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Evaluation of CD47 Expression in Solid Pancreatic Tumors Diagnosed with Endosonography Guided Fine Needle Aspiration Biopsy

Endosonografi Eşliğinde İnce İğne Aspirasyon Biyopsisi ile Tanı Konulan Solid Pankreas Tümörlerinde CD47 Ekspresyonunun Değerlendirilmesi

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ABSTRACT

Background: CD47, which is also known as integrin-associated protein, is a membrane protein of the immunoglobulin superfamily. Its expression was shown to be elevated in hematologic and many solid organ malignancies like pancreatic tumors.

Materials and Methods: The materials of 80 patients diagnosed with endosonography (EUS)-guided fine needle aspiration biopsy (FNAB) and 42 patients diagnosed after resection were evaluated retrospectively. EUS guided FNAB specimens of all cases and slides prepared from blocks that had been selected from the subsequently obtained resection materials were stained with CD47 immunohistochemical staining. According to the CD47 expression level, it was divided into two groups as low and high, and the results were compared with clinicopathological and prognostic factors.

Results: Eighty patients (male=36, female=44, mean age=61.32 years) were included in our study. In 80 EUS biopsy material, 53 (66.75%) cases were diagnosed with adenocarcinoma and this diagnosis was confirmed in resected patients. We found that 32 of 80 patients who underwent EUS-guided FNAB and 32 of those who underwent resection had positive staining with CD47. Considering high and low staining levels with CD47, our study showed a significant difference between high CD47 expression and disease-free survival in resection materials ($p<0.005$), but did not find a significant relationship between other clinicopathological prognostic factors.

Conclusion: Although high CD47 expression levels were not detected in most EUS biopsy samples, it was observed that high CD47 expression had a negative prognostic effect on disease free survival.

Keywords: Pancreatic cancer, endoscopic ultrasonography, CD47 protein, prognoses

ÖZ

Amaç: İntegrin ile ilişkili protein olarak da bilinen CD47, immünoglobulin süper ailesinin bir membran proteinidir. Pankreas tümörleri gibi hematolojik ve birçok solid organ malignitesinde ekspresyonunun arttığı gösterilmiştir.

Gereç ve Yöntemler: Endosonografi (EUS) eşiliğinde ince iğne aspirasyon biyopsisi (FNAB) ile tanı alan 80 hasta ve rezeksiyon sonrası tanı alan 42 hastanın materyalleri retrospektif olarak değerlendirildi. Tüm olguların EUS biopsi örnekleri ve rezeksiyon materyallerinden seçilen bloklardan hazırlanan kesitler CD47 immünohistokimyasal boyası ile boyandı. CD47 ekspresyon düzeyine göre düşük ve yüksek olarak iki gruba ayrıldı ve sonuçlar klinikopatolojik ve prognostik faktörlerle karşılaştırıldı.

Bulgular: Çalışmamıza 80 hasta (erkek=36, kadın=44, yaş ortalaması=61,32 yıl) dahil edildi. Seksen EUS biyopsi materyalinde 53 (%66,75) olguya adenokarsinom tanısı konuldu ve bu tanı rezeke edilen materyallerde doğrulandı. EUS eşliğinde FNAB uygulanan

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ÖZ

80 hastanın 32'sinde ve rezeksiyon yapılan hastaların 32'sinde CD47 ile pozitif boyanma saptandı. CD47 ile yüksek ve düşük boyama düzeyleri göz önüne alındığında, çalışmamız rezeksiyon materyallerinde yüksek CD47 ekspresyonu ile hastalıksız sağkalım arasında anlamlı bir fark olduğunu gösterdi (p<0,005), ancak diğer klinikopatolojik prognostik faktörler arasında anlamlı bir ilişki bulunmadı.

Sonuç: EUS biyopsi örneklerinin çoğunda yüksek CD47 ekspresyon düzeyleri saptanmamasına rağmen, yüksek CD47 ekspresyonunun hastalıksız sağkalım üzerinde olumsuz prognostik etkisi olduğu gözlenmiştir.

Anahtar Kelimeler: Pankreatik kanser, endoskopik ultrasonografi, CD47 protein, prognoz

Introduction

According to statistical research, pancreatic cancer ranks third in cancer-related mortality and its rates have been increasing gradually over the years (1). The most common solid tumors of the pancreas are pancreatic ductal adenocarcinomas. Prognostic factors such as clinical stage, depth of tumor invasion, lymph node metastasis, and histological grade are important in the evaluation. These tumors are associated with quite high recurrence and distant metastasis rates, even after curative treatment with surgical resection and subsequent adjuvant chemotherapy. In the resectable group, survival time is usually shorter than two years, even after postoperative adjuvant chemotherapy. This highlights the need for research on potential solutions that can be curative in the early diagnosis and treatment of these tumors.

Endosonography (EUS) guided fine needle aspiration biopsy (FNAB) plays an important role in the grading and histological diagnosis of pancreatic and peripancreatic lesions (2). Evaluations made using EUS were shown to have high diagnostic impact and reliability in cases of pancreatic adenocarcinoma (2,3) with this method, both solid-semisolid and cystic lesions of the pancreas can receive a faster and more reliable diagnosis, accelerating the treatment decision for the patient.

CD47 is a membrane protein of the immunoglobulin superfamily. CD47 is a widely expressed cellular receptor well known for its immunoregulatory functions. By interacting with its ligands such as signal regulatory protein α and thrombospondin-1, it modulates cellular phagocytosis by macrophages, transmigration of neutrophils and activation of dendritic cells, T-cells and B-cells (4). CD47 expression was determined to be high in most solid organ malignancies (5,6,7,8,9,10). Since CD47 secretion in the cancer cell membrane would inhibit the phagocytic activity of immune cells; it is associated with a poor prognosis in many solid organ malignancies (8,11,12,13). Accordingly, the suppression of CD47 results in tumor inhibition. Some studies have shown that various types of cancer express high levels of CD47 to escape from the immune system. Based on these studies, CD47 is currently considered a prominent target in cancer therapy.

Pancreatic carcinomas are highly destructive tumors that progress rapidly. Although there are few studies in the literature on solid pancreatic tumors, longer disease-free survival can be expected for these devastating tumors as the number of relevant studies increases and new antitumorigenic immunotherapy agents are developed. In this study, it was aimed to evaluate the CD47 expression in determining treatment planning and prognostic factors in pancreatic tumors diagnosed with EUS-FNAB and resected afterwards, and to compare CD47 expression levels with clinical and prognostic parameters.

Material and Methods

Endoscopic and Pathological Evaluation

EUS-FNAB was planned for 80 cases with a preliminary diagnosis of pancreatic solid tumor. The endoscopy of the patients was performed using the Fujinon EG 530WR endoscopy device in the endoscopy unit of our hospital. All patients were starved for 6 hours before endoscopy, and the endoscopy procedure was performed after local pharyngeal xylocaine anesthesia. The cell blocks and slide preparations prepared from the samples taken were sent to the pathology laboratory. The pathological diagnosis were made under light microscopy and immunohistochemical evaluations.

Biopsy materials of 42 cases diagnosed with EUS biopsy and subsequently resected in our hospital were extracted from the block and side archives of the pathology laboratory and re-evaluated. CD47 staining was made immunohistochemically to EUS biopsy materials and sections of blocks selected from tumor resections.

Tissue Immunohistochemistry Staining and Pathological Evaluation

New sections were obtained from EUS cell blocks and resection materials and mounted onto polylysine slides, which were then stained with the CD47 (Abcam CD47 antibody ab3283, Cambridge, UK) monoclonal antibody using the Ventana immunohistochemical staining device.

All slides were evaluated for CD47 expression using a Nikon light microscope with H&E slides in a controlled manner. In the evaluation of CD47; cytoplasmic and membranous brown staining in the cells were considered and the extent of expression was also graded besides its intensity. The extent of CD47 expression in the cells was graded as; "0" if there was no staining, "1" if there was staining in up to 10% of the cells, "2" if there was staining in 11-25% of the cells, and "3" if there was staining in 50% or more. Accordingly, those with staining graded as 0 and 1 were evaluated within the low CD47 expression group ($CD47^{low\ exp}$) and those with staining graded 2-3 were evaluated within the high CD47 expression group ($CD47^{high\ exp}$), thus categorizing the cases into two groups.

CD47 expression was evaluated in 80 EUS samples. At the same time, both EUS biopsy materials and tumor resection materials were evaluated for CD47 low and high expression in 42 cases. Statistically, CD47 expressions were compared. Also, the post-treatment clinical follow-up records of the patients were reviewed to record the known survival times. It was also investigated whether or not the states of CD47 expression were correlated with survival times.

Statistical Analysis

The chi-squared test was used to analyze the association between candidate CD47 expression and clinicopathological characteristics of pancreatic lesions. Survival curves were evaluated using the Kaplan-Meier method, and differences between survival curves were tested by the log-rank test. Only significantly different variables in univariate analysis were included in the multivariate analysis. Statistical signifcance was based on two-tailed tests at $p<0.05$. SPSS 22.0 (IBM Corp.) and GraphPad Prism 6 (San Diego, CA, USA) software was used for statistical analyses and graphical representation.

Results

Eighty cases diagnosed with EUS-guided FNAB in our hospital were included in the study. The mean age of the 80 patients included in this study was 61.32 years. The minimum age was 16 years and the maximum age was 87 years. Out of 80 patients diagnosed with EUS-guided FNAB in our hospital, the diagnoses of 42 patients who were operable were confirmed at resection material. Of the 42 resection patients, 33 had a final diagnosis of pancreatic ductal adenocarcinoma, 7 had a final diagnosis of neuroendocrine tumor, and 2 had a final diagnosis of solid pseudopapillary neoplasm in their reports. The pathological tumor stage was pT3 in 25 cases, pT2 in 8 cases, pT1 in 6 cases, and pT4 in 3 cases. Of the resection patients, 25 demonstrated lymph node metastasis, 31 perineural invasion, and 26 lymphovascular invasion. All clinicopathological parameters are summarized in Table 1.

CD47 staining was observed in 32 of 80 patients who underwent EUS-guided FNAB and 32 of 42 patients who underwent resection (Table 2).

When CD47 staining levels are evaluated Of the 42 resection materials, 22 showed $CD47^{high\ exp}$ (52.38%) (Figure 1a, b) and 10 showed $CD47^{low\ exp}$ (23.82%) (Figure 2a, b) staining. Out of a total 80 EUS- FNAB materials, 19 showed

Table 1. Clinicopathological features of patients

Operation	**(n)**	**(%)**
EUS-FNAB	80	100
Pancreatic resection	42	52.5
Age (16-87)		
Median	61.32	-
Sex		
Male	36	45
Female	44	55
Diameter (1-13 cm)		
Median	3.95	-
Pathology		
Adenocarcinoma	53	66.25
NET	19	23.75
SPPN	3	3.75
GIST	3	3.75
Adenosquamous	2	2.5
Pathologic grade		
pT1	6	14.29
pT2	8	19.05
pT3	25	59.52
pT4	3	7.14
CD47 positivity		
EUS-FNAB	32 (n=80)*	42.10
Pancreatic resection	32 (n=42)**	76.90
Lymphovasculer invasion		
Yes	26	61.9
No	16	38.0
Perineural invasion		
Yes	31	73.8
No	11	26.19
Lymph node metastasis		
Yes	25	59.52
No	17	40.48

*Number of all cases belonging to EUS-FNAB, **Number of cases to resection materials, EUS: Endosonography, FNAB: Fine needle aspiration biopsy, NET: Neuroendocrine tumor

CD47$^{high\ exp}$ (23.75%) (Figure 3a, b) and13 showed CD47$^{low\ exp}$ (16.25%) (Figure 4a, b). 50% of the cases with resection materials in the CD47$^{low\ exp}$ group and 62.5% of the cases in the CD47$^{high\ exp}$ group had grade 2 tumors (p=0.884) (Table 2). Neither the resection material nor the EUS materials were painted with CD47 in any of the solid pseudopapiller neoplasm.

Our study showed that increased CD47 expression in resection materials. It was observed that as CD47 staining level increased in patients who underwent resection for pancreatic cancer, disease-free survival time decreased. When the levels of tumor CD47 expression in the resection materials were compared with survival times, a statistically significant relationship was determined between these two parameters (p<0.005) (Figure 5).

Discussion

The most common solid tumors of the pancreas are pancreatic ductal adenocarcinomas. Pancreatic ductal adenocarcinoma is a highly destructive tumor with a five-year survival rate lower than 5% (14).The EUS guided FNAB method is widely used in the diagnoses of solid, semisolid and cystic lesions identified in the pancreas, with high accuracy rates and low morbidity and mortality rates. Its diagnostic value is particularly high for lesions of small size (2,3).

Table 2. Summary of variables and analysis results related to CD47 expression in EUS-FNAB samples and pancreatic tumors, before resection

	CD47 tumor expression			
	Positive	**Negative**	**N of patiens (%)**	**p**
pT	**32**	**10**	**42**	
1	2 (6.25%)	4 (40.00%)	6 (14.28%)	0.363
2	6 (18.75%)	2 (20.00%)	8 (19.05%)	
3	21(65.62%)	4 (40.00%)	25 (59.52%)	
4	3 (9.37%)	0 (0.00%)	3 (7.14%)	
Hystologic grade				
1	3 (9.75%)	3 (30.00%)	6 (14.28%)	0.519
2	20 (62.50)	5 (50.00%)	25 (59.52%)	
3	9 (28.12%)	2 (20.00%)	11 (15.49%)	
Lymphovascular invasion				
Yes	21 (16.00%)	5 (50.00%)	26 (61.90%)	0.236
No	11 (40.00%)	5 (50.00%)	16 (38.09%)	
Perineural invasion				
Yes	24 (75.00%)	7 (70.00%)	31 (73.81%)	0.381
No	8 (25.00%)	3 (30.00%)	11 (26.19%)	
Lymph node metastasis				
Present	20 (62.50%)	5 (50.00%)	25 (59.52%)	0.072
Absent	12 (37.50%)	5 (50.00%)	17 (40.47%)	
CD47 resection				
High exp	22 (68.75%)	0 (0.00%)	22 (52.38%)	*0.005
Low exp	10 (31.25%)	10 (23.80%)	20 (47.62%)	
Pathology				
Adenoca	30 (69.04%)	3 (30.00)	33 (78.57%)	0.194
NET	2 (4.76%)	5 (50.00%)	7 (16.66%)	
SPPN	0 (0.00%)	2 (20.00%)	2 (4.76%)	
CD47 EUS-FNAB (before resection)	**17**	**25**	**42**	
High exp	7 (41.18%)	0 (0.00%)	7 (16.66%)	0.286
Low exp	10 (58.82%)	25 (100%)	35 (83.33%)	

Data given as frequency (percentage), *chi-square test significant p-value, NET: Neuroendocrine tumor, EUS: Endosonography, FNAB: Fine needle aspiration biopsy

CD47 is a novel prognostic biomarker of certain malignant tumors. CD47 is a widely expressed cell surface protein that regulates phagocytosis, which is mediated by innate immune system cells such as macrophages and dendritic cells (15). Numerous studies in the literature have shown elevated CD47 expression in various solid organ malignancies (5,8,12,14,16). In our study, in accordance with the literature, the final diagnosis was resulted as pancreatic adenocarcinoma in most forty-two resection cases (thirty-three). Of these, 32 had positive staining with CD47. Statistically significant high CD47 expression was observed in 22 of CD47 positive stained cases.

Yuan et al. (9) in patients with hormone receptor negative breast cancer and Lascorz et al. (17) colorectal carcinomas showed that overexpression of CD47. In a study conducted by Olcucuoglu et al. (7) high CD47 expression was reported to contribute to the evaluation of bladder tumors at various stages. These studies also showed that increased CD47 expression was a poor prognostic factor (7,9,17). Moreover, studies have shown that this increased expression promotes the escape of cancer cells from phagocytosis (8,18). Majeti et al. (10) determined that cancer cells promoted tumorigenesis and metastasis in this way. Accordingly, CD47 is considered a biomarker of cancer, and its high expression is an indicator

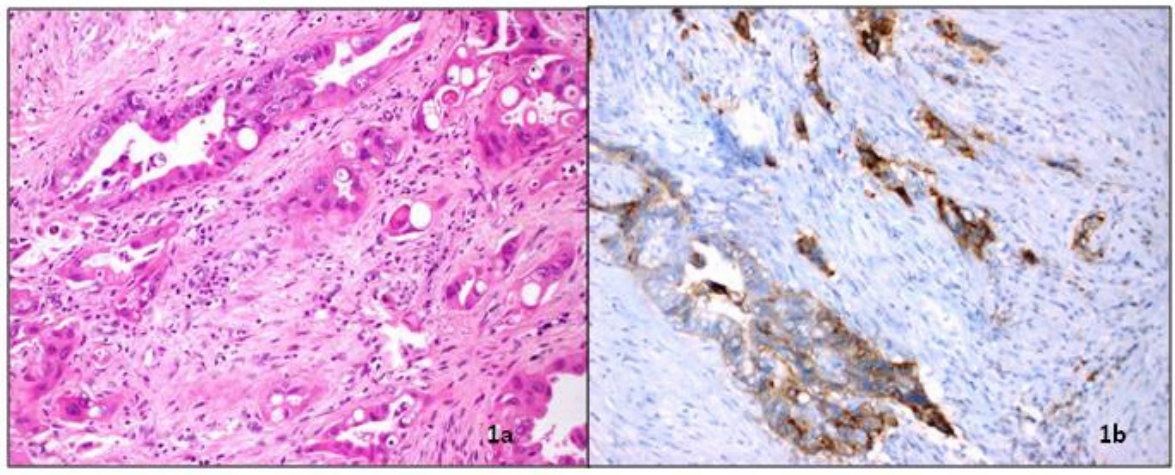

Figure 1. Pancreatic ductal adenocarcinoma (H&E section,x200) (a), CD47 high stainign (b)

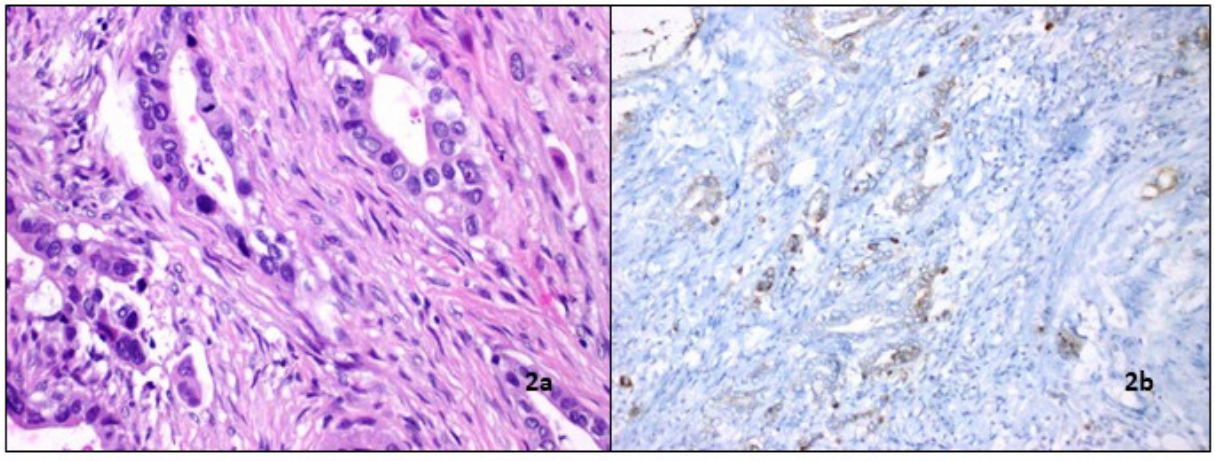

Figure 2. Pancreatic ductal adenocarcinoma (H&E section,x200) (a), CD47 low stainign (b)

of poor clinical prognosis. Edris et al. (13) demonstrated that anti-CD47 therapy inhibits the high tumor cell phagocytosis and tumor growth in cancer cell series. Similarly, Ye et al. (12) reported that CD47 could serve as a biomarker of oral precancer and cancer progression.

Patients with early-stage pancreatic cancers who undergo surgical resection and adjuvant chemotherapy have a median survival time of two years, predominantly due to the presence of micrometastatic disease in the liver that goes undetected and the consequent progression of this disease. In a study conducted by Michaels et al. (6), liver macrophages were shown to significantly stall the progression of pancreatic cancer micrometastases in a pre-clinical mouse model. The suppression effect of the macrophages was augmented by blocking CD47 on pancreatic cancer cells, leading to a decrease in metastatic burden and extending survival times. Therefore, these data support a clinical trial of CD47 blockade as an adjuvant immunotherapy for pancreatic cancer (6).

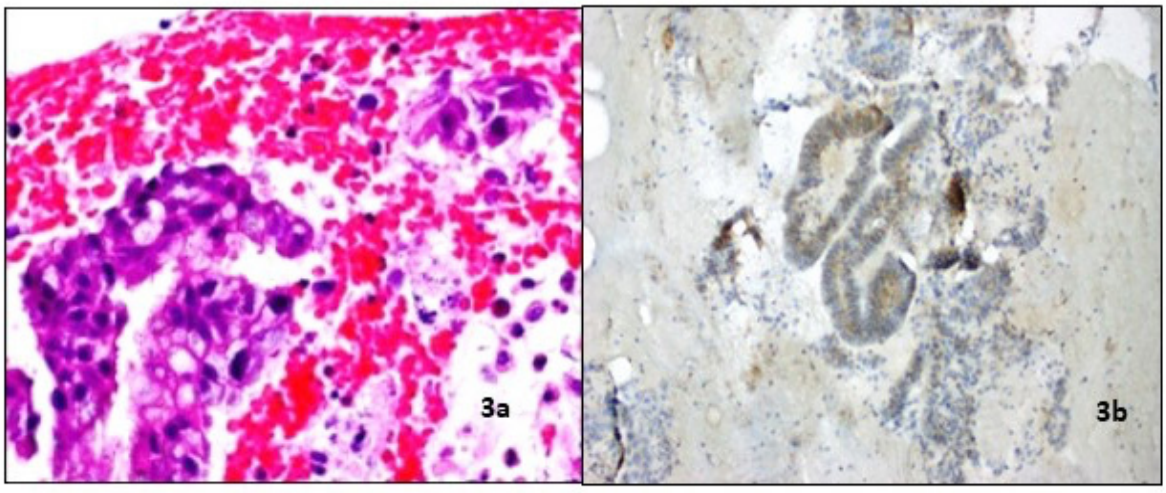

Figure 3. Cell block sections of the EUS biopsy material performed before resection of the pancreatic ductal adenocarcinoma case in figure (2a), (H&E section, x200), (b) High CD47 expression in the EUS material in the same case (respectively)

EUS: Endosonography

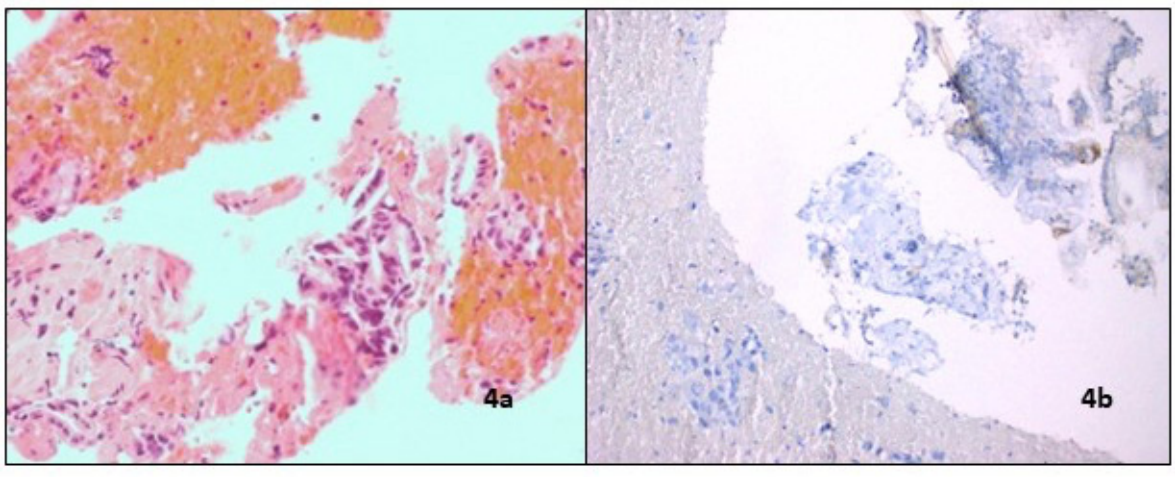

Figure 4. Cell block sections of the EUS biopsy material performed before resection of the pancreatic ductal adenocarcinoma case in figure (3a), (H&E section, x200), low CD47 expression in the EUS material in the same case (respectively) (3b)

EUS: Endosonography

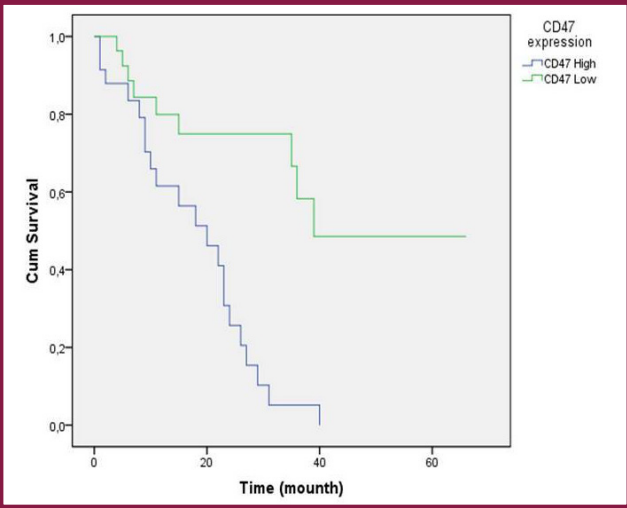

Figure 5. Kaplan-Meier survival analysis according to CD47 over expression

In order to grow and metastasize, solid tumors must escape phagocytosis with tumor-associated macrophages (19). This evidence indicates that the cell surface expression of CD47 is a common mechanism by which the cells avoid phagocytosis (20). With regard to solid organ tumors, a study done by Willingham et al. (5) showed that monoclonal antibodies that block CD47 were effective in the *in vitro* and *in vivo* treatment. Studies on experimental mouse models determined that adjuvant immunotherapy that achieved CD47 blockade resulted in increased progression free and overall survival times in resectable pancreatic cancers (6,16).

In our study, no significant staining was detected with CD47 in EUS biopsy material of eighty total cases. Evaluation of CD47 expression in EUS biopsy materials before resection was not statistically significant. This result might be explained by the limited amount of tumor cells in the cell-block materials. In this study, cell blocks obtained from 80 patients by EUS FNAB were CD47 stained, and 19 of these produced positive results. Although this number is low, these positive staining rates determined by EUS guided FNAB can guide future processes.

Study Limitations

We have some limitations in our study. Because some cell blocks contain very few tumor cells and these are the strengths of our work. Although there are a limited number of tumor cells in some cell blocks, staining detected in EUS guided FNAB may be considered important.

Pancreatic carcinomas are highly destructive tumors that progress rapidly. Although there are few studies in the literature on solid pancreatic tumors, longer disease-free survival can be expected for these devastating tumors as the number of relevant studies increases and new antitumorigenic immunotherapy agents are developed. In this study, it was aimed to evaluate CD47 expression in determining treatment planning and prognostic factors in pancreatic tumors diagnosed with EUS-FNAB and resected afterwards, and to compare CD47 expression levels with clinical and prognostic parameters.

Our study showed that increased CD47 expression in resection materials. In our study, a significant correlation was determined between elevated CD47 expression in resectable pancreatic tumors and disease free survival times. But our study did not find a significant relationship between CD47 expression levels and other clinicopathologic prognostic factors. As shown in other clinical trials, increased expression of CD47 was evaluated as a poor prognostic factor. When adjuvant immunotherapy is given and CD47 blockade is achieved, an increase in progression-free and overall survival times in resectable pancreatic cancers can be expected.

Conclusion

In our study no significant staining was detected with CD47 in EUS biopsy materials. In the resectable group, a significant correlation was found between increased CD47 expression and disease-free survival, and it was evaluated as a poor prognostic factor. As more studies on this subject increase, we think that pancreatic tumors, which are very challenging to diagnose and treat after diagnosis, may have a chance to be treated with immunotherapy according to the CD47 expression results to be applied to EUS biopsy samples before resection.

Ethics

Ethics Committee Approval: To conduct this study, ethical approval was obtained from the ethics committee of our hospital. All the applied procedures were complied with the ethical standards of human testing committee of our institution and the Helsinki Declaration. Ethical approval (study number: 12.2017/20) was obtained from the Local Ethics Committee of the Bezmialem Vakıf University of Medical School, Turkey.

Informed Consent: Informed consent was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: N.Ü., N.Ş., Z.G., D.S.A, Concept: N.Ü., D.S.A, Design: N.Ü., G.Ç., Data Collection or Processing: G.Ç., N.Ş., Z.G., H.Ş., Analysis or Interpretation: N.Ü., Z.G., D.S.A., Literature Search: Z.G., D.S.A., Writing: N.Ü.

Conflict of Interest: No conflict of interest was declared by the authors.

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