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Evaluation of Gastroenteropancreatic Neuroendocrine Tumors for Ki 67, p16 and Cyclin D1 Expression

Gastroenteropankreatik Nöroendokrin Tümörlerin Ki 67, p16 ve Siklin D1 Ekspresyonu Açısından Değerlendirilmesi

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SUMMARY

Objectives: *The aim of this study was to examine the roles of p16/cyclin D1 and Ki 67 proliferation index in gastroenteropancreatic neuroendocrine tumors (GEPNETs), which were classified by the World Health Organization (WHO).*

Methods: *A series of 41 cases of GEPNETs including 16 well-differentiated endocrine tumors (WDET), 17 well-differentiated endocrine carcinomas (WDEC) and 8 poorly differentiated endocrine carcinomas (PDEC) were searched for p16, cyclin D1 and Ki 67 expression.*

Results: *Overexpression of p16 and cyclin D1 was observed in 8 (50%) and 8 (50%) of 16 WDET cases, 12 (70%) and 5 (29%) of 17 WDEC cases and 5 (63%) and 5 (63%) of 8 PDEC cases. Low Ki 67 index (≤2%) was found in 15 (94%) of WDETs, 13 (76%) of WDECs and none of PDECs.*

Conclusion: *Ki 67 index was low in WDECs as in WDETs. High Ki 67 index (>10%) is related to poor prognosis, but low proliferation activity is not an indicator of benign behavior. There were no significant differences between the tumor groups with respect to p16 and cyclin D1 expression in our study. More objective results can be obtained in such studies that include retinoblastoma protein and analyze all of the components of the Rb pathway.*

Key words: *Cyclin D1; GEPNET; Ki 67 index; p16.*

ÖZET

Amaç: Bu çalışmanın amacı, Dünya Sağlık Örgütü'ne göre sınıflandırılmış gastroenteropankreatik nöroendokrin tümörlerde (GEPNET) p16 ve siklin D1'in rolünü ve Ki 67 proliferasyon indeksini incelemektir.

Gereç ve Yöntem: Toplam 41 olguluk GEPNET serisinin 16'sı iyi diferansiye nöroendokrin tümörden (İDNET), 17'si iyi diferansiye nöroendokrin karsinomdan (İDNEK) ve 8'i az diferansiye nöroendokrin karsinomdan (ADNEK) oluşmaktaydı. Bu tümör grupları p16, siklin D1 ve Ki 67 ekspresyonu açısından incelendi.

Bulgular: p16 ve siklin D1 over ekspresyonu İDNET grubunun 8'inde (%50) ve 8'inde (%50), İDNEK grubunun 12'sinde (%70) ve 5'inde (%29), ADNEK grubunun 5'inde (%63) ve 5'inde (%63) izlendi. Düşük Ki 67 indeksi (≤ %2) İDNET grubunun 15'inde (%94), İDNEK grubunun 13'ünde (%76) mevcuttu.

Sonuç: Bu çalışmada Ki 67 indeksi, İDNEK grubunda benign gruptaki gibi düşük bulunmuştur. Yüksek Ki 67 indeksi kötü prognoz ile ilişkilidir, ancak proliferatif aktivitenin düşük olması benign davranışın göstergesi olarak değerlendirilmemelidir. Çalışmamızda p16 ve siklin D1 ile ilgili anlamlı bir sonuç elde edilmemekle birlikte daha çok olgudan oluşan, retinoblastom (Rb) proteinin de dahil edildiği ve p16/siklin D1/Rb yolağının tümüyle incelendiği çalışmalarda daha anlamlı sonuçlar elde edilebilir.

Anahtar sözcükler: Siklin D1; GEPNET; Ki 67 indeks; p16.

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INTRODUCTION

Gastroenteropancreatic neuroendocrine tumors (GEPNETs) represent 2% of all tumors of the gastrointestinal tract. These tumors range from incidental benign lesions to highly metastatic tumors. Factors that determine the biologic behavior of endocrine tumors are complex and multifaceted.[1] Currently GEPNETs are classified as well differentiated endocrine tumors with benign (WDETB) or uncertain behavior (WDETUB), well differentiated endocrine carcinomas (WDECs) and poorly differentiated endocrine carcinomas (PDECs) according to the World Health Organization (WHO) 2000 guideliness.[2]

Ki 67 index, indicators of proliferative activity, is significant prognostic parameter. It is noticed that Ki 67 index ≤%2 in WDETs, %2 -%10 in WDECs and >%10 in PDECs in WHO 2000 guidelines. Genetic alterations and molecular mechanisms in these neoplasms are largely unknown. The role of p16/cyclin D1/Rb pathway in GEPNET tumor pathogenesis is not fully understood yet.[3,4]

The aim of this study was to examine the roles of p16/cyclin D1 and Ki 67 proliferation index in GEPNETs which were classified by WHO. We showed that Ki 67 index was low in WDECs as in WDETs. High Ki 67 index (>10%) is related to poor prognosis but low proliferation activity is not indicator of benign behavior.

MATERIALS AND METHODS

Cases

Cases of primary endocrine tumors in the GI tract and pancreas diagnosed in Istanbul Training and Research Hospital Pathology Department from January 2001 to June 2009 were reviewed. Pathologic material (H/E sections and paraffin blocks) obtained from archieve. Neuroendocrine differentiation of the lesions can be demonstrated by immunohistochemical studies of neuroendocrine markers: Chromogranin A (LK2H10+PHE5, Neomarkers), Synaptophysin (27G12, Novocastra), Neuron-specific enolase (Clone E 27, Neomarkers). The majority of tumors were diffusely (>%50) and strongly positive for two of three neuroendocrine markers. The tumors were classified as WDETB or WDETUB, WDEC and PDEC according to the WHO 2000 guidelines. In this classification tumor diameter, localisation, depth of invasion, angiolymphatic invasion and metastatic state were considered (Table 1, Table 2).

Table 1. WHO 2000 guidelines in GEPNET'S

Well differentiated endocrine tumor	**Benign:** Limited to mucosa and submucosa, without angioinvasion, ≤1cm in size (for stomach and small intestine), ≤2 cm in size (for colon and rectum)
	Uncertain behavior: Limited to mucosa and submucosa with angioinvasion, >1 cm (for stomach and small intestine), >2 cm (for colon and rectum)
Well differentiated endocrine carcinoma (Low grade malign potential)	Invasion beyond submucosa or metastasis
Poorly differentiated endocrine carcinoma (High grade malign potential)	

Table 2. Classification of appendiceal neuroendocrine tumors according to WHO

Well differentiated endocrine tumor	**Benign:** Limited to appendix wall (without extension into mesoappendix), non angioinvasive, ≤2 cm in size
	Uncertain behavior: Limited to subserosa with angioinvasion or >2 cm in size
Well differentiated endocrine carcinoma (Low grade malign potential)	Invasion of mesoappendix and/or metastasis
Poorly differentiated endocrine carcinoma (High grade malign potential)	

Table 3. Primary antibody list

Antibody	Company	Clone	Dilution
P16INK4a	Neomarkers	16P04	1/30
Cyclin D1	Neomarkers	P2D11F11	1/200
Ki-67	Neomarkers	SP6	1/200
Synaptophysin	Novocastra	27G12	1/200
Chromogranin A	Neomarkers	LK2H10 + PHE5	1/200

Immunohistochemical Staining

Immonohistochemical analysis was performed with antibodies against the following proteins: chromogranin A, synaptophysin, NSE, p16, cyclin D1, Ki 67. We studied chromogranin A, synaptophysin and NSE to verify the diagnosis, p16 and cyclin D1 to pose their roles in pathogenesis, Ki 67 to show proliferation activity. Clones, antigen retrieval methods and commercial sources of antibodies listed in Table 3. Immunohistochemical studies were performed with the streptovidin-avidin- biotin method according to standard procedures in Bond fully integrated IHC and ISH system. Tissue specimens of neuroendocrine tumors were formalin-fixed and paraffin-embedded, and cut into 5 μm thick sections for staining. All sections were kept in the incubator to be deparafinize for a night.

All immunohistochemical preparations were evaluated by two pathologists without knowledge of the diagnosis. Only nuclear staining was considered for the assessment of p16 and Rb reactivities.

Proliferation zone cells that locate in gastrointestinal mucosal basal layer were regarded as positive internal control for Ki 67. In maximum nuclear staining area we counted 2000 cells (average 4 HPF) and set its percentage.[2]

While p16 was assessed; squamous cell carcinoma of cervix was used for positive control. Even though both cytoplasmic and nuclear expression existed, only nuclear expression was concerned (100). Distribution of nuclear expression was graded as 0, <10%; +1, 10% to 30%; +2, 31% to 60%; +3, >60%. A tumor was considered positive if more than 10% of the tumor cell were stained.[5,6]

Non-tumoral gastrointestinal mucosal cells and lymphoid tissue were used as positive control for cyclin D1 expression. In the assessment of cyclin D1 staining, the staining distribution was graded as 0, 1, 2, 3 when <5%, 5% to 10%, 11% to 50%, >50% tumor cells stained, and the staining intensity was graded as 1, 2, 3 when the staining was weak, moderate or strong. A tumor with a staining score (distribution score + intensity score) of 2 or greater was acknowledged as positive for cyclin D1.

Statistical Analysis

The association between immunohistochemical staining and histopathologic data was estimated by using the Pearson χ^2 test, as appropriate.

RESULTS

Patients

We studied 41 cases of gasrointestinal tract and pancreatic endocrine tumors; 10 cases were WDETB, 6 cases were WDETUB, 17 cases were WDEC and 8 cases were PDEC.

The most common localization were appendix including 15 cases, the other localizations with decreasing frequency were stomach (7 cases), colon (5 cases), rectum (4 cases), caecum (3 cases), pancreas (3 cases), ileum (2 cases), duedonum (1 case) and eusophageus (1 case). There were 21 women and 20 men. The youngest patient was 17 years old, the oldest one was 83. The mean of the patients age was 48. There was no statistically significant difference in ages and genders of the groups.

Immunohistochemical Staining for Ki 67, p16 and Cyclin D1

In our 41 cases there were 28 tumors that Ki 67 index was equal or less than 2% and included in WDETB, WDETUB and WDEC. In those 41 tu-

Table 4. The distribution of Ki 67 index between all tumor groups

Groups	=%2	%3-10	>%10	Total
WDETB	9	1	0	10
WDETUB	6	0	0	6
WDEC	13	4	0	17
PDEC	0	0	8	8
Total	28	5	8	41

Table 5. The distribution of p16 expression between all tumor groups

	WDETB	WDETUB	WDEC	PDEC
p16 expression				
(+)	3	5	12	5
(–)	7	1	5	3
Total	10	6	17	8

Table 6. The distrubition of cyclin D1 expression between all tumor groups

	WDETB	WDETUB	WDEC	PDEC
Cyclin D1 expression				
(+)	6	2	5	5
(–)	4	4	12	3
Total	10	6	17	8

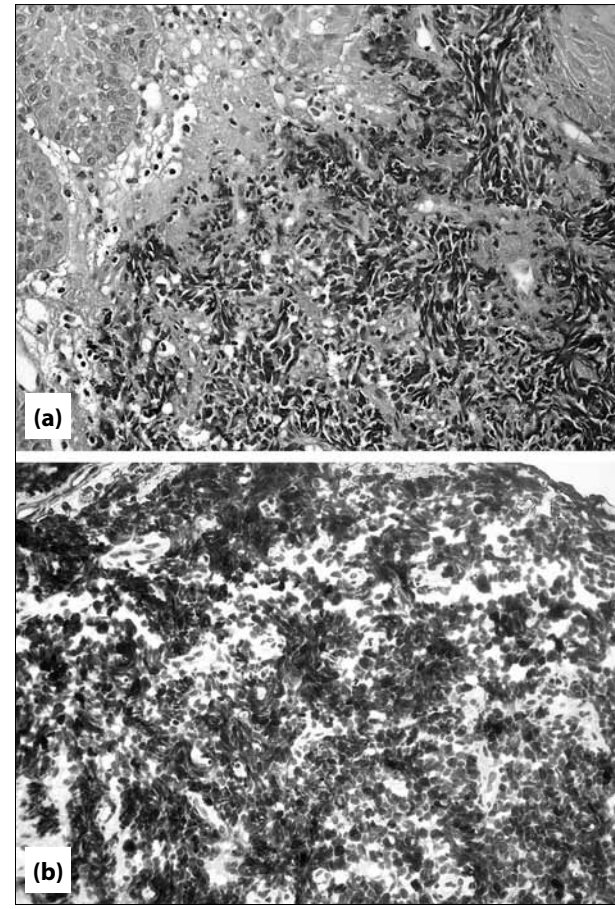

Fig. 2. **(a)** Esophagial PDEC. **(b)** Esophagial PDEC with high Ki 67 proliferation index.

mors; there were 5 (12%) cases where Ki 67 index was between 3% to 10%. 4 of those tumors was in WDEC group and 1 of them was in WDETB group (Fig. 1). In 8 (19.5%) PDEC group, Ki 67 index is over 10% (Fig. 2).

Between WDET (WDETB + WDETUB) and WDEC groups, there was not any significant differences with regard to Ki 67 index ($p>0.05$), in PDECs this parameter was relatively higher than other groups ($p<0.001$). The association between Ki 67 index and tumor groups is summarized in Table 4.

Overall, 25 (61%) of all 41 cases showed p16 expression. In all these cases, 3 of 10 WDETB group, 5 of 6 WDETUB group, 12 of 17 WDEC group, 5 of

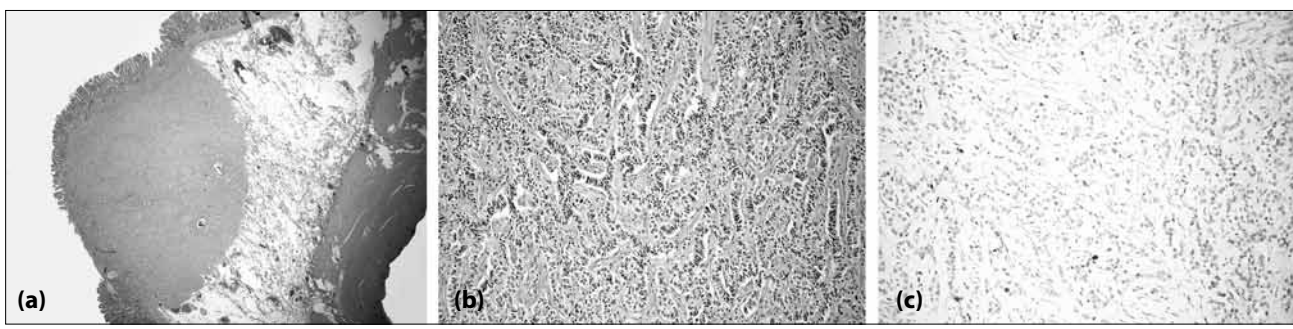

Fig. 1. **(a)** Gastric WDEC which localized in submucosa, smaller than 1 cm and showed lymph node metastasis. **(b)** Gastric WDETB with trabecular pattern. **(c)** Gastric WDEC with low Ki 67 proliferation index.

8 PDEC showed p16 expression (Table 5) (Fig. 3).

The same cases were searched for cyclin D1 as well, in 17 of 41 cases showed cyclin D1 expression. In these cases 6 of 10 WDETB group, 2 of 6 WDETUB group, 5 of 17 WDEC group, 5 of 8 PDEC group showed cyclin D1 expression (Table 6) (Fig. 4).

Between benign and malign groups and between WDEC and PDEC groups there were not significant differences with respect to p16 and cyclin D1 expression ($p>0.05$). At the same time there were not any significant differences for expression phenotype as well ($p>0.05$) (Table 7, Table 8)

DISCUSSION

The gastroenteropancreatic neuroendocrine tumors (GEPNETs) are rare neoplasms derived from cells with a neuroendocrine phenotype. Oberndorfer used the term of "carcinoid" for these tumors firstly in 1907. Actually the malignant potential of GEPNETs is difficult to predict.[7-9] To determine their clinical behavior, these neuroendocrine tumors are classified on the basis of their clinicopathological features including size, local invasion, angioinvasion, proliferative activity, histological differentiation and metastases. The 2000 WHO classification divides GEPNETs into well differentiated endocrine tumors with benign

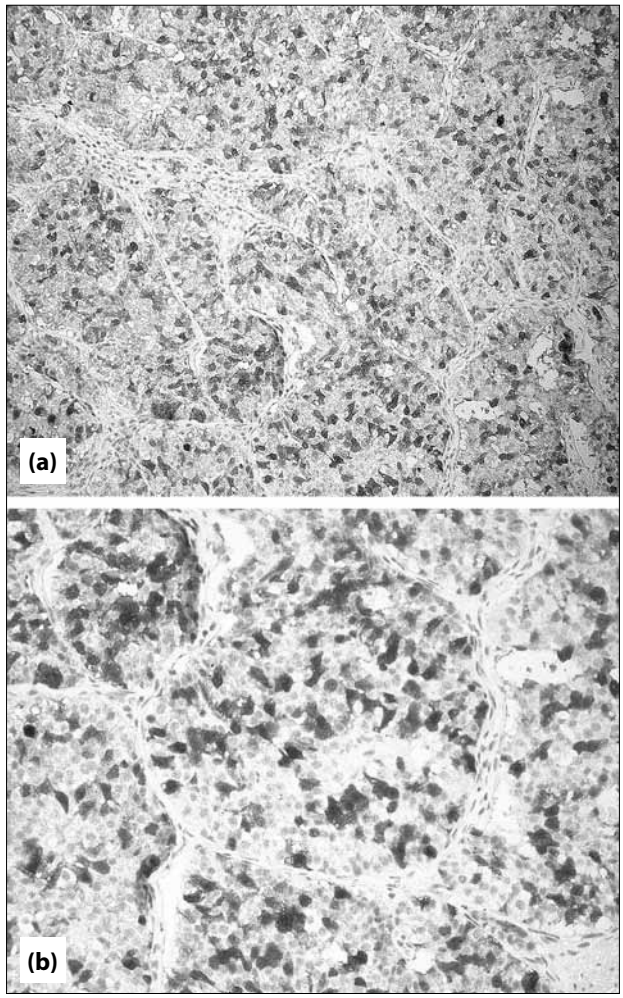

Fig. 3. **(a)** Duedonal WDETB, positive p16 immunostaining with more than 60% positive cells, positive for p16 over expression (+3). **(b)** Gastric WDEC, positive p16 immunostaining with 50% positive cells, positive for p16 over expression (+2).

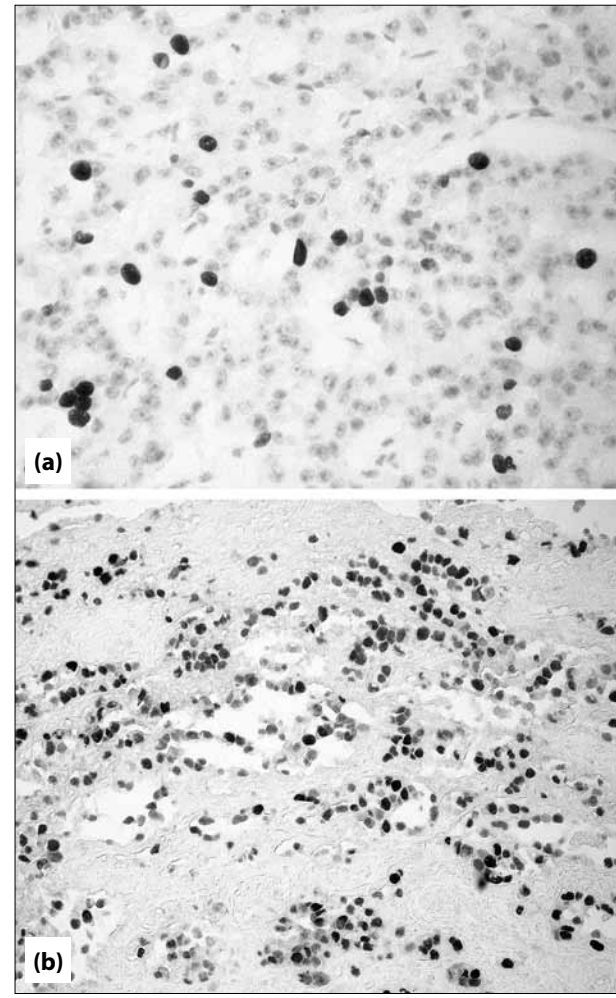

Fig. 4. **(a)** Caecal WDEC, positive cyclin D1 immunostaining with 15% positive cells (+2) and strong nuclear staining (+3) (Score: 5). **(b)** Gastric PDEC, positive cyclin D1 immunostaining with more than 50% positive cells (+3) and strong nuclear staining (+3) (Score: 6).

Table 7. p16 and cyclin D1 expression in benign and malignant groups and different expression phenotye

	Benign		Malignant			
	n	%	n	%	X^2	p
p16						
Negative	8	50.0	8	32.0		
Positive	8	50.0	17	68.0	1.32	0.249
D1						
Negative	7	43.8	15	60.0		
Positive	9	56.3	10	40.0	1.03	0.309
Combined						
P16(-)D1(-)	2	12.5	6	24.0		
P16(+)D1(-)	5	31.3	9	36.0		
P16(-)D1(+)	6	37.5	2	8.0		
P16(+)D1(+)	3	18.8	8	32.0	5.71	0.126

Table 8. p16 and cyclin D1 expression in WDECs and PDECs and different expression phenotype

	PDEC		WDEC			
	n	%	n	%	X^2	p
p16						
Negative	3	37.5	5	29.4		
Positive	5	62.5	12	70.6		–
Cyclin D1						
Negative	3	37.5	12	70.6		
Positive	5	62.5	5	29.4		0.194
Combined						
P16(-)D1(-)	1	12.5	5	29.4		
P16(+)D1(-)	2	25.0	7	41.2		
P16(-)D1(+)	2	25.0				
P16(+)D1(+)	3	37.5	5	29.4	–	–

or uncertain behavior, well differentiated endocrine carcinomas and poorly differentiated endocrine carcinomas.[2]

Mitosis and Ki 67 index, indicators of proliferative activity, are significant prognostic parameters. Ki-67 immunostaining can detect a larger proportion of cells in the replicative pool rather than just mitotic figure frequency. In fact it can detect all phases of the cell cycle (G1, S, G2, and M phases) except the G-zero phase.[10,11]

It is noticed that Ki 67 index is ≤%2 in WDETs, %2 -%10 in WDECs and >%10 in PDECs in WHO 2000 guidelines. We found that Ki 67 index was high (>%10) in PDECs compared to other groups, but there was not any statistically significant difference between WDET (benign and uncertain behavior) and WDEC groups regard to Ki 67 index. Alexiev at all didn't find statistically significant correlation between tumor grade and Ki-67 index and Ki-67 index and metastatic behavior in their study which was included in 38 GEPNET cases.[12] Kawahara at al. detected that Ki 67 staining was not correlate with malignant behavior in their study based on analyses of 41 cases, similarly.[13] According to our study high Ki 67 index is associated with poor prognosis but low proliferative activity should not be evaluated as predict of benign behavior.

The malignant potential of endocrine tumors is difficult to predict. It has shown that small, low grade NETs with low proliferative index which met the criteria of the WHO classification criteria for the benignity could made metastasis. According to WHO guidelines it is not possible to evaluate the certain criteria for malignancy as the presence of metastasis and muscularis propria invasion in biopsi materials. Factors that determine the biologic behavior of endocrine tumors are complex and multifaceted. Recent studies on neuroendocrine tumors have focused on predicting of prognostic factors, but genetic alterations and molecular mechanisms in these neoplasms are largely unknown. The p16 and cyclin D1 genes are components of the p16/cyclin D1/Rb pathway that controls G1-S checkpoint of the cell cycle. The control of the progression of G1 phase and G1-S checkpoint is abnormal in tumors and this resuts in endless cell cycle entrance and cell proliferation. The role of p16/cyclin D1/Rb pathway in GEPNETs pathogenesis is not fully understood yet.[3,4]

It has shown that p16 overexpression is associated with HPV in cervical and tonsillar carcinomas.[14] Most of the studies on neuroendocrine tumors have focused on small cell lung carcinomas and carcinoids of the respiratory system. There is a small number of study about p16/cyclin D1 expression in GEPNETs in the literature. Li at al. demonstrated that overexpression of p16 in 73% of the PDECs and none of the well differentiated endocrin neoplasms (WDENs) in a series of 57 cases of gastrointestinal tract endocrine tumors. According to their study overexpression of p16 is the major etiologic factor in PDECs.[14] Nevertheless, they have found over-expression of p16 in 76% of PDECs, on the other hand they didn't find any over-expression of p16 in WDETs in their study which composed of 76 cases.They have detected overexpression of cyclin D1 was significantly associated with WDENs.[15]

We didn't find any significant differences regard to p16 and cyclin D1 expression between all tumor groups in our study. Similar to our study Kawahara et al.[13] didn't find any statistically meaningfull differences between benign and malignant groups respect to cyclin D1 expression in their study that composed of 41 cases. Igarashi et al.[5] didn't detect any significant differences between typical and atypical pulmonary carcinoids according to loss of p16 and expression of cyclin D1. Nevertheless, in Beasley's[6] study there was not statistically meaningfull results between typical and atypical pulmonary carcinoids according to loss of p16.

In our study p16 expression was seen 12 (80%) of 15 cases with appendiceal localization. In none of these 12 cases, except one, loss of cyclin D1 striking. As it mentioned before, in previous studies, these results might have occured due to the differences of tumors in these locations. Our statistical results related to p16 are not consistent with some studies of literature because of insufficient number of cases, methodological differences or use of different antibodies. More fair results could be obtained in such studies that include Retinoblastoma protein and analyze all of the components of Rb pathway.

The biological behavior of these tumors is still largely unknown. Nowadays, a large series of immunohistochemical, molecular and genetic studies are needed which can detect the biologic behavior of GEPNET's.

REFERENCES

1. Grame-Cook F. Neuroendocrine tumors of the gastrointestinal tract and appendix. In: Odse RD, Goldblum JR, editors. Surgical pathology of the GI tract, liver, biliary tract and pancreas. 2nd ed. Philadelphia: 2009. p. 653-93.
2. Klöppel G, Rindi G, Anlauf M, et al. Site-spesific biology and pathology of gastroenteropancreatic neuroendocrine tumors. Virchows Arch 2007;451 (Suppl 1):S9-S27.
3. The cell cycle and programmed cell death. In: Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P, editors. Molecular biology of the cell. 4th ed. New York: Taylor and Francis Group; 2002. p.983-1026.
4. Kishimoto I, Mitomi H, Ohkura Y, et al. Abnormal expression of p16(INK4a), cyclin D1, cyclin-dependent kinase 4 and retinoblastoma protein in gastric carcinomas. J Surg Oncol 2008;98:60-6.
5. Igarashi T, Jiang SX, Kameya T, et al. Divergent cyclin B1 expression and Rb/p16/cyclin D1 pathway aberrations among pulmonary neuroendocrine tumors. Mod Pathol 2004;17:1259-67.
6. Beasley MB, Lantuejoul S, Abbondanzo S, et al. The

P16/cyclin D1/Rb pathway in neuroendocrine tumors of the lung. Hum Pathol 2003;34:136-42.

7. Alexiev BA, Drachenberg CB, Papadimitriou JC. Endocrine tumors of the gastrointes-tinal tract and pancreas: grading, tumor size and proliferation index do not predict malig-nant behavior. Diagn Pathol 2007;2:28.
8. Modlin IM, Shapiro MD, Kidd M. Siegfried Oberndorfer: origins and perspectives of carcinoid tumors. Hum Pathol 2004;35:1440-51.
9. Abraham SC, Carney JA, Ooi A, et al. Achlorhydria, parietal cell hyperplasia, and multiple gastric carcinoids: a new disorder. Am J Surg Pathol 2005;29:969-75.
10. Key G, Becker MH, Baron B, et al. New Ki-67-equivalent murine monoclonal antibod-ies (MIB 1-3) generated against bacterially expressed parts of the Ki-67 cDNA contain-ing three 62 base pair repetitive elements encoding for the Ki-67 epitope. Lab Invest 1993;68:629-36.
11. McCormick D, Chong H, Hobbs C, et al. Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB1. Histopathology 1993;22:355-60.
12. Alexiev BA, Drachenberg CB, Papadimitriou JC. Endocrine tumors of the gastroin-testinal tract and pancreas: grading, tumor size and proliferation index do not predict malignant behavior. Diagn Pathol 2007;2:28.
13. Kawahara M, Kammori M, Kanauchi H, et al. Immunhistochemical prognostic indicators of gastrointestinal carcinoid tumours. Eur J Surg Oncol 2002;28:140-6.
14. Li AF, Tsay SH, Liang WY, et al. Clinical significance of p16INK4a and p53 over-expression in endocrine tumors of the gastrointestinal tract. Am J Clin Pathol 2006;126:856-65.
15. Li AF, Li AC, Tsay SH, et al. Alterations in the p16INK4a/cyclin D1/RB pathway in gastrointestinal tract endocrine tumors. Am J Clin Pathol 2008;130:535-42.