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Expression of p16 Protein and Cyclin D1 in Periampullary Carcinomas

Periampuller Karsinomlarda p16 ve Siklin D1 Ekspresyonu

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ABSTRACT

Objective: The majority of the tumors in periampullary region are pancreatic and ampulla of Vater carcinomas. The aim of this study was to compare histopathological features of ampulla of Vater carcinomas with pancreatic ductal adenocarcinomas and to determine diagnostic and predictive values of p16 protein and cyclin D1 expression.

Material and Method: Tissue samples from pancreatic ductal adenocarcinomas and ampulla of Vater carcinomas were obtained from 31 patients who underwent pancreticoduodenectomy for periampullary carcinoma. The study group was composed of 12 women and 19 men. Their median age was found to be 62.32 years (range 26-85 years). The parameters analyzed in the study included lymph node metastases, perineural invasion, differentiation, duodenal invasion, grade of intraepithelial neoplasia and p16 and cyclinD1 expression in tumoral and peritumoral pancreatic tissues.

Results: In both tumor groups, the loss of p16 protein expression was significantly correlated with perineural invasion (p=0.0001). Perineural invasion was more frequent in the pancreatic ductal adenocarcinoma group than the ampulla of Vater carcinoma group (p=0,01). When desmoplasia and lymphoplasmacytic stromal infiltration were examined, desmoplastic reaction was significantly higher in pancreatic ductal adenocarcinomas than ampulla of Vater carcinomas (p=0.01). No significant difference was observed between tumor groups for Cyclin D1 (p>0.05).

Conclusion: The results suggest that loss of p16 protein expression may be a sign for poor prognosis in periampullary cancers that is correlated mainly with perineural invasion. Desmoplastic stromal reaction may be a distinctive feature for pancreatic ductal adenocarcinoma compared with ampulla of Vater carcinoma.

Key Words: Carcinoma, p16, Cyclin D1, Ampulla of Vater, Histopathology, Pancreatic ductal carcinoma

ÖZ

Amaç: Periampuller bölgedeki tümörlerin çoğunluğu pankreatik ve ampulla Vater karsinomlarıdır. Bu çalışmanın amacı, pankreatik duktal adenokarsinomlar ile ampulla Vater karsinomlarının histopatolojik özelliklerini karşılaştırmak ve p16 ve siklinD1 ekspresyonunun tanısal ve prediktif değerini tespit etmektir.

Gereç ve Yöntem: Pankreatik duktal adenokarsinom ve ampulla Vater karsinomlarına ait doku örnekleri periampuller karsinoma tanısı ile pankreatikoduodenektomi yapılan 31 hastadan elde edildi. Hasta grubu 12 kadın ve 19 erkekten oluşmaktaydı. Ortalama yaş 62,32 (26-85 yaş arası) olarak saptandı. Tümöral ve peritümöral pankreatik dokuda lenf düğümü metastazı, perinöral invazyon, diferansiyasyon, duodenal invazyon, intraepitelyal neoplazi derecesi ve p16 ve siklin D1 ekspresyonunu içeren parametreler analiz edildi.

Bulgular: p16 protein ekspresyon kaybı, her iki tümör grubunda perinöral invazyon ile anlamlı şekilde korele idi (p=0,0001). Pankreatik duktal adenokarsinomlar grubunda perinöral invazyona, ampulla Vateri karsinomları grubuna göre daha sık rastlandı. (p=0,01). Desmoplastik reaksiyon bulgusu, pankreatik duktal adenokarsinomlarda ampulla Vater karsinomlarına göre daha anlamlı saptandı (p=0,01). Siklin D1 için gruplar arasında anlamlı farklılık bulunmadı (p>0,05).

Sonuç: Bu bulgular, perinöral invazyonla korele olan p16 protein ekspresyon kaybının, periampuller kanserlerde kötü prognoz göstergesi olabileceğini düşündürmektedir. Desmoplastik stromal reaksiyon pankreatik duktal adenokarsinomda ampulla Vater karsinomu ile kıyaslandığında ayırıcı tanı için ayırd ettirici özellik olabilir.

Anahtar Sözcükler: Karsinom, p16, Siklin D1, Ampulla Vater, Histopatoloji, Pankreatik duktal karsinom

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INTRODUCTION

The term "periampullary cancer" defines the tumors located in the region of the ampulla of Vater although the tumors of this region may originate from different anatomic locations (1,2). The majority of the tumors seen in the periampullary region originates from the pancreas and ampulla of Vater. Pancreatic ductal adenocarcinoma (PDAC) is the most common pancreatic tumor and one of the most fatal human cancers (3). Ampulla of Vater carcinomas (AVCs) are rare but they are second among the periampullary tumors after the most commonly seen pancreatic head tumors (1).

Cyclins were revealed in some studies to show different abnormalities in the progression of the pancreatic carcinomas. Cyclin D1 associates with CDK4/6 and plays an important role during the early G1 phase of cell cycle. The cyclin D1- CDK4/6 complex is inhibited by p16, the cell cycle regulator gene that has been most exhaustively studied in pancreatic ductal cancer (4-6). p16 (also called $p16^{INK4}$, CDKN2 or MTS1) is a tumor suppressor gene located on chromosome 9p21 and involved in the pathogenesis of many tumors. It belongs to the cyclin-dependent kinase inhibitor family and inactivates the complex of D-type-cyclin and cyclin-dependent-kinase-4 and blocks the progression of cells through G1 phase (1). Several studies have shown that inactivated p16 tumor suppressor protein expression among these gene abnormalities was markedly important in the development of the pancreatic adenocarcinomas (1,7-14).

The aim of this study was to compare the histopathological features of AVC with PDAC in the periampullary region and to determine diagnostic and predictive values of p16 protein, cyclinD1 expression and pancreatic intraepithelial neoplasia (PanIN) lesions in tumoral and peritumoral pancreatic tissues.

MATERIAL and METHOD

Paraffin-embedded tissue samples of PDAC and AVC were obtained from patients who underwent pancreticoduodenectomy for periampullary carcinoma in the Pathology Departments of Pamukkale University and Cumhuriyet University The defining criteria for the two subgroups (PDAC vs. AVC) was anatomic allocation. 20 of the samples were diagnosed as PDAC and 11 were diagnosed as AVC. The group of patients included 12 women and 19 men. Their median age was 62.32 years (range 26-85 years). Tissue samples were first examined as Hematoxylin-Eosin stained sections for the tumor characteristics such as lymph node status, perineural invasion, differentiation, duodenal invasion and grade of PanIN.

Immunohistochemistry

Histological sections (5 μm) were prepared from the specimens, mounted on poly-l-lysine-coated slides, and dried for 12–24 hours at 37 °C. Immunohistochemistry was performed with the avidin–biotin complex (ABC) kit using the following primary antibodies. Monoclonal antibodies of p16 (Dilution: 1/100, Clone: 6H12, Novocastra, Norwell, MA, USA) and cyclin D1 (Dilution: 1/100, Clone: SP4, Neomarker, Freemont, CA, USA) were used as immunohistochemical markers.

The sections were deparaffinized in xylene and then rehydrated in graded alcohol solutions. The tissues were then treated for 15 minutes with a 3% (v/v) solution of H_2O_2 and rehydrated. Slides were then exposed to microwave pre-treatment by immersion in 10 mM EDTA (pH 8.0), followed by 15 minutes of cooling at room temperature. Afterwards, the slides were rinsed with phosphate-buffered saline (PBS), and 100 μl of the appropriate dilution of antibody was added for one and a half hours and rinsed in PBS solution. Later, slides were stained with the Universal LSAB peroxidase kit (Lab Vision USA) and were counterstained with Mayer's hematoxylin and covered with slide covers. The slides were then examined with light microscopy.

During immunohistochemical analysis, p16 evaluation was as negative: no cytoplasmic and nuclear staining was present, score 1: weak diffuse cytoplasmic and weak nuclear expression in only one lobe, score 2: weak diffuse cytoplasmic and weak nuclear expression in only two lobes, score 3: weak diffuse cytoplasmic and weak nuclear expression in only five lobes, score 4: strong nuclear expression and score 5: strong nuclear and cytoplasmic expression.

The cyclin D1 immunohistochemical analysis was scored as negative if no nuclear expression was present, score 1 was 0-5% nuclear expression, score 2 6-25% nuclear expression, and score 3 26-100% nuclear expression.

Statistical analysis

Statistical analysis of the data was performed with the SPSS 10.0 statistical package software (SPSS Inc., Chicago, IL, USA) using the Chi-square, Mann-Whitney and Student's T tests. The results were considered as statistically significant when $p<0.05$.

RESULTS

Out of the 20 cases with PDAC, 12 cases had cyclinD1 expression, while 8 cases were not found to have expression. As concerns the 11 cases with AVC, 7 cases expressed cyclinD1, while 4 cases did not. No significant difference

was observed between the groups for Cyclin D1 (p>0.05). In 20 cases with PDAC, only one case showed p16 protein expression, while 19 cases had p16 protein loss (Figure 1). In 10 cases with AVC, 8 cases expressed p16 protein, while 2 cases had p16 protein loss (Figure 2)

PanIN distribution in peripancreatic region neighbouring and associated with PDAC and AVC were explored. Among 20 PDACs, only seven cases were found to be neighboured and associated with PanIN lesions while 13 cases showed no association with PanIN lesions. When those cases were categorized according to PanIN types, five cases were together with PanINb, one case was together with PanIN2 and one case was together with PanIN3. None of the cases had an association with PanIN1a. When AVCs were considered, no PanIN was detected in eight cases in the peripancreatic region while two cases had PanIN1b and only one case had PanIN2 association (Table I). There were no PanIN1a and PanIN3 lesions neighbouring pancreatic tissue in the AVC cases. In this respect, when PDAC and AVC were compared according to PanIN lesion association, there was no statistically significant difference between these two major tumor groups (p>0.05). Among 20 PDACs, 12 (60%) had PanIN lesions including two cases (10%) of PanIN1a, four cases (20%) of PanIN2 and 6 cases (30%) of PanIN3.

The presence of desmoplasia and lymphoplasmacytic inflammation was found to be significantly higher in PDACs than AVCs (p=0.001) (Figure 3A, B). Among AVC cases, these findings were absent in 63.6% of cases. Thus, desmoplasia and lymphoplasmacytic inflammation were found to be important features in PDACs.

Perineural invasion in the PDACs was highly statistically significant compared with AVCs (p=0.001). Among 20 cases

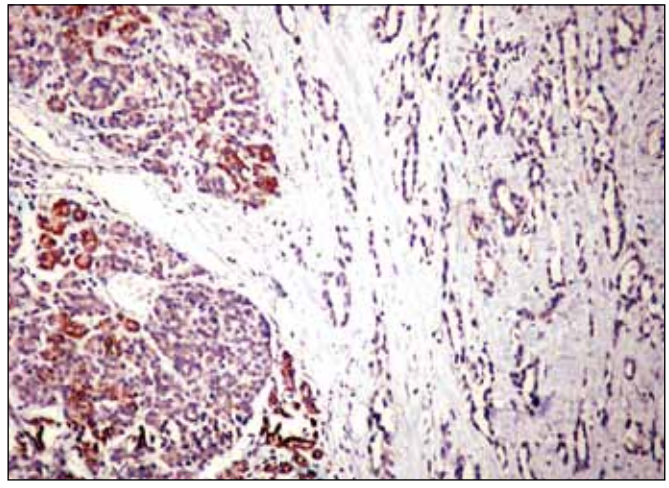

Figure 1: Loss of p16 protein expression in pancreatic ductal adenocarcinoma. Note that normal pancreatic tissue expresses p16 protein on the left but the tumor on the right does not (H&E; x100).

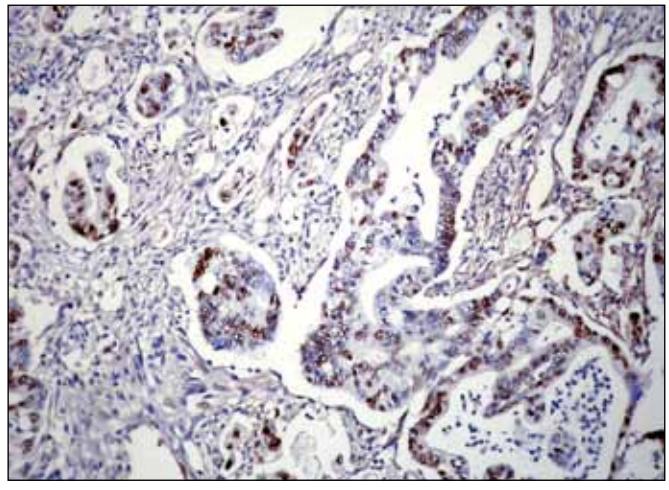

Figure 2: Expression of p16 protein in ampulla of Vater carcinoma (H&E; x200).

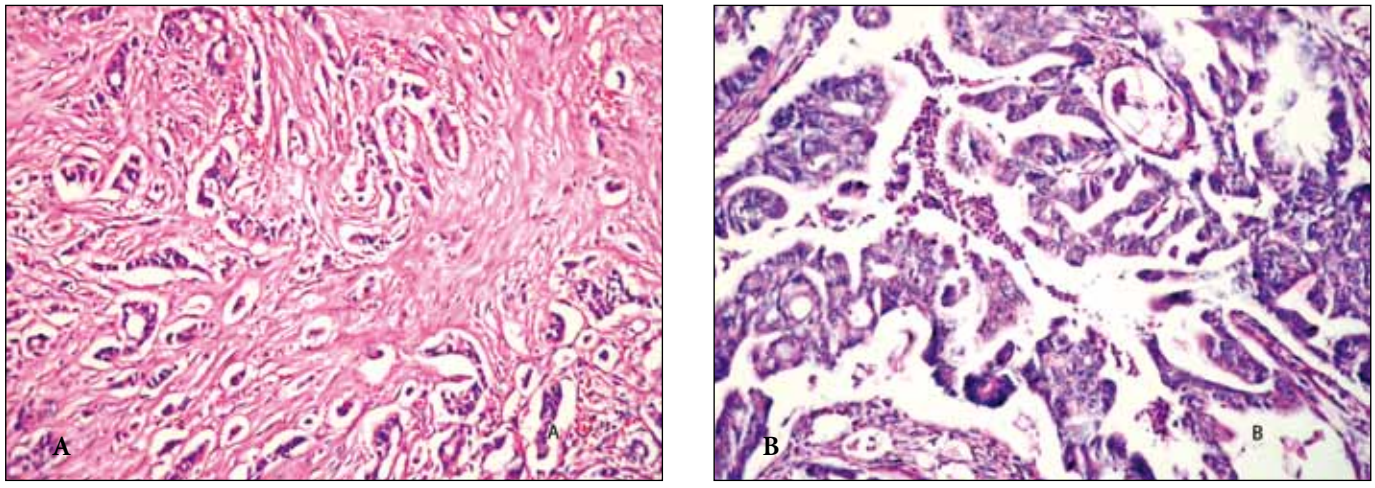

Figure 3: A) Highly invasive pancreatic ductal adenocarcinoma with prominant desmoplasia and some inflammation (H&E, x100), **B**) ampulla of Vater carcinoma having no desmoplasia but moderate inflammation (H&E; x200).

Table I: Distribution of PanIN

	PanIN1a	PanIN1b	PanIN2	PanIN3	None	Total
The normal pancreatic tissue neighbouring PDAC	0	5	1	1	13	20
The normal pancreatic tissue neighbouring AVC	0	2	1	0	8	11
Associated with tumor in PDAC	2	0	4	6	8	20

PanIN, pancreatic intraepithelial neoplasia; **PDAC**, pancreatic ductal adenocarcinoma; **AVC**, ampulla of Vater carcinoma.

Table II: Distribution of perineural invasion, peritumoral desmoplasia and lymphoplasmacytic inflammation and p16 expression in PDAC and AVC

	Total (N =31)	Perineural invasion		p* value	Peritumoral desmoplasia and lymphoplasmacytic inflammation		p* value	p16 expression (**)		p* value
		Present	Absent		Present	Absent		Present	Absent	
Tumor group				0.01			0.01			0.0001
PDAC	20	17	3		14	6		1	19	
AVC	11	4	7		4	7		8	2	

PDAC, pancreatic ductal adenocarcinoma; **AVC**, ampulla of Vater carcinoma.
* χ^2.
**In one tumor case p16 expression could not be evaluated due to technical reasons and the evaluation was therefore performed for 30 cases instead of 31 by excluding the defectively stained case.

of PDACs, 17 (85%) showed perineural invasion, but only 4 (50%) AVCs had perineural invasion. In 20 cases with perineural invasion, 19 (95%) were found to have p16 loss. Among 10 of the cases with no perineural invasion, only 2 (20%) had p16 loss. In conclusion, the loss of p16 protein expression was significantly correlated with perineural invasion considering all cases (p=0.0001) (Table II).

DISCUSSION

The majority of the tumors in the periampullary region are PDAC and AVC (1,2). Although they are cured surgically by similar procedures, the features and prognoses of these two tumors are rather different. This then raises the question as to which mechanisms in their carcinogenesis leads to these different behavioral patterns (4).

Expressions of cell cycle proteins are altered in the carcinogenetic pathway. Among them, the D-cyclin–cdk4/6–INK4– RB pathway is universally disrupted. The frequency of cyclin D-cdk4/6–INK4 pathway alterations suggest that acceleration of G1 progress provided a proliferative and perhaps survival advantage to cancer cells (4,6,15,16). Cyclin D1 expression has been studied in pancreas and ampulla vateri tumors in many studies (4-6). Xu et al. (6) showed cyclin D1 gene amplification in one cell line and alteration of cyclin D1 gene in pancreas adenocarcinomas. Qiao et al. (16) and Gansauge et al. (15) also revealed not only the overexpression of cyclin D1 but that this overexpression in human pancreatic carcinoma is associated with a poor prognosis. In the study of Chang et al. (4), the overall immunostaining rates were observed to be significantly different for cyclin D1 in AVCs and pancreatic head ductal carcinomas (PHDC). They noted significantly higher cyclinD1 expression in AVCs than PHDCs. In this study cyclinD1 expression was nearly the same in the both tumors and not consistent with the other studies in the literature.

p16 (also called $p16^{INK4}$, CDKN2 or MTS1) belongs to the cyclin-dependent kinase inhibitor family and inactivates the complex of D-type-cyclin and cyclin-dependent-kinase-4 by blocking the progression of cells through the G1 phase (1,17). Schutte et al. (11) showed that the p16 tumor suppressor gene is abrogated in virtually all infiltrating ductal adenocarcinomas of the pancreas, in 40% by homozygous deletion, in 40% by intragenic mutation coupled with loss of the remaining allele, and in 15% to 20% by aberrant methylation of the p16 promoter.

Esposito et al. (1) and Yuan et al. (14) also showed p16 mutations in ampullary carcinomas. However, loss of p16 expression and heterogenecity in many of these studies seemed to be a late mutation in the sequence of pancreatic cancer development because intensity of loss of p16 expression increases starting from PanIN1 to progression into invasive PDAC, which means PanIN1a has been shown to have a less p16 loss when compared with invasive PDAC (7,9,12,13). It may not be surprising that extensive p16 loss may be related with worse prognosis since p16 loss is a late finding in PDAC progression. In the present study, loss of p16 gene expression was significantly higher in all cases of either AVC or PDAC with perineural invasion which is one of the criteria for a bad prognosis. In the same cases with perineural invasion, p16 loss was significantly more when compared with cyclinD1 expression. This finding was important in thinking that p16 expression is more valuable than cyclinD1 when one considers the prognosis of tumors in periampullary region.

PanINs are commonly found in the pancreatic ducts adjacent to invasive PDAC, and have been regarded as precursor lesions of PDAC (10,18,19). In our study, 12 (60%) of the 20 PDAC cases had PanIN lesions. The distribution of the PanIN lesions was as follows: two cases (10%) with PanIN1a, four (25%) cases with PanIN2 and six cases (30%) with PanIN3. PanIN1b was observed in none of the cases and also no PanIN was present in 8 cases. PanIN lesions in the peritumoral pancreas in 20 PDACs were divided as five (25%) PanIN1b, one (5%) PanIN2 and one (5%) PanIN3. No PanIN1a (0%) was observed and also the remaining 13 lesions (65%) had no PanIN. In the AVC group two PanIN1b (18%) and one PanIN2 (9%) were observed in a total of 11 cases in the peritumoral pancreas. We observed no PanIN1a (0%) and no PanIN3 (0%) lesions and the remaining eight cases (73%) had no PanIN. When both groups were compared in respect to the presence of PanIN lesions, no statistically significant difference was observed.

PanIN lesions can be seen not only in PDAC but also in areas with inflammation especially in areas of chronic pancreatitis. In some studies, chronic pancreatitis is defined as a potential lesion for PDAC (3,10,13). All cases of either PDAC or AVC peritumoral areas included several degrees of inflammatory reactions, and PanIN lesions were almost always present in the pancreas neighboring the tumor. These findings confirm that high-grade PanINs are more often associated with PDAC than AVC.

Fibrosis is a common morphological finding of the human pancreatic adenocarcinoma and chronic pancreatitis. The source of fibrosis in the pancreas is thought to be fibroblast-like cells located in the peri-acinar region, called pancreatic stellate cells (PSCs), which remain quiescent under normal conditions. During inflammation, activating signals from cytokines, such as TNFα, and growth factors, such as TGF-β1, TGFα and PDGF, stimulate these cells to proliferate and differentiate into myofibroblasts (20). Fibrosis was significantly prominent in this study in PDACs when compared with AVCs. We suggest that PSCs present in the pancreas tissue but not found in ampulla Vateri of the duodenum may be responsible to massive fibrosis.

Perineural invasion is one of the most common characteristics of PDAC due to the high affinity of pancreatic cancer cells to neural tissue and the anatomic abundance of nerves surrounding the pancreas (21). Meng et al. (22) proposed that beta-NGF and TrKA might play a role as beta-NGF and TrKA may have a mutual effect in signal transduction, leading to perineural invasion of pancreatic carcinoma. Ben et al. (21) found strong associations between L1 cell adhesion molecule (L1-CAM) and glial cell line-derived neurotrophic factor (GDNF) expression in PDAC correlated with neural invasion and concluded that perineural invasion was one of the poorest prognostic factors after curative resection. In our study, perineural invasion was one of the main striking and statistically significant differences between PDAC and AVC. Perineural invasion was rather prominent in PDAC and this may partly explain the prognostic differences between these two tumor types theoretically because we had no information about prognosis and follow-up of the patients in our study.

In conclusion, these results suggest that the loss of p16 protein but not cyclinD1 expression may be a sign of poor prognosis of periampullary cancer being correlated mainly with perineural invasion. PDAC and AVC may be distinguished by the presence of a desmoplastic stromal reaction. The existence of PanIN lesions in the peritumoral pancreatic area does not seem to be a specific finding for PDAC.

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