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Papiller Tiroid Karsinomunun Hematojen Yayılım Potansiyelini Öngörmek Mümkün mü?: P27 ile İmmünohistokimyasal Çalışma

Is It Possible to Predict the Haemotogenous Metastatic Potential of Papillary Thyroid Carcinomas?: An Immunohistochemical Study with P27

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ÖZ

Giriş: Papiller tiroid karsinomu (PTC) en sık görülen tiroid malignitesidir. Genel olarak olumlu bir seyir izlediği için mevcut yaklaşım daha az agresif tedavi yöntemlerinin kullanılmasıdır. Ancak uzak metastaz gelişen olgularda hastalığın ilerlemesi ve mortalitenin daha yüksek olması söz konusudur. Buradan çıkan sonuç, bu olguların hangilerinin uzak organlara metastaz yapacağının anlaşılmasının önemli olduğunu göstermektedir. Hücre döngüsü inhibitörü p27'nin ekspresyon kaybı, birçok tümördeki malign davranışla pozitif korelasyon göstermektedir. Biz bu çalışmada PTC'de tümör p27 ekspresyonu ile uzak metastazlar arasındaki ilişkiyi araştırmayı amaçladık.

Yöntem: Çalışmamıza endokrinoloji bölümünde takip edilen 15'i uzak organ metastazı, 12'si lenf nodu metastazı ve 17'si metastazı olmayan toplam 44 olgu dahil edildi. Tüm vakalara p27'nin immünohistokimyasal boyaması yapıldı. Sonuçlar pozitif boyanma yüzdesine göre değerlendirildi.

Bulgular: Metastazı olmayan olguların 16'sında (%94,1) 2+ veya 3+ pozitif boyanma görülürken, uzak organ metastazı olanların sadece 5'inde (%33,3) görüldü. Uzak organ metastazı olan olgularda p27 ekspresyonunda istatistiksel olarak anlamlı azalma saptadık (p=0,002).

Sonuç: PTC'lerde p27 ekspresyonunun değerlendirilmesinin tümörün biyolojik davranışının belirlenmesinde yardımcı olabileceği sonucuna vardık.

Anahtar Kelimeler: papiller tiroid karsinomu, p27, uzak metastaz

ABSTRACT

Objective: Papillary thyroid carcinoma(PTC) is the most common thyroid malignancy. Since it generally has a favorable course, the current approach is to use less aggressive treatment modalities. However, patients with distant metastatic disease experience progression of disease with a higher mortality. This leads to the conclusion that it is crucial to understand which of these malignancies will metastasize to distant organs. The expression loss of the cell cycle inhibitor p27 is positively correlated with malignant behavior in many tumors. We aimed to investigate the relationship between tumor p27 expression and distant metastases in PTC.

Method: Our study included a total of 44 cases, 15 with distant organ metastases, 12 with lymph node metastases, and 17 without metastases, who were followed up in the endocrinology department. Immunohistochemical staining of p27 was performed for all the cases. The results were evaluated according to the percentage of positive staining.

Results: While 2+ or 3+ staining was observed in 16(94.1%) of the cases without metastases, it was observed in only 5(33.3%) of those with distant organ metastasis. We found a statistically significant decrease in p27 expression in cases with distant organ metastasis(p = 0.002).

Conclusion: We concluded that the evaluation of p27 expression in PTCs could be helpful in determining the biological behavior of the tumor.

Keywords: papillary thyroid carcinoma, p27, distant metastasis

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INTRODUCTION

Thyroid carcinomas are among the most frequently encountered tumors, accounting for 1% of all cancers but only 0.2% of cancer deaths (1). Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy. This tumor generally tends to be multifocal and to metastasize to the lymph nodes of the neck by lymphatic invasion. However, even in cases of regional lymph node metastases, the prognosis is mostly favorable, with a 20-year survival rate of over 90% (2,3). Venous invasion is extremely rare, and distant organ metastases are observed in approximately 5% to 7% of the cases (4,5). The 20-year survival rate has been reported to be 51.2% in cases with distant organ metastasis (6). These rates also show how important distant organ metastasis is in terms of predicting the prognosis and determining the appropriate follow-up and treatment modalities. Studying which factors could affect the metastatic potential of PTC cases, which rarely metastasize to distant organs, is therefore an important research topic. Although it is known that some factors, such as molecular markers and histological subtypes, may be associated with metastatic potential, immunohistochemical markers, which are much more cost-effective than molecular methods, are thought to be useful in prognosis as well as diagnosis (7,8).

p27 is a tumor suppressor gene located on chromosome 12p13, which encodes a nuclear protein that inhibits the cyclin D1/CDK complex during the cell cycle at the G0 or early G1 phase. It prevents inactivation of the pRb region and halts the cell cycle at the G1 to S phase. Contact inhibition, which occurs as a result of two cells touching each other after proliferation, has been found to increase p27 expression. p27 is also known to be effective in apoptosis. Genetic studies have demonstrated multiple organ hyperplasia and tumor development in mice after the loss of the p27 gene (9). Since p27 is normally located in the nucleus, its expression is generally observed immunohistochemically. Previous studies in the literature show that a decrease in p27 expression is associated with poor prognosis in many cancers, including breast, colon, and prostate carcinomas, non-small cell carcinomas of the lung, malignant melanomas, gastric cancers, and parathyroid cancers (10–13).

The mechanism by which p27 affects prognosis and metastasis is of concern. The hypothesis that p27 mutation causes the lower p27 expression has been proposed; however, it has been found that p27 mutations are very rare in tumors. The difference in p27 expression is thought to be due to post-translational targeted alterations. Visone R. et al. found evidence that the mechanism leading to decreased p27 expression in thyroid papillary carcinomas might be related to micro-RNAs (14). An increase in micro-RNA-221 and micro-RNA-222 has been found to decrease p27 expression in papillary thyroid carcinomas. This makes immunohistochemistry a valuable method to evaluate the effect of p27 on tumors.

Since p27 is known to be a cell cycle inhibitor, a decrease in its expression has been thought to lead to an excessive proliferation of cells. However, in studies of colon cancers and breast cancers that investigated this relationship, the p27 and Ki-67 proliferation indices were evaluated together and no evidence was found to support this hypothesis (15,16). It was found that a decrease in p27 expression, but not in the Ki-67 proliferation index, was associated with poor prognosis.

p27 is also known to be associated with cell-to-cell attachment (16). Loss of p27 expression is thought to provide resistance to changes in the extracellular matrix and intercellular attachment in tumors, thereby contributing to the spread of tumors. In a study on papillary carcinoma, Garcia-Rendueles AR et al. found that the oncoprotein p27 reorganizes the effects of TGF-β in thyroid cancer, explaining the slow proliferation but lack of apoptosis in PTCs (17).

Decreased expression or loss of p27 is known to be associated with poor prognosis in many cancers, such as those of the breast and pancreas (10). In addition, the cytoplasmic mislocalization of p27, which is generally known to be located in the nucleus, has also been found to have prognostic significance in some breast cancers. There are also some studies in the literature indicating that a decrease in p27 expression is associated with lymph node metastasis in papillary thyroid carcinoma (18,19). There is also one previous study about the relationship between p27 expression and distant metastasis in PTC cases (20). However, there is no analysis of the detailed morphological features concerning the alteration of p27 expression in PTC cases with distant metastasis. We therefore integrated the clinical and pathological features to assess the alteration of p27 expression in PTC cases with different metastatic status to fill this knowledge gap. The aim of this study was to investigate the role of p27 immunoexpression as a prognostic factor in the identification of the subgroup of papillary carcinomas with the potential to spread to distant organs.

MATERIALS AND METHODS

Study design

The study was conducted on a total of 44 cases with histopathologically diagnosed PTC, who underwent total thyroidectomy and were followed up in the endocrinology department of our hospital for at least six years. Of these PTC cases, 15 had iodine-positive distant organ metastases radiologically detected at the time of diagnosis, 12 had only lymph node metastases, and 17 had no metastases. Cases in the groups without metastases and with only lymph node metastases were known to have not developed distant organ metastases during follow-up for at least six years and were similar in terms of tumor diameter, tumor multicentricity, and patient age to the cases that developed distant organ metastases, to reduce possible bias between the groups. Since the age break point in the American Joint Committee on Cancer (AJCC) staging system for papillary thyroid cancer is 55 years, the cases were grouped as those under and over 55 years of age, and it was ensured that the cases in the three groups were in a similar age range, based on this age limit. Detailed information about the cases is presented in Table 1.

Pathological review

All of the hematoxylin-eosin (H&E)-stained slides were re-evaluated by two independent pathologists (BO, YSG) concerning tumor size, foci number, the presence of a capsule, capsular invasion, extrathyroidal extension, vascular invasion, lymph node invasion, distant metastasis, necrosis, mitosis, surgical margins, and morphological findings of the special subtypes which have more aggressive course. We have excluded the patients with aggressive subtypes of PTC to reduce the bias between groups. So, subtypes known to be associated with poor prognosis, such as solid variants and hobnail variants, were not included in the study.

Table 1. Clinicopathologic Characteristics of the Study Cases

Clinicopathologic Characteristics	Non-Metastatic PTC (n=17)	PTC with Only Lymph Node Metastases (n=12)	PTC with Distant Metastases (n=15)	p value
Age (%) < 55 years old ≥ 55 years old	 7 (41.76%) 10 (58.82%)	 6 (50%) 6 (50%)	 4 (26.66%) 11 (73.33%)	0.448
Primary tumor size (cm)	4.09 ± 2.03	3.14 ± 2.03	4.29 ± 2.46	0.283
Histopathological subtype (%) Classical variant Follicular variant Oncoytic variant Warthin-like variant	 3 (17.6%) 9 (52.9%) 4 (23.5%) 1 (5.8%)	 7 (58.3%) 3 (25%) 2 (16.6%) 0	 10 (58.8%) 5 (24.4%) 0 0	0.079
Multicentricity Unifocal Multifocal	 14 3	 12 0	 10 5	0.083
Lymphovascular invasion Yes No	 1 16	 1 11	 4 11	
Disease status at the last follow-up - Free of disease - Dead of disease -Dead of unknown reason -Alive with disease	 17 0 0 0	 12 0 0 0	 5 3 2 5	

PTC; Papillary Thyroid Carcinoma

Immunohistochemistry

In all cases, a representative paraffin block that contained both tumor and normal thyroid tissue was chosen for p27 immunohistochemical analysis. After a block representing the tumor was selected for immunohistochemical study, 4 µ-thick sections of the paraffin block were prepared for slides coated with poly-L-lysine. The sections were first deparaffinized with xylene, and their grades were progressively rehydrated by treatment with an ethanol solution. The antigen retrieval procedure was performed by keeping the sections in a pressure cooker with sodium citrate buffer solution (pH 6) at 100ºC for 4 minutes. Endogenous peroxidase activity was prevented by keeping the sections in a 3% solution of H2O2 for 15 minutes. The sections were then incubated with an anti-p27 antibody (p27, clone: R7, 1/150, Thermo Fischer Scientific, Cheshire, UK) for 1.5 h. After incubation, the primary antibody was made visible using a secondary antibody conjugated with a streptavidin-biotin complex (Lab VisionTM UltraVisionTM Detection System Large Volume anti-polyvalent HRP system, Fisher Scientific, Fremont, CA, USA) and a chromogen (Lab VisionTM Ready-To-Use AEC Substrate System, Thermo Fisher Scientific, CA, USA).

Normal thyroid tissue adjacent to the tumor was used as an internal positive control, and nonimmune serum was used as a negative control. Only the nuclear positivity of p27 was accepted as positive. The results were evaluated in a blinded fashion with no knowledge of the clinical data of the patients. Both the percentage of positively stained cells and the intensity of staining were evaluated. In this study, tumors were assigned a score of 0 (no staining), 1+ (≤ 10% of neoplastic cells staining), 2+ (11%–50% of neoplastic cells staining), or 3+ (> 50% of neoplastic cells staining). We compared the staining intensity of tumor cells with that of normal thyroid follicular cells. Tumor cells that had a staining intensity similar to normal thyroid follicular cells were classified as “strong staining”; cells seen only at high magnification (x400) were classified as “weak staining”; the cells with in-between staining intensity, as “moderate staining”; and cells with no staining were classified as “none.” Cases with no staining were included in the weak staining category.

Statistical Analysis

Statistical analysis was performed with the IBM SPSS 13.0 program (SPSS Inc., Chicago, IL, USA). To analyze the differences between groups, the Kruskal-Wallis test was used for quantitative data, and the

Pearson chi-square test was used for qualitative data; $p < 0.05$ was considered statistically significant.

RESULTS

A total of 44 cases, 14 males and 30 females, with an age range of 24–84 years, were included in the study. Of these, 15 cases had distant organ metastasis, 12 had only lymph node metastasis, and 17 cases were in the non-metastasis control group. In 7 of the 15 cases with distant metastases, concomitant lymph node metastasis was found. The most common metastatic sites were bone (8/15, 53.3%), followed by lung (6/15, 40%) and kidney (1/15, 6.7%). The distribution of PTC subtypes by histopathological evaluation of the cases is summarized in Table 1. Of the cases with distant organ metastasis, 10 had classical-variant papillary thyroid carcinoma, and 5 had follicular-variant papillary thyroid carcinoma. No distant organ metastasis was detected in the oncocytic variant of PTC cases, and only lymph node metastasis was detected in 2 cases with this morphology.

The mitotic index was examined in H&E-stained sections and was observed to be quite low in all of the cases (less than 2 mitoses per 2 mm2). No necrosis was observed. Hobnail morphology was observed at 5–10% in 3 of the cases with distant organ metastasis and 3% in only 1 of the cases without metastasis (Figure 1 A). H&E sections revealed lymphovascular invasion in 4 of the 15 cases (26.7%) with distant organ metastasis, 1 of the 12 (8.3%) cases with only lymph node metastasis, and 1 case (5.9%) without metastasis. A capsular structure around the tumor was observed in 9 of the 15 cases with distant organ metastasis, and 8 of the 9 cases had capsular invasion. The capsular structure was also observed in 8 of the 12 cases with only lymph node metastasis, of which 7 showed capsular invasion, and in 8 of the 17 cases without metastasis, of which 7 showed capsular invasion. Papillary endothelial hyperplasia was observed in one case with distant organ metastasis (Figure 1 B).

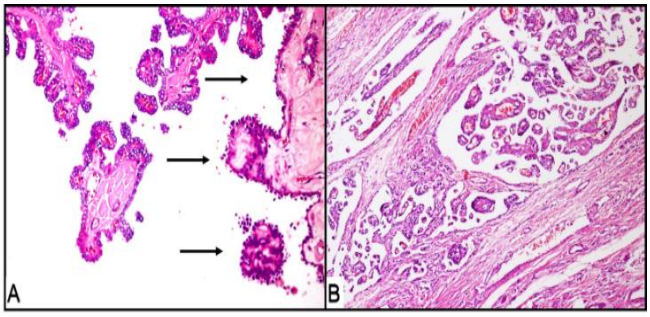

Figure 1.

A- Classical papillary thyroid carcinoma with focal hobnail morphology (black arrow) (H&E x400).

B- Papillary endothelial hyperplasia was observed in one case with distant organ metastasis (H&E x200).

At the same time, immunohistochemical staining using the p27 antibody was carried out for all cases. The surrounding thyroid tissue served as an internal positive control. The results of the immunohistochemical evaluation of p27 are summarized in Table 2.

Table 2. Relationship Between Metastatic Status and P27 Expression

	p27			
Metastatic status	**0**	**1+**	**2+**	**3+**
No Metastasis (n = 17)	0 (0%)	1 (5.9%)	9 (52.9%)	7 (41.2%)
Only Lymph Node Metastasis (n = 12)	1 (8.3%)	0 (0%)	5 (41.7%)	6 (50%)
Distant Organ Metastasis (n = 15)	3 (20%)	7 (46.7%)	5 (33.3%)	0
p = 0.002				

While 0 or 1+ staining was observed in 10 (66.7%) of the cases with distant organ metastasis (Figure 2 A-B), 16 (94.1%) of those without metastasis showed 2+ or 3+ staining (Figure 2 C-D), and 2+ or 3+ staining was also observed in 11 (91.7%) of the cases with only lymph node metastasis. It was noted that the staining pattern of p27 expression in cases without metastases and with only lymph node metastases were similar, but there was a marked loss of staining in those with distant organ metastases. The results of the statistical evaluation indicated that there was a significant relationship between metastasis status and p27 expression, with a decrease in p27 expression in cases with distant organ metastasis ($p = 0.002$). The results from the evaluation of p27 staining intensity are summarized in Table 3.

Table 3. Relationship Between Metastatic Status and P27 Staining Intensity

	p27 staining intensity		
Metastatic status	**Weak**	**Moderate**	**Strong**
No Metastasis (n=17)	2 (11.8%)	13 (76.4%)	2 (11.8%)
Only Lymph Node metastasis (n=12)	6 (50%)	4 (33.3%)	2 (16.7%)
Distant Organ Metastasis (n=15)	10 (66.7%)	4 (26.7%)	1 (6.6%)
p = 0.020			

Cases with no staining were included in the weak staining category. A statistically significant relationship was found between staining intensity and metastasis status ($p = 0.02$). Weak staining was observed in 10 of the cases (66.7%) with distant organ metastasis, while strong staining was observed in only 1 case (6.7%). Moderate or strong staining was observed in 88.2% of the cases without metastasis.

Regarding the relationship between p27 expression and extrathyroidal extension, 2+ or 3+ p27 staining was observed in 6 of the 14 cases (42.86%) with extrathyroidal extension and in 26 of the 30 cases (86.67%) without extrathyroidal extension This demonstrated a link between extrathyroidal extension and decreased p27 expression. The result was also statistically significant (p = 0.003) (Table 4). Both cytoplasmic and nuclear p27 expression were detected in a case with distant metastasis (Figure 2 E). In addition, cytoplasmic cross-reactions were encountered in cases with an oncocytic subtype (Figure 2 D).

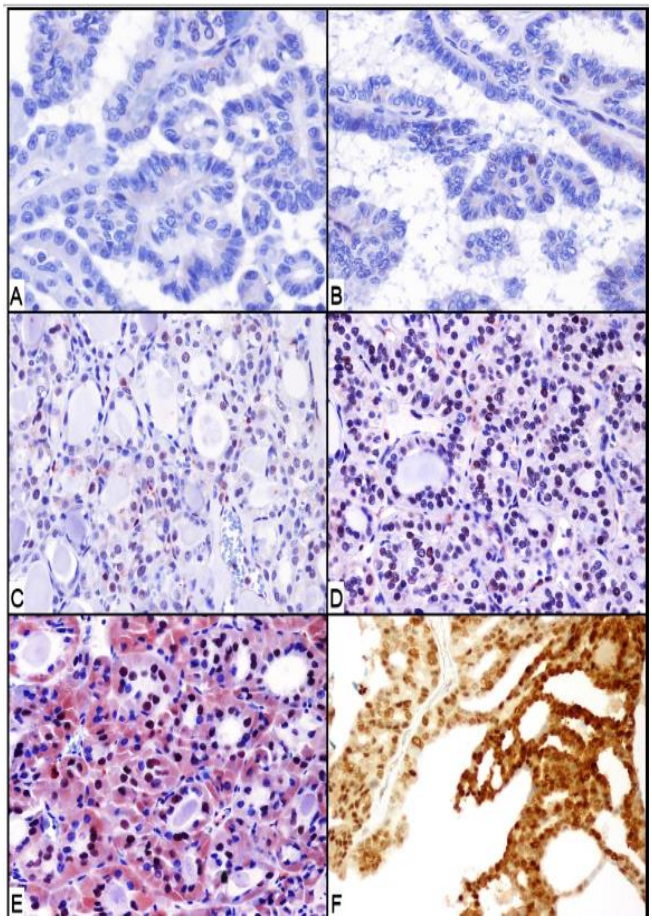

Figure 2. Immunohistochemical staining of p27.

A- Loss of p27 expression in one case with distant metastasis (p27x400).
B-1+ staining (p27x400).

C-2+ staining (p27x400).

D- 3+ staining (p27x400).

E-Cytoplasmic cross-reaction was encountered in the cases with the oncocytic (Hurthle) cell subtype (p27x400).

F-Both cytoplasmic and nuclear p27 expression were detected in a case with distant metastasis (p27x400).

Table 4. Relationship Between Extrathyroidal Extension and P27 Expression

	p27			
Extrathyroidal Extension	**0**	**1+**	**2+**	**3+**
Present	2 (14.3%)	6 (42.9%)	1 (7.1%)	5 (35.7%)
Absent	2 (6.7%)	2 (6.7%)	18 (60%)	8 (26.7%)
p = 0.003				

DISCUSSION

Based on reports from Western Europe, Canada, Australia, and the United Staes, the incidence of papillary thyroid carcinoma is globally increasing (21–25). However, it is uncertain if this increase is due to an actual rise in papillary tumors, or if it is the result of novel diagnostic techniques that have led to the detection of even occult papillary carcinomas. Although PTC has an indolent course (5–7%), distant organ metastasis has been reported in 9–10% of cases in some studies (1–5,26).

Since the relationship between certain PTC subtypes and a poor prognosis is a well-known fact, the aggressive subtypes should be mentioned in diagnostic reports for their prognostic significance. The hobnail variant, which is a papillary tumor with a hobnail morphology of more than 30%, is correlated with poor prognosis (27). Lino Silva LS et al. claimed that the presence of hobnail morphology of 5% and above directly affected the prognosis negatively and had to be mentioned (28). In our series, there were no cases that met the diagnostic criteria for hobnail variants, but a lower-rate hobnail morphology of 5–10% was observed in 3 of the cases with distant organ metastasis, while one non-metastatic case had a hobnail morphology of 3%. Even though it seems correlated with the previous knowledge of the literature, we do not have enough cases with hobnail morphology to evaluate the prognostic effect of this morphological feature. In the current study, bone (53.3%) and lung (40%) were the most common metastatic sites, similar to results found in the literature (29). Renal metastasis, which is extremely rare for papillary carcinomas, was detected in one case with a classical papillary thyroid carcinoma morphology.

Khoo MLC et al. examined p27 expression in a study of 125 PTC cases, 36 with lymph node metastasis and 89 without metastasis. They took into consideration the staining intensity while scoring. They observed a decrease in p27 expression in 12 of the 36 cases (33.3%) with lymph node metastasis and in 6 of the 80 cases (16.7%) without metastasis and concluded that decreased p27 expression was associated with lymph node metastasis in PTCs ($p < 0.001$) (18). Khoo ML et al. also evaluated papillary microcarcinomas in another study, and their data were similar to their previous study; the effect of p27 on the risk of metastasis in 55 papillary thyroid microcarcinoma cases—22 with lymph node metastasis and 33 without metastasis—was observed (30). They also carried out scoring by taking into consideration the staining intensity in their study. They observed that while 15 of the 22 cases (68.2%) with lymph node metastases showed

weak staining with scores of 0 and 1, these scores were also found in 6 of the 33 cases (18.2%) without metastases. They concluded that a decrease in p27 expression was statistically associated with lymph node metastasis in papillary microcarcinomas ($p < 0.005$) (30). In our study, approximately 83.3% of the cases with lymph node metastasis showed weak or moderate staining. No statistically significant relationship was observed between the decrease in p27 expression and lymph node metastasis based on our results ($p = 0.497$). However, we think that the small number of cases with lymph node metastasis may be the reason for this result.

Extrathyroidal extension is known to be a poor prognostic factor in PTCs. Do SI et al. suggested that a decrease in p27 expression was also associated with extrathyroidal extension in papillary thyroid carcinomas (31). In our study, 86.67% of the cases without extrathyroidal extension showed 2+ or 3+ staining with p27. We found a statistically significant relationship between a decrease in p27 expression and extrathyroidal extension ($p = 0.003$).

A series of recent studies have indicated that the cytoplasmic reaction of p27 has prognostic significance in malignancies (32,33). This effect is suggested to occur by inducing an epithelial-mesenchymal transition. We observed cytoplasmic staining in only one case with lung metastasis that had classical variant morphology. Cytoplasmic staining was considered only as observational data and was not taken into account in scoring, which was based only on nuclear staining. However, this case had a highly differentiated tumor, and there was no sarcomatoid differentiation. Two cases with the oncocytic subtype were observed to show pale intracytoplasmic staining in some areas. This was thought to be a common cross-reaction in oncocytic (Hurthle) cell tumors rather than the cytoplasmic expression of the protein. We believe that it may be useful to keep in mind this characteristic of the oncocytic variant when evaluating the cytoplasmic expression of p27 by the immunohistochemical method.

We found only one study in the literature comparing the correlation between p27 expression and distant organ metastasis of PTC. Liang H et al. investigated a total of 58 thyroid papillary carcinomas (27 with distant metastases and 31 without metastases) and found that a decrease in p27 expression was significantly related to metastasis ($p = 0.031$) (20). In that study, an anti-p27 antibody (clone: DCS-72.F6, Maixin Bio, China) was used, and evaluation was done by the electronic Pathological Visual Analysis Program. Both the staining percentage and intensity were taken into consideration. In this study, p27 expression was found to be high in the cases with no metastasis. Similar to the literature, decreased p27 expression was observed in the cases with distant organ metastasis ($p = 0.002$), and even 3 cases had a complete loss of p27 expression in our study. We reached similar results with this study conducted by Liang H et al. They have included the clinical features of the cases like our study. Even though they had more cases than our study, they did not specify the histopathological subtypes and histomorphological findings of the cases, as detailed in our study. We have excluded the patients with aggressive subtypes of PTC to reduce the bias between groups. In our study, two pathologists (BO, YSG) have also carefully evaluated the histomorphological features of tumors which will help us to have a better understanding of the tumors. We think that our results will make a good contribution to the previous data, with the meticulous pairing between the groups and the detailed examination of the histopathological findings.

In our study, we found a statistically significant decrease in p27 expression in cases with distant organ metastasis. We have made a detailed histomorphological analysis. However, some limitations of the current study warrant discussion. Firstly, we have had a small number of cases. We also did not have the genetic profiles and microRNA alterations of the cases in this study. We think that further studies of a larger number of cases and their genetic alterations will more clearly reveal the relationship between metastatic potential and p27 expression, as well as the possible mechanism. PTC is the most common thyroid malignancy, with a generally indolent clinical course. Distant metastasis, which is the most important criterion directly related to prognosis, is very rare. Therefore, the current approach is to use less aggressive treatments in papillary carcinomas.

Thus, the determination of the biological behavior of papillary carcinomas and the development of tailored treatments are very important. In this study, we evaluated p27 expression in PTCs with distant organ metastasis and compared it to similar tumors without metastasis. We found a significant decrease in staining intensity ($p = 0.02$) and ratio ($p = 0.002$) in PTCs with distant organ metastasis. In conclusion, immunohistochemical detection of p27 expression in PTCs may be used as a practical and cost-effective method to establish a better therapeutic approach.

In summary, this is among the first studies in PTC cases to evaluate associations between the alteration of p27 expression and metastatic status. In addition, to the best of our knowledge, we are the first that integrate the pathological and clinical features to evaluate p27 expression in PTC cases with distant metastasis.

Ethics Committee Approval: The present study was approved by the local ethics committee (Ethical Committee Approval Date: 05.07.2017 Number: GOKAEK 2017/191).

Author contributions: The study was designed by BO, ZOA, and YSG. BO, ZOA, and YSG collected the data. The study was analyzed by BO and YSG. BO, ZOA, YSG, and SS wrote the manuscript. YSG and SS supervised the manuscript. All authors contributed substantially to its review. BO takes responsibility for the paper as a whole.

Conflict of Interest: The authors have no commercial associations or sources of support that might pose a conflict of interest.

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