastasis, stage, nuclear grade, histological grade, lymphatic and neural invasion, or age.

When the MK expression at mRNA level was evaluated through real-time relative quantification in 40 samples chosen randomly, presence of expression at mRNA level was seen in 3 subjects (7.5%). The immunohistochemical characteristics of the breast tissues of the patients who were found to have mRNA expression were also positive. One patient had positive immunohistochemical expression (25%–50%) in her breast tissue and the other two patients had strongly positive expression (50%).

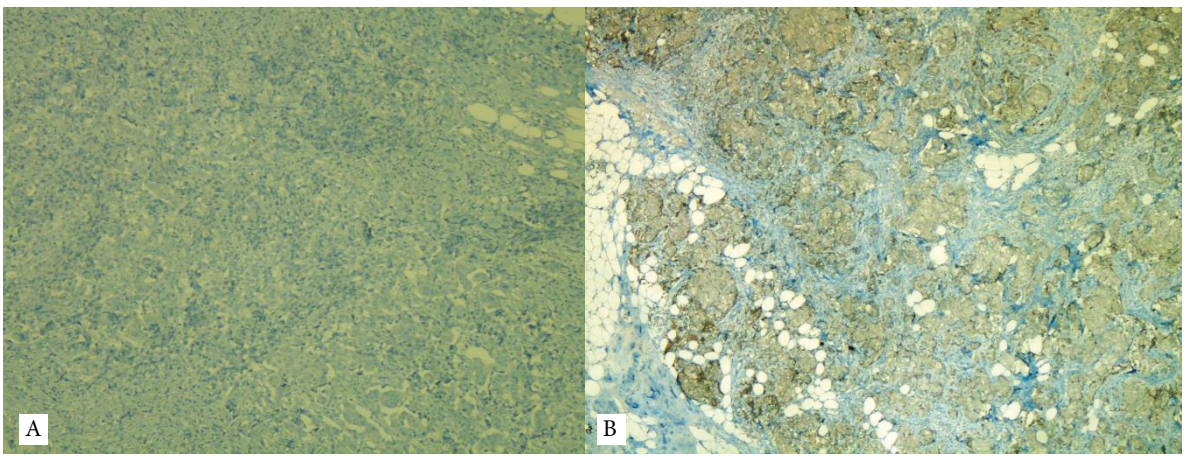

Figure 1. MK expression in breast tissues. Breast tissue samples that did not show immunohistochemical staining (A) 40× and that showed weak staining (B) 40×.

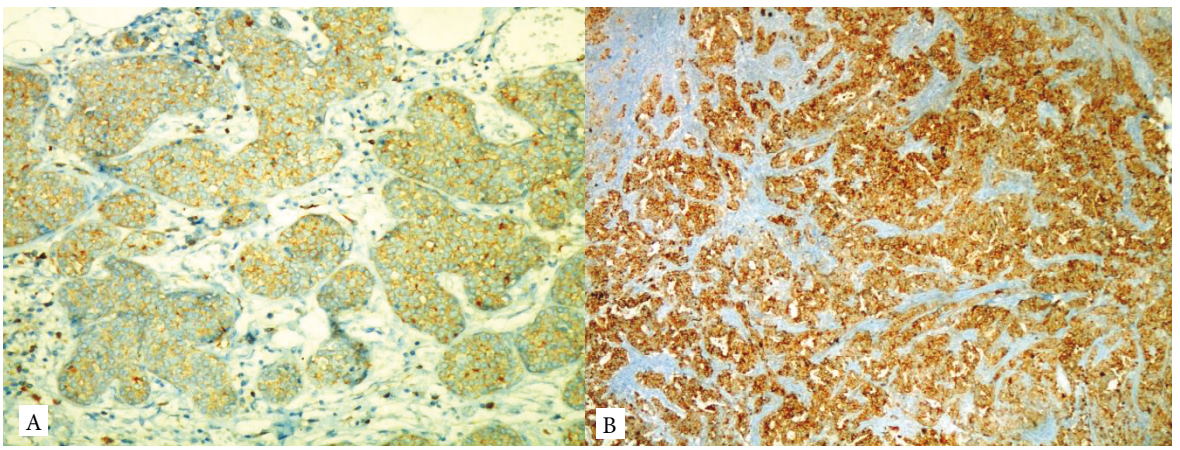

Figure 2. Samples that had positive (A) 100× and strongly positive (B) 40× immunohistochemical MK expression in breast tissues.

Table 1. Immunohistochemical staining statuses of breast cancer patients with respect to clinical parameters.

Clinical parameters		- (0%–5%)	+ (5%–25%)	++ (25%–50%)	+++ (>50%)	Total
Tumor size (cm)	≤2	5 (19.2%)	9 (19.2%)	4 (15.4%)	8 (30.8%)	26 (100%)
	>2	12 (34.3%)	8 (34.3%)	7 (20.0%)	8 (22.9%)	35 (100%)
Lymph node metastasis	-	8 (28.6%)	7 (25%)	5 (17.9%)	8 (28.6%)	28 (100%)
	+	9 (27.3%)	10 (30.3%)	6 (18.2%)	8 (24.2%)	33 (100%)
Stage	I	9 (24.3%)	10 (27%)	8 (21.6%)	10 (27%)	37 (100%)
	II	5 (25%)	6 (30%)	3 (15%)	6 (30%)	20 (100%)
	III	3 (75%)	1 (25%)	0 (0%)	0 (0%)	4 (100%)
Nuclear grade	2	3 (20%)	3 (20%)	5 (33.3%)	4 (26.7%)	15 (100%)
	3	14 (30.4%)	14 (30.4%)	6 (13.0%)	12 (26.1%)	46 (100%)
Histological grade	1	0 (0%)	2 (66.7%)	0 (0%)	1 (33.3%)	3 (100%)
	2	7 (25.9%)	8 (29.6%)	7 (25.9%)	5 (18.5%)	27 (100%)
	3	10 (32.3%)	7 (22.6%)	4 (12.9%)	10 (32.3%)	31 (100%)
Lymphatic and perineural invasion	-	9 (23.7%)	12 (31.6%)	9 (23.7%)	8 (21.1%)	38 (100%)
	+	8 (34.8%)	5 (21.7%)	2 (8.7%)	8 (34.8%)	23 (100%)
Age	<50	9 (23.1%)	12 (30.8%)	7 (17.9%)	11 (28.2%)	39 (100%)
	≥50	8 (36.4%)	5 (22.7%)	4 (18.2%)	5 (22.7%)	22 (100%)
Total		17	17	11	16	61

Staining statuses: 0%–5% negative (-), 5%–25% weak positive (+), 25%–50% positive (++), and >50% strongly positive (+++).

However, when the MK expression in breast tissues was evaluated immunohistochemically, we could not obtain any mRNA expression although we had other patients who had positive and strongly positive staining.

The mean serum MK level was 1.77 ± 0.38 ng/mL (mean ± SD) in the healthy individuals who took part in our study and formed the control group. When the individuals who formed the control group were assessed for their menopausal status, no difference was seen between the serum MK levels of the patients in menopause (1.65 ± 0.22 ng/mL) and those of the patients in premenopausal period (1.84 ± 0.44 ng/mL) (P = 0.978).

When we compared the mean preoperative serum MK level of the patient group (3.68 ± 2.13 ng/mL) with the mean serum MK level of the control group (1.77 ± 0.38 ng/mL), we found that the serum MK level of the patient group was considerably higher and it was statistically highly significant. A comparison of the serum MK level of the patient group before the operation (3.68 ± 2.13 ng/mL) with that after the removal of the tumor (2.47 ± 1.00 ng/mL) showed that postoperative MK level dropped and this was statistically highly significant (P = 0.000) (Figure 3). There was not any statistical significance when the preoperative serum MK levels of the patients who had a tumor diameter of 2 cm or less and of the patients who had a tumor diameter larger than 2 cm were compared (P = 0.924). Again, no statistical significance was found when the mean serum MK level of the patients who had lymph node metastasis was compared with the mean serum MK level of the patients who had no lymph node metastasis (Table 2).

When we grouped our patients by cancer stages, we had one patient at stage III and three patients at stage IV. Since the number of patients at these two stages was small, we considered them to be in the same group and grouped them to be at stage III. The serum MK levels of the patients who were at stage III before their tumors were removed were higher than the serum MK levels of the patients at both stage I and stage II and this was statistically significant. The postoperative serum MK levels were close to each other in all stages and this was not statistically significant. As there was only one nuclear grade stage 1 patient, she was included in stage 2, and thus nuclear grade stages 2 and 3 were assessed. When MK levels were analyzed in terms of nuclear and histological grades, no statistical significance was seen. When we explored the mean MK levels of the patients who had a lymphatic invasion and/or neural invasion, they showed an increase in those who had a lymphatic invasion and the increase was higher in those who had a neural invasion. The serum MK levels of

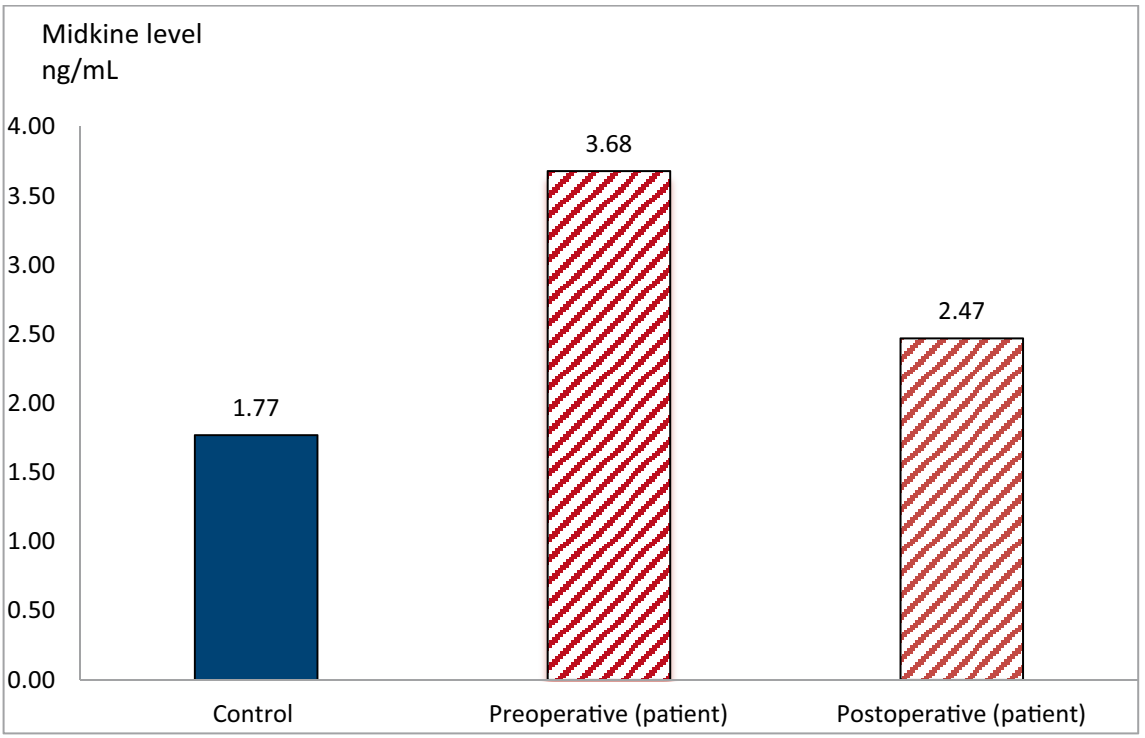

Figure 3. Serum MK levels of control and patients groups.

Table 2. Serum MK levels of breast cancer patients with respect to their clinical parameters.

Clinical parameters		(n)	*Preop. (ng/mL)	P-values	**Postop. (ng/mL)	P-values
Tumor size (cm)	≤2	26	3.71 ± 2.16	n.s.	2.64 ± 1.09	n.s.
	>2	35	3.65 ± 2.14		2.34 ± 0.93	
Lymph node metastasis	-	28	3.69 ± 2.26	n.s.	2.53 ± 1.00	n.s.
	+	33	3.66 ± 2.06		2.41 ± 1.02	
Stage	I	37	3.71 ± 2.09	I-III, P = 0.050	2.57 ± 1.03	n.s.
	II	20	3.09 ± 1.49	I-II, P = 0.520	2.26 ± 0.96	
	III	4	6.29 ± 3.61	II-III, P = 0.016	2.58 ± 1.10	
Nuclear grade	2	15	3.86 ± 1.84	n.s.	2.37 ± 0.89	n.s.
	3	46	3.62 ± 2.24		2.50 ± 1.05	
Histological grade	1	3	2.52 ± 0.39	n.s.	2.04 ± 0.20	n.s.
	2	27	3.77 ± 1.78		2.60 ± 1.17	
	3	31	3.71 ± 2.50		2.39 ± 0.88	
Lymphatic and perineural invasion	–	32	3.37 ± 1.98	n.s.	2.28 ± 0.80	n.s.
	Lymph +	14	3.68 ± 1.85		2.86 ± 1.32	
	Neural +	6	4.22 ± 2.12		3.02 ± 1.15	
	Lymph+neur+	9	4.40 ± 3.06		2.17 ± 0.85	
Age	<50	39	3.80 ± 2.31	n.s.	2.48 ± 1.05	n.s.
	≥50	22	3.46 ± 1.81		2.44 ± 0.94	

(Lymph+neur+: lymph and perineural invasion +). n.s.: not significant (P > 0.05).
*Preop.: preoperative mean serum MK levels ± SD; **Postop.: postoperative mean serum MK levels ± SD.

the patients who had both lymphatic and neural invasions were the highest. The serum MK levels of the patients who did not have any lymphatic or neural invasion were the lowest, but when they were compared with those of the patients who had lymphatic and neural invasions, this was not statistically significant (Table 2).

Our patients who were diagnosed with breast cancer had one or more of the following breast cancer types: invasive ductal carcinoma (IDC), ductal carcinoma in situ (DCIS), lobular carcinoma in situ (LCIS), and mucinous carcinoma (Mca). There were 18 (29.5%) patients who had IDC alone and 32 (52.5%) patients who had both the IDC and DCIS types. All three types of IDC, DCIS, and LCIS were found in 2 (3.3%) patients. IDC and DCIS together with Mca were seen in 3 (4.9%) patients. One (1.6%) person had Mca, 2 (3.3%) had LCIS, and 2 (3.3%) had DCIS types of breast cancer. There was 1 (1.6%) patient who had both IDC and Mca types.

We assessed our patients as the IDC and DCIS groups with respect to their breast cancer types. In the IDC group, the serum MK levels of the patients who had no lymph node metastasis were lower than those of the patients who had lymph node metastasis, but this was not statistically significant. The mean serum MK level of the DCIS group patients was higher than that of the IDC group patients with or without lymph node metastasis. While a significant drop was seen in the postoperative mean serum MK level of the DCIS patients, the drop in the serum MK level of IDC patients was less (Table 3).

4. Discussion

When expressed by tumor cells and/or stromal cells, growth factors contribute to a large extent to tumor formation and development. A large number of studies have shown that the level of growth factors in circulation increases in cancer patients compared to healthy individuals and such growth factors may stimulate focuses of metastasis in an endocrinal way (23). MK is a multifunctional growth hormone that can induce various effects in the targeted cells including nerve cells, neutrophils, macrophages, smooth muscle cells, fibroblasts, and tumor cells. It plays a role in tumor development, growth, invasion, angiogenesis, and metastasis (4,5). For this reason, some researchers have focused on investigating the MK expression as one of the growth factors and on the relationship of this expression with tumor types and sizes and its serum values (13,14,20,22,24).

MK mRNA and protein expressions have been frequently seen to rise in many human carcinomas such as lung, breast, stomach, colorectal, urinary bladder, prostate, glioblastoma, neuroblastoma, and Wilms tumors (25–31). It has been reported that MK expression is higher in cancer tissues than in normal cervical tissues and 88.1% of cancer tissues show reaction to anti-MK antibody (22).

MK expression was seen in 78.6% of osteosarcoma tissue samples (32), 54% of samples from patients with gastrointestinal stromal tumors (20), 89% of samples from patients with thyroid invasive papillary carcinoma (33), and 76.4% of gastric cardiac adenocarcinoma samples (34). It has also been reported that excessive MK is secreted in cancerous breast tissues (35) and positive MK expression is seen in 86% of IDC breast cancer cases (21). In our study, we found MK expression in 44 (72.1%) of the breast tissue samples of 61 breast cancer patients through the immunohistochemical method. We observed immunohistochemical MK expression in 42 breast tissues (73.6%) from 58 IDC cases. The immunohistochemical findings of our study are similar to those of previous studies.

It has been stated that in cervical cancer cases MK protein expression is associated with cancer stages, histology, and tumor diameter (22), and there is no relation between tumor diameter and MK expression in gastrointestinal stromal tumors but it has an important association with proliferative index (20). Qin et al. pointed out that high MK expression in breast cancer is associated with lymph node metastasis and TNM stages but not with age, menopausal status, or tumor diameter (21). We found

Table 3. Pre- and postoperative mean serum MK levels and immunohistochemical staining statuses of breast cancer patients with respect to their cancer types.

Breast cancer types	n	*Preop. (ng/mL)	P-values	**Postop. (ng/mL)	P-values	IHC -	IHC +
IDC lymph node metastasis -	25	3.50 ± 1.67		2.61 ± 1.04		7 (28%)	18 (72%)
IDC lymph node metastasis +	33	3.66 ± 2.06	P = 0.611	2.41 ± 1.02	P = 0.617	9 (27.3%)	24 (72.7%)
DCIS lymph node metastasis -	3	5.26 ± 5.63		1.93 ± 0.08		1 (33.3%)	2 (66.7%)

*Preop.: preoperative mean serum MK levels ± SD.
**Postop.: postoperative mean serum MK levels ± SD.

in our study that when MK expression was assessed in an immunohistochemical way in the cancerous breast tissues with respect to the patients' clinical parameters, it did not show any statistical significance.

We were able to find expression of MK at mRNA level in only 3 (7.5%) of the randomly selected 40 subjects in our study. Moon et al. found the mRNA expression of MK at a rate of 88.1% in patients with cervical cancer (22). We think that the reason for finding a low mRNA expression in our study is that the sample tissues studied were obtained from paraffin blocks. Obtaining the mRNA expression level from fresh tissues of the patients with breast cancer could have allowed us to reach more accurate results.

It has been reported that since MK is a secretion protein, it can also be found in the peripheral blood of patients who have tumors that release a lot of MK. The MK expression in esophageal carcinoma tissue has a significant relationship with its increase in serum level (5,36,37).

It has been stated that the plasma MK level is higher in patients with breast cancer when compared to controls and the MK level is significantly higher in metastatic cancer patients than the levels in primary invasive cancer and DCIS. However, it has been reported that there is no significant difference with respect to tumor diameter, nodal status, clinical stage, vascular invasion, hormone receptor status, and HER2 status (24). It was reported in the study of Ikematsu et al. that the serum MK level of cancer patients was higher than that of the control group. However, it was also reported that the increase in the serum MK level in various cancer types was not associated with tumor diameter or stage. They reported that the serum MK level increased even at an early stage (stage I) in patients with stomach cancer and lung cancer. It was observed that after surgically removing the tumor tissues of 5 patients with liver cancer, the serum MK level dropped significantly in 4 patients (14). When we compared the serum MK levels of healthy individuals and our patients diagnosed with cancer in our study, we found that it was statistically highly significantly different. When we measured the postoperative serum MK levels of our patients, we saw that they decreased considerably and there was statistical significance. When we examined our patients with respect to their stages, we found that the mean serum MK level of the stage III group was significantly higher than those of the stage I group and stage II group. The mean serum MK levels of the patients in stages I and II were significantly higher than the mean serum MK level of healthy individuals. Our study is similar in this respect to the study of Krzystek-Korpacka et al., who stated that the MK level went up depending on the cancer stages in patients with colorectal cancer and it was significantly high even at an early stage. It was also reported in the same study that the serum MK level was higher in patients with lymph node metastasis (38). Although we also found in our study that the serum MK level was higher in patients with lymph node metastasis, we did not find this to be statistically significant. The serum MK level also did not show any significance with respect to tumor diameter, age, histological and nuclear grade, or lymphatic and neural invasions. The serum MK levels of the IDC patients who had lymph node metastasis were high in our study, but this was not statistically significant. In this respect, our study differs from that of Tanabe et al., who stated that the serum MK level was significantly correlated with lymph node metastasis in endometrial carcinoma (39). Nevertheless, the fact that the postoperative decrease in the serum MK levels was low in our IDC patients suggests that metastasis may have occurred in these patients.

In conclusion, it has been seen in our study and other studies that there is an increase in MK expression in the presence of tumors. The fact that this expression increases at the serum level even at early stages and that there is a decrease in the serum MK level after the tumor has been removed shows that MK can be used as a cancer marker. However, we found in our study that the postoperative serum MK level dropped only a little in some of the patients. There is a need for further studies that will monitor patients who had serum MK levels that did not drop and investigate whether or not such serum MK levels that remained high were associated with metastasis. Such studies to be carried out will provide important contributions to the use of MK as a cancer marker.

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