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ORIGINAL ARTICLE

The role of PEDF, VEGF, and Ki-67 in tissue invasion and tumor angiogenesis of medullary thyroid carcinoma

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ABSTRACT

Objective: This study investigated the role of pigment epithelium-derived factor (PEDF), vascular endothelial growth factor (VEGF), nuclear factor kappa B (NF-kB), and Ki-67 proliferation index (PI) factors in Medullary thyroid carcinoma (MTC) tissue progression and their relationship with tumor angiogenesis. Immunohistochemical (IHC) analyses of PEDF expression in various human tumor samples and healthy tissues have shown that high levels of PEDF expression are associated with a positive prognosis, and low levels of PEDF suggest a poorer prognosis. With the completion of further studies showing the antitumor effects of PEDF on different types of cancer, PEDF's potential as a therapeutic agent has become increasingly promising. To the best of our knowledge, the role of PEDF in tumor angiogenesis in MTC has not been previously investigated.

Material and Methods: Thirty-seven tissue samples were retrospectively analyzed and sent/removed for pathology after total or subtotal thyroidectomy and archived between 01.01.2000 and 31.12.2015 in the Pathology Department of Hacettepe University. Relationships between categorical variables are analyzed using Pearson Chi-squared or Fisher's Exact tests.The correlation between two numerical variables is analyzed using Spearman's Rho correlation coefficient, and a comparison of numerical variables between two groups is made using the Mann-Whitney U test.

Results: The clinicopathological features of the patients were evaluated as sporadic or hereditary, tumor node metastasis (TNM) staging, single or multifocal focus, recurrence or regional or distant metastasis, progression-free survival time (PFS), and appropriate pathology tissue samples were determined as PEDF, VEGF, NF-kB, Ki-67 staining was compared. As a result of this comparison, a moderate negative correlation was found between VEGF expression level and PFS (rho = - 0,407, p = 0,019). No statistically significant correlation was found between single or multifocal groups and Ki-67 proliferation index (PI) expression levels (p = 0.070), but this is a small p value, a variable that is likely to find significant differences in studies with larger sample groups.The relationship between PEDF, VEGF, NF- kB, Ki-67 PI factors and tumor angiogenesis as reflected by microvessel density (MVD) was also investigated. To this end, we correlated the expression levels of PEDF, VEGF, NF-kB, and Ki-67 staining of suitable pathology tissue samples of the patients with the results obtained from the staining with CD 31. As a result of this comparison, no statistically significant relationship was found.

Conclusions: We demonstrated and described for the first time PEDF expression patterns in MTC tumor tissues. A direct relationship between PEDF expression level and angiogenesis, angiogenetic and clinicopathological factors does not seem to exist, at least as it pertains to the group studied. Further studies are required to fully elucidate mechanisms for tumor angiogenesis and invasion in MTC.

Keywords: Medullary thyroid carcinoma (MTC), Pigment epithelium-derived factor (PEDF), Vascular endothelial growth factor (VEGF), Nuclear factor kappa B (NF-kB), Ki-67 proliferation index, Microvessel density (MVD), angiogenesis, invasion.

INTRODUCTION

Medullary thyroid carcinoma (MTC) is a malignant tumor of parafollicular C cells arising from the neural crest. MTC accounts for a significant portion of thyroid cancer morbidity and mortality. At presentation, the rates of regional and distant metastases are as high as 35% and 13%, respectively, and there has been no trend towards earlier diagnosis or improvement in overall survival in recent years (1).

Pigment epithelium-derived factor (PEDF) is a glycoprotein belonging to the serpin family. The gene encoding PEDF, also known as serpin peptidase inhibitor, clade F, member 1 (SERPINF1), is located on chromosome 17p13.3, contains eight exons and seven introns, and codes for a glycoprotein of molecular weight 50 kDa consisting of 418 amino acids (2). PEDF was initially identified as a neuroprotective and antiangiogenic factor secreted by the human fetal retina pigment epithelium. PEDF expression has been reported in various organs and tissues such as the brain, spinal cord, eyes, lungs, heart, liver, uterus, ovary, prostate, pancreas, bone, and plasma (3). Immunohistochemical analyses of PEDF expression in various human tumor samples and healthy tissues have shown that high levels of PEDF expression are associated with a positive prognosis, and low levels of PEDF suggest a poorer prognosis. With the completion of further studies showing the antitumor effects of PEDF on different types of cancer, PEDF's potential as a therapeutic agent has become increasingly promising (4). To the best of our knowledge, the role of PEDF in tumor angiogenesis in MTC has not been previously investigated.

CD 31, also known as platelet endothelial cell adhesion molecule (PECAM-1), is highly expressed on the surface of endothelial cells and is used to detect vessel density in tumor tissue (5).

A high microvessel density (MVD) number is a marker of tumors that are more prone to metastasis and is a sign of poor prognosis (6)

The family and receptors of vascular endothelial growth factor (VEGF) create the most important signaling pathways in tumor angiogenesis. Recognition of the VEGF pathway as a key regulator of angiogenesis has led to the development of various VEGF-targeted agents used in cancer treatment today, including agents that prevent the binding of VEGF-A to its receptors, antibodies that directly block vascular endothelial growth factor receptor-2 (VEGFR-2), and small molecules that inhibit kinase activity (7).

Nuclear factor kappa B (NF-kB) is a family of transcription factors that play critical roles in inflammation, immunity, cell proliferation and apoptosis. Inactive NF-kB is found in most cell types in the cytoplasm and can be activated by various extracellular stimuli such as proinflammatory cytokines, bacterial lipopolysaccharides, viral RNA and DNA via activation of membrane and cytoplasmic receptors. Several experimental studies have shown that the NF-kB transcription factor plays a role in the development or progression of human cancers (9).

Ki-67 is an antigen associated with cell proliferation expressed in all cell cycle stages except G0 (10). Ki-67 has been studied in many types of cancer, including cervical, lung, breast, and thyroid. It has been reported that Ki-67 is an independent prognostic factor in thyroid cancer patients (11).

This study investigated the role of PEDF, VEGF, NF-kB, and Ki-67 proliferation index (PI) factors in the progression of MTC tissue and their relationship with tumor angiogenesis. The study evaluated the correlation between PEDF, VEGF, NF-kB, and Ki-67 proliferation index (PI) factors and Microvessel density (MVD) concerning tumor angiogenesis by analyzing the expression levels of PEDF, VEGF, NF-kB, and Ki-67 in appropriate pathology tissue samples obtained from staining.

MATERIAL AND METHODS

Patients and clinicopathological data collection

Thirty-seven tissue samples were retrospectively analyzed and sent/removed for pathology after total or subtotal thyroidectomy and archived between 01.01.2000 and 31.12.2015 in the Pathology Department of Hacettepe University. TNM (tumor node metastasis) staging; pathological tumor, lymph node, metastasis (pTNM) criteria for clinicopathological tumor staging adopted by the International Union for Cancer Control (UICC) and the American Joint Cancer Committee

(AJCC), tumor size and presence or absence of extrathyroidal invasion, local and based on regional lymph node metastases and distant metastases (12). The patients included in the study were evaluated as recurrence or metastasis cases based on the radiological methods used in the postoperative follow-up of the patients.

Immunohistochemical analysis

Formalin fixed, paraffin-embedded thyroid tissue samples diagnosed with MTC were retrieved from the archive and tissue microarray (TMA) was prepared. Three mm diameter punch biopsy needles were used to transfer tumor tissues for creating (TMA) blocks. Then 3-micrometer sections were made.

The antibody clones were Anti-PEDF (1:25, rabbit polyclonal antibody – Immunohistochemical (IHC) - orb339611 product code, Biorbyt brand), Anti-VEGF (1:25, mouse monoclonal antibody - IHC - sc 7269 product code, Santa Cruz brand), Anti-NF-kB p65 (1:25, rabbit monoclonal antibody – IHC - sc 8008 product code, Santa Cruz brand), Anti-Ki-67 (1:300, mouse monoclonal antibody - IHC - DIA-670-P1 product code, Optistain brand), Anti-CD31 (1:100, mouse monoclonal antibody - IHC - MS -353-S product code, Thermo Scientific brand).

For immunostaining with antibodies including anti-PEDF, anti-VEGF and anti-NF-kB p65; the slides were deparaffinized by xylene 2 x 10' in an oven at 60 degrees after a night. The slides were pre-treated in ER1 (Epitope Retrieval Solution 1) for 10 minutes at 100 degrees. A blocking solution of 6% Hydrogen peroxide and 80% Methanol was used for 20 minutes. The slides were incubated with antibodies for 1 hour. The secondary antibody was applied for 30 minutes, streptavidin peroxidase for 30 minutes, and DAB for 2 minutes. The tissues were treated with hematoxylin for 5 seconds before being passed through alcohol and xylene and closed. For immunostaining with antibodies including Ki-67 and CD31; the slides were deparaffinized by xylene 2 x 10' in an oven at 60 degrees after a night. The slides were pre-treated in ER2 (Epitope Retrieval Solution 2) for 20 minutes at 100 degrees. A blocking solution of 6% Hydrogen peroxide and 80% Methanol was used for 20 minutes. The slides were incubated with antibodies for 1 hour. The samples were treated with post-primary for 9 minutes, polymer for 9 minutes, and DAB for 7 minutes, then left in hematoxylin for 8 minutes before being passed through alcohol and xylene and closed. The immunostained slides were evaluated by one pathologist without previous knowledge of clinical features.

Statistical analysis

In the study, descriptive statistics for numerical variables are given as mean±standard deviation or median (minimum-maximum) values, and for categorical variables as numbers and percentages. Whether the numerical variables were normally distributed or not was examined with the Shapiro Wilk test. Relationships between categorical variables are analyzed using Pearson Chi-squared or Fisher's Exact tests. The correlation between two numerical variables is analyzed using Spearman's Rho correlation coefficient, and a comparison of numerical variables between two groups is made using the Mann-Whitney U test. A comparison of numerical variables between more than two groups is made using the Kruskal-Wallis test. In the study, $p<0.05$ is considered statistically significant. Analyses were conducted in IBM SPSS Statistics 23.0 program.

RESULTS

Thirty-seven tissue samples were retrospectively analyzed and sent/removed for pathology after total or subtotal thyroidectomy and archived between 01.01.2000 and 31.12.2015 in the Pathology Department of Hacettepe University. Of the 37 patients, 30 (81.1%) received a diagnosis of sporadic MTC, and 7 (18.9%) received a diagnosis of hereditary MTC. As to the staging of the patients, 19 (51.4%) were diagnosed in stage I, 4 (10.8%) in stage II, 3 (8.1%) in stage III, and 11 (29.7%) in stage IV. Development of MTC was found to be a single focus in 26 (70.3%) of the 37 patients and multifocal in 11 (29.7%). In the post-surgical follow-up of the 37 patients, 21 (56.8%) had no recurrence or regional or distant metastasis, 12 (32.4%) had a recurrence or regional or distant metastasis, and data on recurrence or regional or distant metastasis could not be obtained for 4 (10.8%) patients. The median progression-free survival (PFS) time was 31 months for 33 (89.2%) patients. Data on PFS time could not be obtained for 4 (10.8%) patients (Table 1).

Table 1. Clinicopathological characteristics of patients (n=37)

Feature	n %
Sporadic or Inherited	
Sporadic	30(81,1)
Inherited	7(18,9)
TNM stage	
Stage I	19(51,4)
Stage II	4(10,8)
Stage III	3(8,1)
Stage IV	11(29,7)
Multifocality	
Single focus	26(70,3)
Multiple foci	11(29,7)
Recurrence, regional, or distant metastasis	
No	21(56,8)
Yes	12(32,4)
Loss	4(10,8)
PFS (median, month)	31
Loss	4(10,8)
PEDF	
Negative (< 5%)	6(16,2)
Weak positive (5-30%)	7 (18,9)
Moderately positive (30–60%)	14 (37,8)
Strongly positive (60 < %)	10 (27)
VEGF	
Negative (< %5)	20 (54,1)
Weak positive (%5-30)	12 (32,4)
Moderately positive (%30–60)	1 (2.7)
Strongly positive (%60 <)	4 (10.8)
NF-kB p65	
Negative (< 5%)	20 (54,1)
Weak positive (5-30%)	12 (32,4)
Moderately positive (30–60%)	1 (2.7)
Strongly positive (60 < %)	4 (10.8)
Ki-67	
< 3 %	33(89,2)
3 < %	4(10,8)
CD31 (median number of vessels)	11,3

İmmunohistochemical scoring

The PEDF score was evaluated for tumor cells. The expression levels of PEDF in tumor cells were semi-quantitatively categorized into four groups: negative (0 points), <5% positive cells (Figure 1A); weakly positive (1 point), 5-30% positive cells (Figure 1B); moderately intense positive (2 points), 30-60% positive cells (Figure 1C); and strongly intense positive (3 points), >60% positive cells (Figure 1D).

VEGF score; the tumor cells were evaluated. The levels of VEGF expression in tumor cells were semi-quantitatively categorized into four groups: negative (0 points), <%5 positive cells (Figure 2A); weakly positive (1 point), %5-30 positive cells (Figure 2B); moderately intense positive (2 points), %30-60 positive cells (Figure 2C); strongly intense positive (3 points), %60 < positive cells (Figure 2D).

NF-Kb p65 score; tumor cells were evaluated. The expression levels of NF-Kb p65 in tumor cells were semi-quantitatively categorized into four groups: negative (0 points), <%5 positive cells (Figure 3A); weakly positive (1 point), %5-30 positive cells (Figure 3B); moderately intense positive (2 points), %30-60 positive cells (Figure 3C); strongly intense positive (3 points), %60< positive cells (Figure 3D).

The Ki-67 PI score is evaluated for tumor cells. To calculate the Ki-67 PI, a count of 1000 cells is performed and then expressed as a percentage. The Ki-67 PI score is categorized as <%3 - 1 point (Figure 4A), %3 < - 2 points (Figure 4B).

The CD 31 score was evaluated for tumor cells by selecting the three areas with the highest microvascular concentration (vascular hot spots) stained with CD 31 in the tumor cell area, avoiding lymphocytic infiltration or fibrotic areas. The number of microvessels in the three areas with the highest microvascular concentration was determined, and the determined number of microvessels was added up and divided by 3 to calculate the average number of microvessels (Figure 5).

Evaluation of the correlation between clinical and pathological characteristics and PEDF expression levels of patients.

In 30 (81.1%) of the patients, sporadic MTC was diagnosed, and in 7 (18.9%) of the patients, hereditary MTC was diagnosed. According to the IHC evaluation results, 5 (16.7%) patients from the sporadic patient group showed negative, 6 (20%) showed weak positive, 11 (36.7%) showed moderate positive, and 8 (6.27%) showed strong positive staining. In the hereditary patient group, 1 (14.3%) patient showed negative, 1 (14.3%) showed weak positive, 3 (42.9%) showed moderate positive, and 2 (28.6%) showed strong positive staining. There was no statistically significant relationship between PEDF expression levels and sporadic or hereditary group of patients (p=1,000) (Table 2).

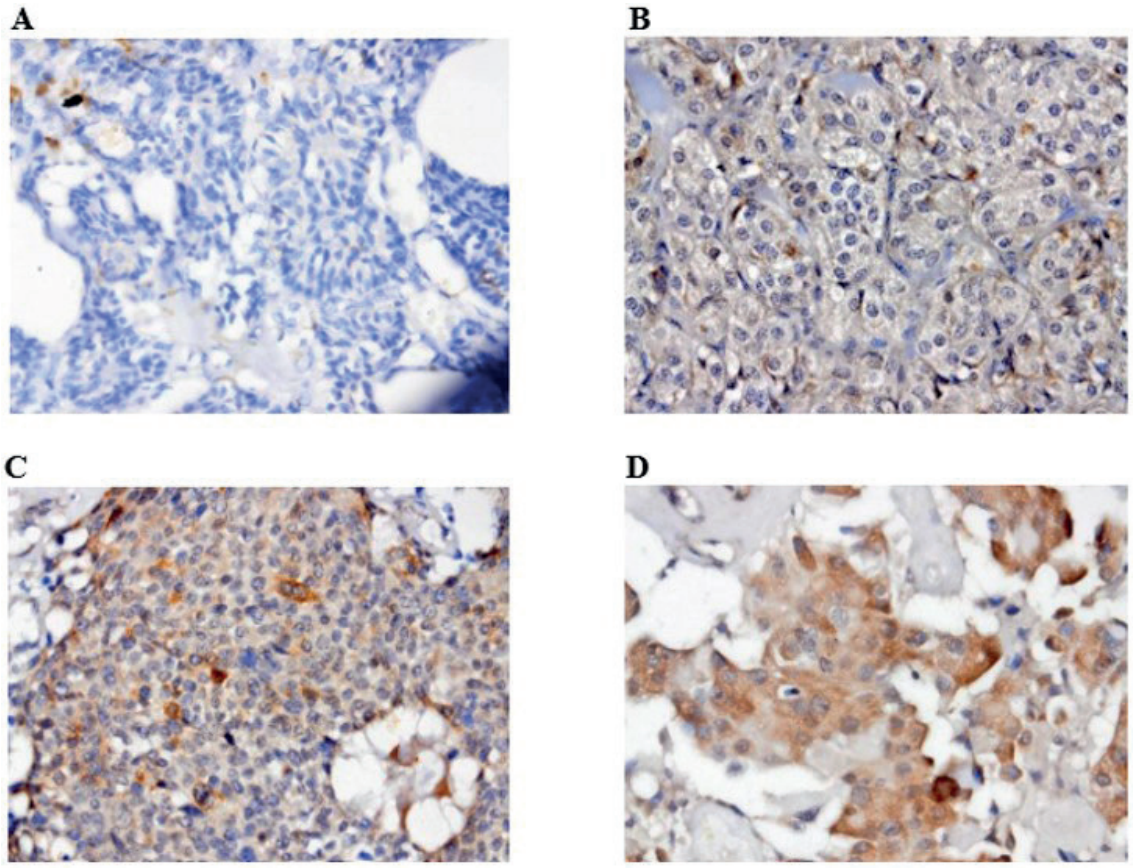

Figure 1. The microscopic image of PEDF evaluated immunohistochemically.
1A – Score 0, 1B – Score 1, 1C – Score 2, 1D – Score 3

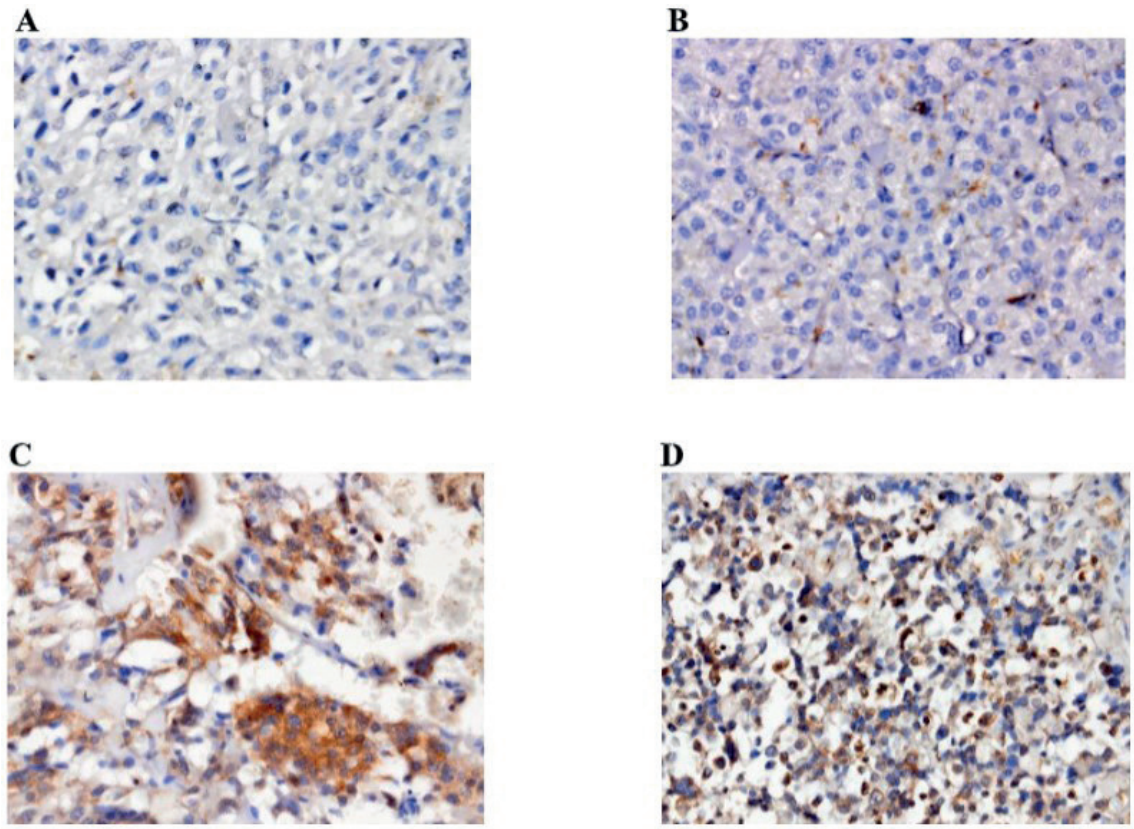

Figure 2. The microscopic image of VEGF evaluated immunohistochemically.
2A – Score 0, 2B – Score 1, 2C – Score 2 and 2D – Score 3

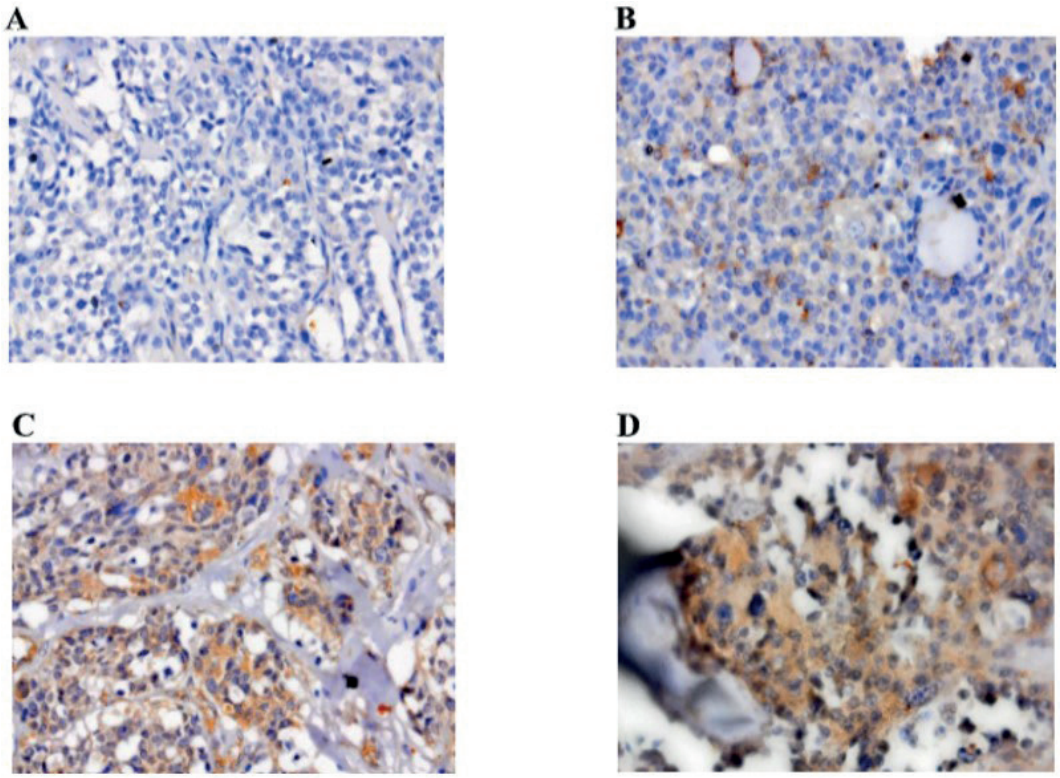

Figure 3. The microscopic image of NF-kB evaluated immunohistochemically.
3A – Score 0, 3B – Score 1, 3C – Score 2 ve 3D – Score 3

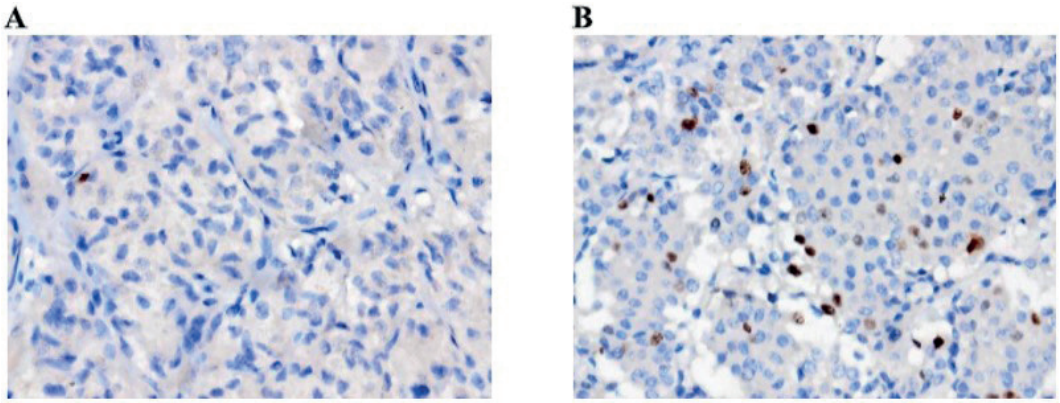

Figure 4. The microscopic image of the Kİ-67 proliferation index evaluated immunohisto chemically.
4A – Score 0, 4B – Score 1

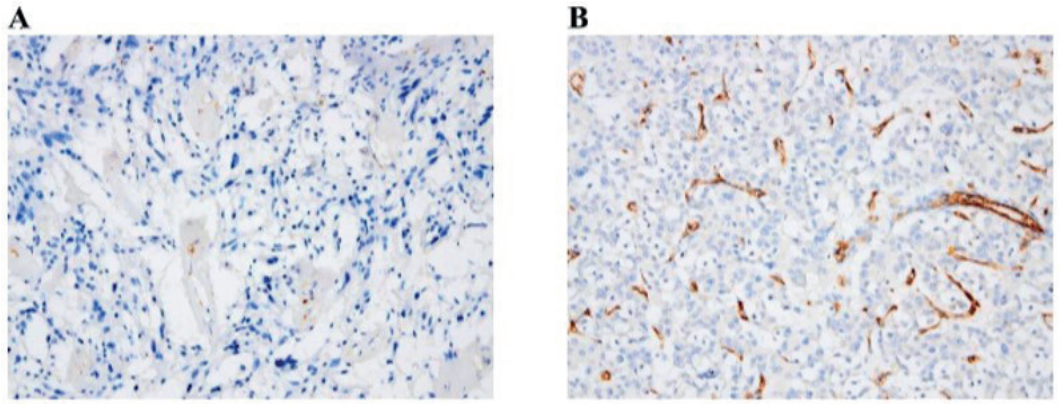

Figure 5. Immunohistochemically evaluated microscopic image of the microvascular area stained with CD-31, with 5A - weak and 5B - strong staining.

According to the TNM staging, 19 (51.4%) patients were diagnosed in stage I, 4 (10.8%) in stage II, 3 (8.1%) in stage III, and 11 (29.7%) in stage IV. Based on the IHC evaluation, 4 (21.1%) of the patients in stage I showed negative, 5 (26.3%) weak positive, 5 (26.3%) moderate positive, and 5 (26.3%) strong positive staining. In stage II, 0 (0%) patients showed negative, 0 (0%) weak positive, 4 (100%) moderate positive, and 0 (0%) strong positive staining. In stage III, 0 (0%) patients showed negative, 1 (33.3%) weak positive, 2 (66.7%) moderate positive, and 0 (0%) strong positive staining. In stage IV, 2 (18.2%) patients showed negative, 1 (9.1%) weak positive, 3 (27.3%) moderate positive, and 5 (45.5%) strong positive staining. No statistically significant relationship existed between the TNM stage groups and PEDF expression levels (p=0.283) (Table 2).

Twenty-six (70.3%) patients had a single focus of MTC, while 11 (29.7%) had a multifocal focus. The IHC evaluation revealed that 5 (19.2%) of the single-focus patients had negative, 6 (23.1%) weakly positive, 8 (30.8%) moderately positive, and 7 (26.9%) strongly positive staining. The multifocal focus patients had 1 (9.1%) negative, 1 (9.1%) weakly positive, 6 (54.5%) moderately positive, and 3 (27.3%) strongly positive staining. No statistically significant correlation existed between single or multifocal focus groups and PEDF expression levels (p=0.544) (Table 2).

In the postoperative follow-up, 21 (56.8%) patients had no recurrence or regional or distant metastasis, while 12 (32.4%) patients had a recurrence or regional or distant metastasis. Data on recurrence or regional or distant metastasis could not be obtained for 4 (10.8%) patients. In the IHC evaluation results, 4 (19.1%) of the patients without recurrence or metastasis showed negative staining, 4 (19%) showed weak positive staining, 9 (42.9%) showed moderate positive staining, and 4 (19%) showed strong positive staining. In the group of patients with recurrence or metastasis, 2 (16.7%) showed negative staining, 1 (8.3%) showed weak positive staining, 4 (33.3%) showed moderate positive staining, and 5 (41.7%) showed strong positive staining. No statistically significant relationship was found between the recurrence or metastasis status and PEDF expression levels (p=0.592) (Table 2).

Evaluation of the correlation between clinical and pathological characteristics and VEGF expression levels of patients.

The results of the IHC assessment showed that in the sporadic patient group, 15 (50%) had negative, 10 (33.3%) had weakly positive, 1 (3.3%) had moderately positive, and 4 (13.3%) had strongly positive staining. In the hereditary patient group, 5 (71.4%) had negative, 2 (28.6%) had weakly positive, 0 (0%) had moderately positive, and 0 (0%) had strongly positive staining. No statistically significant relationship was found between VEGF expression levels and whether the patients were sporadic or hereditary (p=0.772) (Table 2).

The results of the IHC assessment in the TNM stage groups showed that in stage I, 13 (68.4%) had negative, 4 (21.1%) had weakly positive, 0 (0%) had moderately positive, and 2 (10.5%) had strongly positive staining. In stage II, 1 (25%) had negative, 2 (50%) had weakly positive, 0 (0%) had moderately positive, and 1 (25%) had strongly positive staining. In stage III, 2 (66.7%) had negative, 1 (33.3%) had weakly positive, 0 (0%) had moderately positive, and 0 (0%) had strongly positive staining. In stage IV, 4 (36.4%) had negative, 5 (45.5%) had weakly positive, 1 (9.1%) had moderately positive, and 1 (9.1%) had strongly positive staining. No statistically significant relationship was found between TNM stage groups and VEGF expression levels (p=0.464) (Table 2).

The results of the IHC assessment in single-focus MTC patients showed that 13 (50%) were negative, 9 (34.6%) were weakly positive, 1 (3.8%) were moderately positive, and 3 (11.5%) were strongly positive. In multifocal focus MTC patients, 7 (63.6%) were negative, 3 (27.3%) were weakly positive, 0 (0%) were moderately positive, and 1 (9.1%) was strongly positive. No statistically significant relationship was found between single or multifocal focus groups and VEGF expression levels (p=0.910) (Table 2).

The results of the IHC assessment in recurrent or metastasis-free patients showed that 12 (57.1%) were negative, 7 (33.3%) were weakly positive, 0 (0%) were moderately positive, and 2 (9.5%) were strongly positive. In recurrent or metastasis patients, 5 (41.7%) were negative, 5 (41.7%) were weakly positive, 1 (8.3%) was moderately positive, and 1 (8.3%) was strongly positive. No statistically significant relationship was found between recurrent or metastasis and VEGF expression levels (p=0.595) (Table 2).

Table 2. Evaluation of the correlation between clinical and pathological characteristics of the patients and the expression levels of PEDF, VEGF, NF-kB p65, Ki-67 proliferation index

Features	Sporadic/İnherited		TNM stage				Multifocality		Recurrence or metastasis	
PEDF staining intensity	Sporadic	İnherited	stage1	stage2	stage3	stage4	Single focus	Multiple foci	No	Yes
Negative N(%)	5(16.7)	1(14.3)	4(21.1)	0(0)	0(0)	2(18.2)	5(19.2)	1(9.1)	4(19.1)	2(16.7)
Weakly positive N (%)	6(20)	1(14.3)	5(26.3)	0(0)	1(33.3)	1(9.1)	6 (23.1)	1(9.1)	4(19)	1(8.3)
Moderately positive N (%)	11(36.7)	3(42.9)	5(26.3)	4(100)	2(66.7)	3(27.3)	8(30.8)	6(54.5)	9(42.9)	4(33.3)
Strongly positive N (%)	8(6.27)	2(28.6)	5(26.3)	0(0)	0(0)	5(45.5)	7(26.9)	3(27.3)	4(19)	5(41.7)
p value	1.000		0.283				0.544		0.592	
VEGF staining intensity	Sporadic	İnherited	stage1	stage2	stage3	stage4	Single focus	Multiple foci	No	Yes
Negative N(%)	15(50.0)	5(71.4)	13(68.4)	1(25)	2(66.7)	4(36.4)	13(50)	7(63.6)	12(57.1)	5(41.7)
Weakly positive N (%)	10(33.3)	2(28.6)	4(21.1)	2(50.0)	1(33.3)	5(45.5)	9(34.6)	3(27.3)	7(33.3)	5(41.7)
Moderately positive N (%)	1(3.3)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(9.1)	1(3.8)	0(0.0)	0(0.0)	1(8.3)
Strongly positive N (%)	4(13.3)	0(0.0)	2(10.5)	1(25)	0(0.0)	1(9.1)	3(11.5)	1(9.1)	2(9.5)	1(8.3)
p value	0.772		0.464				0.910		0.595	
NF-Kb staining intensity	Sporadic	İnherited	stage1	stage2	stage3	stage4	Single focus	Multiple foci	No	Yes
Negative N(%)	7(23.3)	3(42.9)	5(26.3)	2(50.0)	0(0.0)	3(27.3)	6(23.1)	4(36.4)	7(33.3)	3(25)
Weakly positive N (%)	15(50)	3(42.9)	9(47.4)	2(50.0)	2(66.7)	5(45.5)	11(42.3)	7(63.6)	9(42.9)	7(58.3)
Moderately positive N (%)	5(16.7)	1(14.3)	2(10.5)	0(0.0)	1(33.3)	3(27.3)	6(23.1)	0(0.0)	3(14.3)	2(16.7)
Strongly positive N (%)	3(10)	0(0.0)	3(15.8)	0(0.0)	0(0.0)	0(0.0)	3(11.5)	0(0.0)	2(9.5)	0(0.0)
p value	0.856		0.775				0.223		0.799	
Ki-67 staining intensity	Sporadic	İnherited	stage1	stage2	stage3	stage4	Single focus	Multiple foci	No	Yes
< 3 %	26(86.7)	7(100)	18(94.7)	4(100)	3(100)	8(72.7)	26(96.2)	8(72.7)	20(95.2)	10(83.3)
3 < %	4(13.3)	0(0.0)	1(5.3)	0(0.0)	0(0.0)	3(27.3)	1(3.8)	3(27.3)	1(4.8)	2(16.7)
p value	0.570		0.316				0.070		0.538	

Evaluation of the correlation between the clinical and pathological characteristics of the patients and the expression levels of NF-kB p65.

The results of the IHC evaluation showed that of the sporadic patients, 7 (23.3%) were negative, 15 (50%) were weakly positive, 5 (16.7%) were moderately positive, and 3 (10%) were strongly positive. Of the hereditary patients, 3 (42.9%) were negative, 3 (42.9%) were weakly positive, 1 (14.3%) was moderately positive, and 0 (0%) were strongly positive. There was no statistically significant relationship between the sporadic or hereditary group and the expression levels of NF-kB (p=0.856) (Table 2).

In the TNM stage groups, of the patients in stage I, 5 (26.3%) were negative, 9 (47.4%) were weakly positive, 2 (10.5%) were moderately positive, and 3 (15.8%) were strongly positive. Of the patients in stage II, 2 (50%) were negative, 2 (50%) were weakly positive, 0 (0%) were moderately positive, and 0 (0%) were strongly positive. Of the patients in stage III, 0 (0%) were negative, 2 (66.7%) were weakly positive, 1 (33.3%) were moderately positive, and 0 (0%) were strongly positive. Of the patients in stage

IV, 3 (27.3%) were negative, 5 (45.5%) were weakly positive, 3 (27.3%) were moderately positive, and 0 (0%) were strongly positive. There was no statistically significant relationship between the TNM stage groups and the expression levels of NF-kB (p=0.775) (Table 2).

Of the patients with single-focus MTC, 6 (23.1%) were negative, 11 (42.3%) were weakly positive, 6 (23.1%) were moderately positive, and 3 (11.5%) were strongly positive. Of the patients with multifocal focus MTC, 4 (36.4%) were negative, 7 (63.6%) were weakly positive, 0 (0%) were moderately positive, and 0 (0%) were strongly positive. There was no statistically significant relationship between the single or multifocal focus groups and the expression levels of NF-kB (p=0.223) (Table 2).

Of the patients without recurrence or metastasis, 7 (33.3%) were negative, 9 (42.9%) were weakly positive, 3 (14.3%) were moderately positive, and 2 (9.5%) were strongly positive. Of the patients with recurrence or metastasis, 3 (25.0%) were negative, 7 (58.3%) were weakly positive, 2 (16.7%) were moderately positive, and 0 (0%) were strongly positive. There was no statistically significant relationship between the absence or presence of recurrence or metastasis and the expression levels of NF-kB (p=0.799) (Table 2).

Evaluation of the correlation between clinical and pathological characteristics of the patients and the expression levels of the Ki-67 proliferation index

The results of the IHC evaluation showed that in the sporadic patient group, 26 (86.7%) patients had a Ki-67 PI of <3%, and 4 (13.3%) patients had 3%<. In the hereditary patient group, 7 (100%) patients had a Ki-67 PI of <3%, and 0 (0.0%) patients had 3%<. There was no statistically significant relationship between Ki-67 PI expression levels and sporadic or hereditary groups (p=0.570) (Table 2).

In the TNM stage groups, 18 (94.7%) of stage I patients were evaluated as <3%, and 1 (5.3%) as 3%<. All patients in stage II were evaluated as <3%, and no patients were evaluated as 3%<. All patients in stage III were evaluated as <3%, and no patients were evaluated as 3%<. In stage IV, 8 (72.7%) patients were evaluated as <3%, and 3 (27.3%) as 3%<. There was no statistically significant relationship between TNM stage groups and Ki-67 PI expression levels (p=0.316) (Table 2).

In single-focus MTC patients, 25 (96.2%) were evaluated as <3%, and 1 (3.8%) as 3%<. In multifocal focus MTC patients, 8 (72.7%) were evaluated as <3%, and 3 (27.3%) as 3%<. As a result of evaluating the correlation between single or multifocal focus groups and Ki-67 PI expression levels, no statistically significant correlation was found (p = 0.070), but this is a small p value, a variable that is likely to find significant differences in studies with larger sample groups (Table 2).

In recurrent or metastasis-free patients, 20 (95.2%) were evaluated as <3%, and 1 (4.8%) as 3%<. In recurrent or metastatic patients, 10 (83.3%) were evaluated as <3%, and 2 (16.7%) as 3%<. No statistically significant relationship existed between recurrent or metastatic and non-recurrent or metastatic groups and Ki-67 proliferation index expression levels (p=0.538) (Table 2).

The evaluation of the correlation between the expression levels of PEDF, VEGF, and NF-kB p65 and PFS in the patients.

The results of the analysis between PEDF expression levels (0, 1, 2, 3) and PFS showed no statistically significant relationship (p=0.193) (Table 3). The analysis results between VEGF expression levels (0, 1, 2, 3) and PFS showed a negative, moderate correlation with a Spearman correlation coefficient value of rho = -0.407, p=0.019, and a statistically significant relationship was found (Table 3). No statistically significant relationship was found between NF-kB p65 expression levels (0, 1, 2, 3) and PFS (p=0.835) (Table 3).

Table 3. Correlation between expression levels of PEDF, VEGF, NF-kB P65 and PFS in patients (Spearman's Rho)

	Correlation with PFS*	p-value
PEDF 0, 1, 2, 3	- 0,232	0,193
VEGF 0, 1, 2, 3	- 0,407	0,019
NF-kB 0, 1, 2, 3	- 0,038	0,835

(*Spearman rho correlation coefficient)
PEDF staining intensity: 0 – negative, 1 – weak positive, 2 – moderately positive, 3 – strongly positive;
VEGF staining intensity: 0 – negative, 1 – weak positive, 2 – moderately positive, 3 – strongly positive;
NF-kB staining intensity: 0 – negative, 1 – weak positive, 2 – moderately positive, 3 – strongly positive;

Evaluation of the correlation between the Ki-67 proliferation index expression levels and PFS in patients.

Ki-67 variable is divided into two groups as %3 < and <%3. These two groups were compared with the Mann-Whitney U test according to PFS values. No statistical difference was found between the groups (p = 0.837). Data on four patients were not available for statistical analysis (Table 4).

Evaluation of the comparison of PEDF expression levels of the patients with CD 31

PEDF variable expression levels are divided into four groups: 0, 1, 2, 3. These four groups were compared with the Kruskal Wallis test according to their CD 31 values. No statistical difference was found between groups (p = 0.435) (Table 5).

Evaluation of the comparison between the VEGF expression levels of patients and CD 31.

VEGF variable expression levels are divided into four groups: 0, 1, 2, 3. These four groups were compared with the Kruskal Wallis test according to their CD 31 values. No statistical difference was found between groups (p = 0.565). However, since there was only one patient in the 2 (moderately positive) category according to VEGF staining intensity, it was not appropriate to provide the Kruskal Wallis test comparison result (Table 5).

Evaluation of comparison of NF-kB p65 expression levels with CD 31 in patients.

NF-kB variable expression levels are divided into four groups: 0, 1, 2, 3. These four groups were compared with the Kruskal Wallis test according to their CD 31 values. No statistical difference was found between groups (p = 0.413) (Table 5).

Evaluation of comparing patients' Ki-67 proliferation index levels with CD 31.

Ki-67 variable is divided into two groups: <3% and <3%. These two groups were compared with the Mann-Whitney U test according to their CD 31 values. No statistical difference was found between the groups (p = 0.688) (Table 6).

Table 4. Correlation between Ki-67 proliferation index expression levels and PFS in patients

	Ki-67 %	Number of patients (n=33)	p-value
PFS (month)	< %3	30	0,837
	%3 <	3	

Table 6. Comparison of patient's Ki-67 proliferation index levels with CD 31

Ki-67 (%LI)	Number of patients (n=37)	p-value
< %3	33	0,688
%3 <	4	

Table 5. Comparison of PEDF, VEGF, NF-Kb expression levels with CD-31 in patients

PEDF staining intensity	Number of Patients (N = 37)	Median value	Minimum value	Maximum value	P value
0	6	9.3300	4.33	22.66	0.435
1	7	12.3300	7.66	23.66	
2	14	9.6650	4.00	14.00	
3	10	11.4950	4.66	20.00	
VEGF staining intensity	Number of Patients (N = 36)	Median value	Minimum value	Maximum value	
0	20	11.3300	4.33	23.66	
1	12	11.3300	4.33	22.66	0.565
3	4	8.6650	4.00	12.66	
NF-kB staining intensity	Number of Patients (N = 37)	Median value	Minimum value	Maximum value	
0	10	7.6650	4.00	22.66	0.413
1	18	11.6600	4.33	23.66	
2	6	13.1600	5.66	17.66	
3	3	11.3300	6.00	17.00	

PEDF staining intensity: 0 – negative, 1 – weak positive, 2 – moderately positive, 3 – strongly positive;
VEGF staining intensity: 0 – negative, 1 – weak positive, 2 – moderately positive, 3 – strongly positive;
NF-kB staining intensity: 0 – negative, 1 – weak positive, 2 – moderately positive, 3 – strongly positive;

DISCUSSION

Our study investigated the role of PEDF, VEGF, NF-kB, and Ki-67 PI factors in the progression of MTC tissue and their relationship with tumor angiogenesis. We evaluated the clinical and pathological characteristics of 37 patients with MTC. To evaluate the role of PEDF, VEGF, NF-kB, and Ki-67 PI factors in the progression of MTC tissue, we compared the relationship between the expression levels obtained from PEDF, VEGF, NF-kB, and Ki-67 staining of appropriate pathology tissue samples and clinical and pathological data such as sporadic or hereditary, TNM stage, single or multifocal focus, recurrent or regional or distant metastasis, and PFS. A moderate negative correlation was found between VEGF expression levels and PFS, and a close correlation was found between Ki-67 PI expression levels and single or multifocal focus groups.

Although PEDF, VEGF, NF-kB and Ki-67 PI factors were expressed in MTC tumor tissue in the study, a direct relationship could not be demonstrated between the expression levels of PEDF, VEGF, NF-kB and Ki-67 PI factors and tumor angiogenesis.

It has been shown that PEDF plays an important role in tumor angiogenesis, growth, and dissemination. PEDF is one of the strongest natural endogenous inhibitors of angiogenesis (13). Many previous studies have shown that PEDF blocks the proliferation and dissemination of nasopharyngeal carcinoma (14), pancreatic cancer (15), glioma (16), melanoma (17) and breast cancer (18) cells. PEDF has also been significantly associated with the progression and metastasis of hepatocellular carcinoma (19). Recently, Tang et al. reported that PEDF supports the growth of esophageal cancer cells (20). Regarding papillary thyroid carcinomas (PTC), Yichen et al. have investigated the role of PEDF and evaluated the relationship between PEDF and PTC tumor angiogenesis concerning PTC. Their study showed a significant correlation between PEDF expression levels and clinicopathological characteristics such as lymph node metastasis (LNM), extrathyroidal invasion, high TNM stage, the presence of BRAFV600E mutation and tumor size in PTC.

Furthermore, the evaluation of the relationship between PEDF and PTC tumor angiogenesis showed that PEDF might have an antiangiogenic role by affecting the hypoxia-inducible factor 1a (HIF1a)-VEGF pathway (21).

Our study is the first study in the literature to evaluate the role of PEDF expression levels in MTC tissue progression and its relationship with tumor angiogenesis, and although there is PEDF expression in MTC tumor tissue, no direct relationship was found between MTC tissue progression and tumor angiogenesis.

In this regard, it seems that PEDF does not play a direct role in modifying angiogenesis in MTC, unlike PTC, but this observation requires further verification with larger number of patients.

As to the thyroid cancers, Vieira et al. found that VEGF expression was significantly more widespread in papillary thyroid carcinomas (79%) compared to follicular thyroid carcinomas (50%) or indifferent thyroid carcinomas (37%), using immunohistochemical analysis. Kılıçarslan et al. found stronger expression in papillary thyroid carcinomas than in normal thyroid tissues (22). In our study, the role of VEGF expression levels in the progression of MTC tissue was evaluated, and a statistically negative, moderate relationship was found between VEGF expression levels and PFS; however, no statistical relationship was found with other clinicopathological features of the tumor such as sporadic or hereditary, TNM staging, recurrent or regional or distant metastasis, single or multifocal focus, and tumor angiogenesis.

Activation of NF-kB in tumors can be caused by both a response to classic inflammatory stimuli such as infectious and physical or chemical agents and as a result of oncogene activation. A typical example of the latter is the rearranged during transfection (RET) oncogene, which is present in various cancer types, including thyroid cancer. Activating mutations of the RET gene are responsible for medullary thyroid carcinomas (23). Interestingly, activating mutations of the RET proto-oncogene leads to structural activation of NF-kB, which is important for RET-mediated carcinogenesis. Therefore, blocking NF-kB signaling could represent a new therapeutic strategy for thyroid carcinoma, especially for advanced disease (24). However, our study found no relationship between NF-kB expression levels and the progression of MTC tissue or tumor angiogenesis.

Carr et al. showed that anaplastic tumors have higher Ki-67 indices than well-differentiated thyroid tumors in thyroid tumors, while Erickson et al. showed that Hurthle cell carcinomas of the thyroid have higher Ki-67 indices than benign Hurthle cell adenomas. Tisel et al. showed that in metastatic primary MTC tumors, significantly higher Ki-67 indices were found than in primary tumors without metastasis (25). Some authors claim that the Ki-67 PI may not always match the clinical features of the tumor and that sometimes tumors with low expression of Ki-67 may be more aggressive than tumors with higher expression of Ki-67 (26). Our study found a significant correlation between Ki-67 PI levels and single or multifocal foci in MTK tissue progression. However, no relationship was found between the other characteristics of the tumor included in the study, such as sporadic or hereditary, TNM staging, recurrent or regional or distant metastasis, PFS and tumor angiogenesis and Ki-67 PI levels.

In conclusion, although there exists various degrees of PEDF expression in MTC tumor tissues, we could not find a direct relationship between PEDF expression level and tumor angiogenesis, tissue progression and poor prognostic clinicopathological parameters. The results suggest that PEDF seems not to directly modify the tumor angiogenesis observed in MTC, contrary to what has been observed in PTC. Further studies are clearly required to further elucidate mechanisms for tumor angiogenesis and invasion in MTC.

Author contribution

The study was designed by AG and GK. Data collection was done by GK, OK, AA, SB, and CS. Analysis and interpretation of the results were made by JK, AG and GK. The study was compiled into an article by AG and GK.

Ethical approval

The study was approved by Hacettepe University Non-İnterventional Clinical Research Ethics Committee (Protocol no: GO 20/887; approval no: 2020/16-74 /date 06.10.2020).

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Conflict of interest

The authors declare that there is no conflict of interest.

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