

Research Article,

MTHFD2 Expression Profile and Its Prognostic Importance in Invasive Breast Carcinoma

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Abstract:

Background: Breast cancer is the most common cancer among women. Therefore, it is very important to determine prognostic parameters and biomarkers related to these parameters. Recent studies have shown that folate metabolism is effective on cell division and invasiveness. One of the most important regulators of folate metabolism is the methylene tetrahydrofolate dehydrogenase enzyme (MTHFD2).

Method: -This study, immunohistochemical staining with MTHFD2 was performed in 194 cases diagnosed with invasive breast carcinoma. The relationship between the immunohistochemical expression of MTHFD2 in normal ductal areas and invasive tumoral areas of these cases and some clinicopathological parameters was investigated.

Results: While no expression was observed in non-tumor areas with MTHFD2, varying degrees of expression were observed in all tumor areas ($p < 0.0001$). Significant correlations were also observed between important clinicopathological and prognostic parameters such as MTHFD2 nuclear grade, hormonal receptor, Ki67 and etc. (all $p < 0.05$).

Conclusion: -High MTHFD2 expression in breast carcinomas with invasive and poor prognostic values was determined to be very significant. Therefore, it can be predicted that detecting and lowering MTHFD2 levels in these cases may prevent tumor development, invasion and metastasis. We think that more studies on the subject are needed, including the development of chemotherapeutics targeting MTHFD2.

Keywords: Breast carcinoma; MTHFD2; prognosis

Introduction:

Cancer detection and related deaths are increasing across the world day by day. Breast cancer is the most common cancer among women. It accounts for 11.9% of all cancers and 6.6% of deaths each year. Significant survival times are achieved with early diagnosis and treatment. Tumor type, grade, stage, hormone receptor status, HER2 expression, lymphovascular invasion, and lymph node involvement are important prognostic values in the diagnosis and treatment of breast cancer [1,2].

Folate metabolism plays an important role in the proliferation and invasiveness of tumoral cells. MTHFD2 enzyme initiates protein synthesis by

transporting single carbon units from serine to tetrahydrofolate in folate metabolism. It is a mitochondrial enzyme. One-carbon metabolism usually takes place in the mitochondria and cytosol [3,4]. Cancer cells show rapid cell proliferation. Mitochondrial folate metabolism is thought to be important for the proliferation of cancer cells. The single carbon units required for this proliferation are transported by the mitochondrial MTHFD2 [5,6].

It has been reported in many studies that the MTHFD2 enzyme is highly expressed in various human tumors and is necessary for the survival of cancer cells [4,7]. On the other hand, sudden

decreases in MTHFD2 levels have been shown to cause embryonic death in pregnant mice. This finding indicates that MTHFD2 is indispensable for healthy embryonic development. All these reasons make MTHFD2 interesting for cancer research. [8,9]. Furthermore, there are many researchers suggesting that MTHFD2 reaches high levels in cancer cells and indicates poor prognosis [10-12]. However, there are a few studies have investigated the important role of MTHFD2 in breast cancer in the literature [13]. MTHFD2 has also been associated with poor prognosis in breast cancer [13]

So that we aimed to determine the MTHFD2 expression status in tumor and normal breast tissues in cases diagnosed with breast cancer and investigate the relationship between the expression of this enzyme and important clinicopathological data, such as tumor type, nuclear grade, histological grade, hormone receptors (ER, PR), HER2, Ki67 proliferation index, lymphovascular invasion, tumor size, lymph node metastasis, and pathological stage

Materials and Methods:

Patients' General Information and Tissue Specimens

After receiving approval from the ethics committee of Atatürk University Medical Faculty (Date: 16.01.2020, number: 24), all radical mastectomy materials examined between 2018 and 2020 were retrospectively investigated. Patients with missing clinical data at the time of diagnosis and those for whom paraffin slides could not be reached were excluded from the study, and a total of 194 patients were selected for the final evaluation. The clinical information of the patients was obtained from the automation system of our hospital. Prognostic parameters, such as macroscopic tumor size, histological subtype, lymphovascular invasion, perineural invasion, primary tumor (pT), lymph node metastasis (pN), distant organ metastasis (pM), hormone receptor status, and survival rates were obtained for each patient and recorded. The pathology reports, slides and paraffin blocks of the patients were obtained from our archive, and paraffin blocks containing adjacent non-cancerous tissues were selected for the immunohistochemical analysis.

Immunohistochemistry

Five micron sections were taken from the appropriate blocks where the tumor was most dense, and after drying at 70 degrees for 15 minutes, they were placed in the Roche Ventana automatic immunohistochemistry staining device. Tissues were deparaffinized and dehydrated in this device. Then the tissues were treated with Ultra Cell Conditioning Solution, hydrogen peroxide and treated with MTHFD2 antibodies (MTHFD2 antibody, ab56772; 1:100 dilution; mouse monoclonal Abcam, Cambridge, MA, USA).

Immunohistochemical staining was evaluated by two pathologists as described previously [9]. The adjacent tumor-free areas were accepted as negative controls. Nuclear and/or cytoplasmic staining was considered to indicate MTHFD2 positivity. The staining rate of MTHFD2 was evaluated as Grade 0 [no staining], Grade 1 (1-25% staining), Grade 2 (26-50% staining), Grade 3 (55-75% staining), and Grade 3 (76-100% staining). In addition, the staining intensity of MTHFD2 was classified as Grade 0 (no staining), Grade 1 (weak cytoplasmic and nuclear staining), Grade 2 (moderate cytoplasmic and nuclear staining), and Grade 3 (strong cytoplasmic and nuclear staining) (Figure 1). The Immunoreactivity score (HistoScore) was determined from the results obtained by multiplying the extent of staining with the intensity of staining. The histoScore values for MTHFD2 were evaluated as follows: negative, Grade 0; 1-3, Grade 1; 4-6, Grade 2; and 7-9, Grade 3 [12].

Statistical analysis

Whether the data fit the normal distribution was investigated with the D'Agostino Pearson test. The data that were not normally distributed were presented as median values. The MTHFD2 levels in infiltrative areas and adjacent non-cancerous tissues were examined with the chi-squared test. The correlations of the MTHFD2 expression with prognostic parameters were assessed using the Spearman correlation test. For the two-tailed p value, <0.05 was accepted as significant.

Results:

Patients' Demographic and Histopathological Features

The mean age of all cases included in the study was determined as 53 ± 9.13 years. The tumor location was the right breast for 93 patients, the left breast for 95, and bilateral in eight. The average macroscopic diameter of the tumor was determined

as 1.9 ± 1.8 cm. Four (2%) patients died within two years of follow-up. All the patients were treated with surgery and integrated chemotherapy and

radiotherapy. The survival rate ranged from four to 24 months. The demographic and histopathological features of the cases are presented in Table 1.

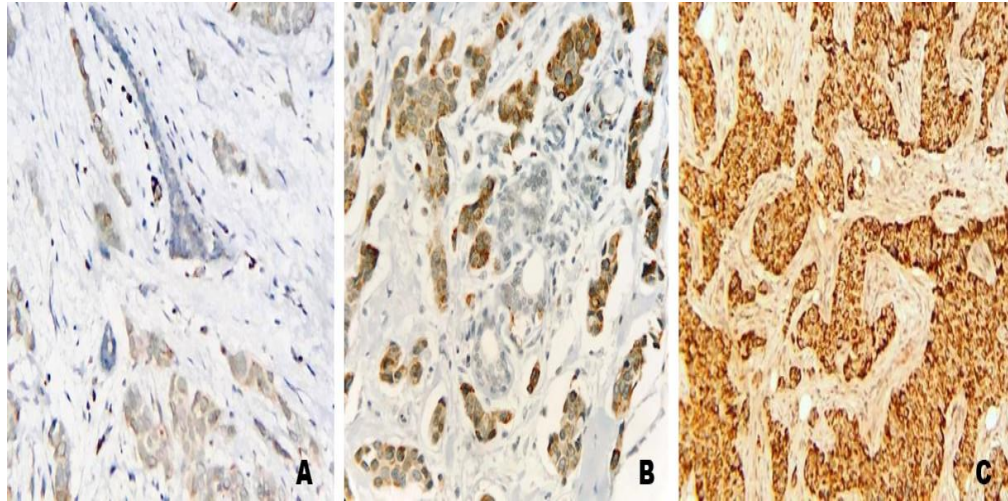


Figure 1-A: Histopathological view of MTHFD2x20 weak staining in infiltrative areas. 1-B: Moderate staining. 1-C: Strong Staining. MTHFD2 x20

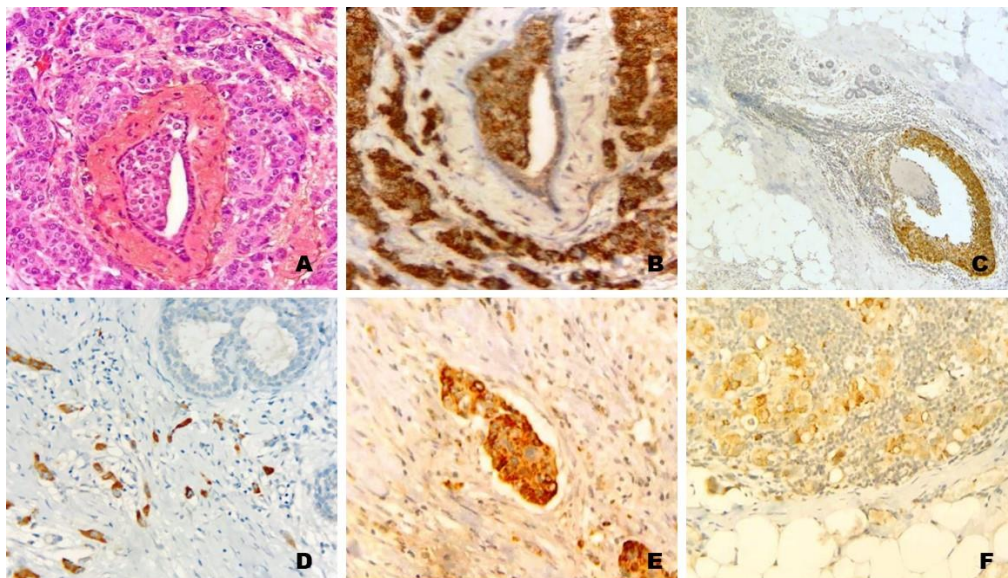


Figure 2- A and B: Invasive and in situ areas in breast cancer (A: H&Ex20, B:MTHFD2 x20), C: MTHFD2 expression is observed in the ductal carcinoma in situ area, but not in the non tumoral area D: MTHFD2x20 expression is observed in the tumoral area, but not in the non tumoral area. E: MTHFD2x20 expression in the area of lymphovascular invasion F: MTHFD2 x20 expression in the area of breast cancer metastasis in the lymph node

Table 1: Demographic and Histopathological Features of the Patients

| | Patients (n = 194) |
|---|---------------------------|
| Age | 53±9,13 |
| Macroscopic Tumor Volume (cm) (n%) | |
| ≤2 cm | 44 (22.6%) |
| 2-5 cm | 110 (56.7%) |
| ≥ 5 cm | 40 (20.6%) |
| Lymphovascular Invasion (n%) | |
| Not identified | 66 (34.0%) |
| Present | 128 (65.9%) |
| Histological Subtype (n%) | |
| Invasive ductal carcinoma | 158 (81.4%) |
| Invasive lobular carcinoma | 12 (6.1%) |
| Others | 24 (12.3%) |
| Nuclear Grade (n%) | |
| G1 | 0 |
| G2 | 122 (62.8%) |
| G3 | 72 (37.2%) |
| Histologic Grade (n %) | |
| Well differentiated (G1) | 0 |
| Moderately differentiated (G2) | 118 (60.8%) |
| Poorly differentiated (G2) | 76 (39.2%) |
| pT (n%) | |
| pTIS | 6 (3.0%) |
| pT1 | 48 (24.7%) |
| pT2 | 116 (59.8%) |
| pT3 | 14 (7.2%) |
| pT4 | 10 (5.1%) |
| pN (n %) | |
| pN0 | 82 (42.2%) |
| pN1 | 74 (38.1) |
| pN2 | 22 (11.3%) |
| pN3 | 16 (8.2%) |
| pM (n%) | |
| pM0 | 174 (89.7%) |
| pM1 | 20 (10.3%) |
| Outcome (n%) | |
| Survived | 190 (97.9%) |
| Died | 4 (2.1%) |
| Estrogen Receptor (n%) | |
| 0-1 (%) | 18 (9.3%) |
| 2-10 (%) | 12 (6.2%) |
| 11-100 (%) | 164 (84.5%) |
| Progesterone Receptor (n%) | |
| 0-1 (%) | 28 (14.4%) |
| 2-10 (%) | 24 (12.3%) |
| 11-100 (%) | 142 (73.2%) |
| HER-2 (n%) | |
| Negative (Score 0 and 1) | 90 (46.4%) |
| Equivocal (Score 2+) | 42 (21.6%) |
| Positive (Score 3+) | 62 (31.9%) |
| Ki67 Proliferation Index (n%) | |
| 0-10 (%) | 22 (11.3%) |
| 11-3 (%) | 56 (28.9%) |
| 31-100 (%) | 116 (59.8%) |

G: Grade, **pT:** Primary Tumor, **pN:** Lymph Node Metastasis, **pM:** Distant Organ Metastasis

Table 2: Relationship between Prognostic Factors and MTHFD-2 Expression (Spearman’s Correlation Test)

| | Histoscore | | | p r |
|------------------------------------|-------------------|------------------|-------------------|---------------|
| | HS 1 (n = 80) | HS 2 (n = 44) | HS 3 (n = 70) | |
| MTD (n) | | | | |
| ≤ 2 cm | 24 | 6 | 14 | 0,7079 |
| 2-5 cm | 40 | 24 | 46 | 0,0385 |
| ≥ 5 cm | 16 | 14 | 10 | |
| Lymphovascular Invasion (n) | | | | |
| Not identified | 38 | 10 | 18 | 0,0408 |
| Present | 42 | 34 | 52 | 0,208 |
| Histological Subtype | | | | |
| Invasive ductal carcinoma | 62 | 34 | 62 | |
| Lobular carcinoma | 8 | 4 | 0 | 0,9998 |
| Others | 10 | 6 | 8 | -0,0028 |
| Nuclear Grade (n) | | | | |
| G1 | 0 | 0 | 0 | |
| G2 | 62 | 24 | 36 | 0,0173 |
| G3 | 18 | 20 | 34 | 0,241 |
| Histological Grade (n) | | | | |
| Well differentiated (G1) | 0 | 0 | 0 | |
| Moderately differentiated (G2) | 62 | 24 | 32 | 0,0041 |
| Poorly differentiated (G2) | 18 | 20 | 38 | 0,289 |
| pT (n) | | | | |
| pTIS | 6 | 0 | 0 | |
| pT1 | 22 | 8 | 18 | |
| pT2 | 40 | 28 | 48 | 0,739 |
| pT3 | 6 | 6 | 2 | 0,0343 |
| pT4 | 6 | 2 | 2 | |
| pN (n) | | | | |
| pN0 | 36 | 12 | 34 | |
| pN1 | 32 | 28 | 14 | 0,639 |
| pN2 | 6 | 0 | 16 | 0,0482 |
| pN3 | 6 | 4 | 6 | |
| pM (n) | | | | |
| pM0 | 70 | 40 | 64 | 0,5744 |
| pM | 10 | 4 | 6 | 0,0577 |
| Outcome | | | | |
| Survived | 80 | 42 | 68 | 0,3650 |
| Died | 0 | 2 | 2 | 0,093 |
| Estrogen Receptor | | | | |
| 0-1% | 6 | 8 | 4 | 0,0071 |
| 2-10 % | 2 | 4 | 6 | -0,272 |
| 11-100 % | 72 | 32 | 60 | |
| Progesterone Receptor | | | | |
| 0 -1% | 8 | 8 | 6 | 0,0279 |
| 2 -10 % | 8 | 9 | 4 | -0,124 |
| 11-100 % | 60 | 30 | 61 | |
| HER2 | | | | |
| Negative (Score 0 and 1) | 52 | 10 | 28 | 0,0273 |
| Equivocal (Score 2+) | 16 | 12 | 14 | 0,224 |
| Positive (Score 3+) | 12 | 22 | 28 | |
| Ki67 Proliferation Index | | | | |
| 0-10 % | 16 | 2 | 4 | 0,0011 |
| 11-30 % | 28 | 8 | 20 | 0,326 |
| 31-100 % | 36 | 34 | 46 | |

MTHFD2: Metylenetetrahydrofolate dehydrogenase 2, HS: Histoscore, MTD: Macroscopic Tumor Diameter, pT: Primary Tumor, pN: Lymph Node Metastasis, pM: Distant Organ Metastasis

Prognostic significance of MTHFD2 expression in breast cancer

In all the cases, MTHFD2 positivity was determined in tumoral areas (100%), while there was no staining in adjacent nontumoral areas ($p < 0.0001$). Also, high expression with MTHFD2 was observed in all areas of carcinoma in situ, lymphovascular invasion and lymph node metastasis (Figure 2). The association between the MTHFD2 expression and clinicopathological parameters of the patients with breast cancer was assessed with the Spearman correlation analysis. The overexpression of MTHFD2 was statistically significantly correlated with the prognostic parameters of lymphovascular invasion, histological grade, nuclear grade, estrogen receptor status, HER-2 expression and Ki67 proliferation index ($p < 0.05$). However, there was no significant association between the MTHFD2 expression and perineural invasion ($p = 0.679$), age ($p = 0.379$), and the other parameters ($p > 0.05$). (Table 2).

For the survival analysis, According to their MTHFD2 expression, Histoscore 1 and 2 were accepted as having low level of MTHFD2 (Group 1) and histoscore 3 were accepted as high expression (Group 2). Kaplan-Meier analysis showed that the two-year survival rates were not significantly different between Groups 1 and 2 (1.61% and 2.86%, respectively, $p > 0.059$).

Discussion:

Breast cancer is the most common type of cancer among women. According to the GLOBOCAN 2020 data, it constitutes 24.5% of all cancers and 15.5% of cancer deaths in women [1]. It is known that histological type, axillary lymph node involvement, presence of lymphovascular invasion, tumor diameter, estrogen/progesterone receptor protein status, HER2 positivity, age, and histological grade are important prognostic parameters that guide the treatment process [2,14,15]. Tumor development in the breast is generally accepted as a multi-stage process that begins benign proliferation, followed by atypical proliferation and carcinoma insitu, further progressing to invasive cancer and metastases [16,17].

As in all cancers, the initiation and progression of breast cancer is under the control of various genetic changes.

Detection of tumor-associated antigenic molecules expressed in cancer tissues is extremely important

in the diagnosis and treatment of breast cancer. MTHFD2 is a bifunctional (metylenetetrahydrofolate dehydrogenase / cyclohydrolase) enzyme responsible for the transfer of single carbon units in folate metabolism. Mitochondrial folate metabolism is thought to be important for the proliferation of cancer cells. These cells show rapid cell proliferation [5].

Therefore MTHFD2 has received special attention in cancer research. It has been reported that MTHFD2 is required for cancer proliferation and may therefore play an important role in tumor development and progression. MTHFD2 is increased in various cancers, but is low or undetectable in developed normal adult tissues [9]. Furthermore, it has been suggested that MTHFD2 reaches high levels in cancer cells and indicates poor prognosis. There are also studies suggesting that a decrease in MTHFD2 in the environment will reduce tumor invasiveness and aggressiveness, and the viability of the tumor cell will disappear [18]. Nilson et al. reported that MTHFD2 protein levels are increased in colon, liver, and breast cancers. In this study, while MTHFD2 was negative or very low in the stroma adjacent to the tumor, it was observed to be high expression in malign cells in tumor and metastatic tissues.

[9]. Similar findings were found in our study. In another similar study, it was reported that high MTHFD2 increased colorectal cancer growth rate and metastasis, and rapid MTHFD2 destruction clearly inhibited tumor growth [10].

Miyo et al., evaluating 117 colorectal cancer cases, reported that the expression of mitochondrial folate metabolic pathway enzymes (MTHFD2 .etc), was increased in human colorectal tumor tissues compared to normal tissues. Also, patients with high enzyme levels had lower overall survival rates [11].

Lin et al. investigated the immunohistochemical expression of MTHFD2 and vimentin in 137 renal cell carcinoma (RCC) cases and showed that the overexpression of both was positively associated with stage, grade, and poor overall survival. The authors noted that after the expression of MTHFD2 was decreased in 786-O cells, there was a decrease in cell proliferation, migration and invasion, suggesting that MTHFD2 could be a potential target for the treatment RCC [12]. In another study conducted by Yu et al., it was stated that the knockdown of MTHFD2 caused a decrease in cell growth and tumor formation in vitro and in vivo. It is claimed that the inhibitory effect of MTHFD2

knockdown on non-small cell lung carcinomas may be mediated through suppression of cell cycle-related genes. [19].

Liu et al. detected a high level of MTHFD2 expression in the tumor tissues of patients with hepatocellular carcinoma (HCC) and found the MTHFD2 expression to be correlated with high stage, metastasis and recurrence. The authors concluded that MTHFD2 was overexpressed in HCC, and it indicated poor prognosis and cellular features associated with metastatic disease [20].

Selçuklu et al. reported that MTHFD2 was in a target position for miR-9 in breast cancer cells, and the knockdown of MTHFD2 increased miR-9, which, in turn, resulted in anti-proliferative, anti-invasive and pro-apoptotic activity. In the same study, tumors expressing MTHFD2 were shown to achieve a higher rate of distant metastasis and reduced survival than those with low or no MTHFD2 expression. It was suggested that the MTHFD2 protein might be an independent prognostic factor and a potential future target for breast cancer therapy [18].

Another group of studies showed that micro-RNA in tumor cells plays an anti-tumor role by inhibiting MTHFD2 expression, such as mir-940 [21], mir-504-3p [22] and mirRNA-92a [23].

Liu et al. showed that the MTHFD2 expression in tumoral areas was much higher than in paratumoral breast tissues. In the same study, they showed that tissue samples showing high MTHFD2 expression compared to tissue samples showing no or low MTHFD2 expression were correlated with tumor size, type, grade, involvement of lymph node, distant metastasis, ER, PR, HER2 [13].

In a study examining breast cancer cell lines, Huang et al. showed that the MTHFD2 level was higher in cancerous tissues compared to the adjacent normal tissues, and MTHFD2 increased the proliferation of cancer cells through the AKT signaling pathway. The authors suggested that MTHFD2 could be a therapeutic target to stop cell proliferation but also emphasized the need for further studies [24]. In another study, Lehtinen et al. showed that MTHFD2 was overexpressed in breast cancer, promoted cellular features associated with metastatic disease, thus suggesting that MTHFD2 was a potential drug target for blocking breast cancer cell migration and invasion [25].

The studies described above show that MTHFD2 is closely involved in the formation and development of tumors. However, data concerning the relationship between MTHFD2 expression and

tumor prognosis and/or clinicopathological parameters are still insufficient and inconclusive. It is clear that there is a need for further studies involving more cases and different organ tumors.

In the current study, the expression of MTHFD2 in the normal ductal epithelium and invasive tumoral areas of the breast was examined. While MTHFD2 was not expressed in the normal ductal epithelium, its expression was at varying degrees and intensity in invasive tumoral areas. On the other hand, it was also observed that there was a significant relationship between the MTHFD2 expression and important prognostic criteria, namely nuclear grade, histological grade, lymphovascular invasion, Ki67 proliferation index, ER and PR receptors, and HER2 staining. However, significant correlation was not observed between the MTHFD2 expression and other important prognostic parameters, such as age, tumor type, number of foci, tumor size, lymph node involvement, and pathological stage.

Inevitably, this study has certain limitations, with the major being the statistically small sample with limited tumor grades and diversity. In addition, investigation with more cases and follow-up period is required. On the other hand, we only used immunohistochemical staining, which is a semi-quantitative method, but other quantitative methods such as Western blot or PCR analysis can provide stronger evidence.

Conclusion:

A high level of MTHFD2 expression in tumor cells gives the tumor a high proliferation rate and invasive character in breast cancer. It is inevitable that this feature results in poor prognosis and is therefore associated with poor prognostic criteria. All these features make the MTHFD2 enzyme a controllable target for cancer development and progression. It can be predicted that the development of drugs that specifically target the reduction of MTHFD2 levels in tumoral cells will provide significant improvements in the course of breast cancer and survival times, as well as playing an important role in the prevention of recurrence. Therefore, there is a need for new studies involving more cases and various tumor morphologies.

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Disclosure Statement

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Author Contributions

RA: Study design, data collection, data analysis, immunohistochemical application and interpretation and etc

OC: Data collection, data analysis, statistical evaluation, shape editing, etc

Conflict of Interest (Disclosure): The authors declare that they have no conflict of interest.

Ethical statement

This research was ethically approved by the Ethics Committee of Atatürk University Faculty of Medicine with the decision number 16.01.2020/24

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