

The prognostic value of CA19-9, D-dimer and expression of TNFAIP3(A20) protein in pancreatic ductal adenocarcinoma patients

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Research

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Abstract

Background: To explore significance of CA19-9, D-dimer with TNFAIP3 (A20) protein and evaluate its prognostic significance in patients suffering from pancreatic ductal adenocarcinoma (PDAC).

Methods: 148 patients suffering from pancreatic ductal adenocarcinoma in Northern Jiangsu People's Hospital affiliated to Yangzhou University between January 2012 to December 2016 were studied. Cutoff values of prognostic factors were predicted by Receiver operating characteristic curve (ROC curve). Kaplan-Meier method was used to describe survival curve of patients. Univariate and multivariate regression analyses were used to analyze prognostic factors of patients.

Results: The recommended cutoff values of the neutrophil-lymphocyte rate (NLR), platelet-lymphocyte rate (PLR), CA19-9 and D-dimer were 2.04 (sensitivity, 0.59; specificity, 0.9; area under the ROC curve (AUC), 0.749; $P < 0.001$), 52.94 (sensitivity, 0.73; specificity, 0.95; AUC, 0.829; $P < 0.001$), 176.66U/ml (sensitivity, 0.7; specificity, 0.9; AUC, 0.794; $P < 0.001$) and 1.18mg/L (sensitivity, 0.82; specificity, 0.9; AUC, 0.845; $P < 0.001$) respectively. The expression of CA19-9 in serum was related with lymph node metastasis ($P = 0.010$), tumor lymph node metastasis (TNM) stage ($P < 0.001$) and survival ratio ($P < 0.001$). The D-dimer was positively related with differentiation grade ($P = 0.014$), tumor size ($P = 0.045$), TNM stage ($P = 0.006$) and survival ratio ($P < 0.001$). A20 was positively related with differentiation grade ($P < 0.001$), BMI ($P < 0.001$), TNM stage ($P = 0.024$) and survival ratio ($P < 0.001$). The Kaplan-Meier curves showed that the patients with pancreatic ductal adenocarcinoma had a significant difference in the expression of CA19-9 group, expression of D-dimer group and expression of A20 ($P < 0.05$). CA19-9, D-dimer, TNM stage, differentiation grade and A20 were independent prognostic markers for patients suffering from pancreatic ductal adenocarcinoma by univariate and COX multivariate analyses.

Conclusions: CA19-9, D-dimer and A20 were independent prognostic markers for pancreatic ductal adenocarcinoma patients.

Background

Patients with pancreatic cancer, as one of the most lethal malignant tumors, were usually found to metastasize to distant organs. The possible reasons may be no specific symptoms in the early stage of pancreatic cancer[1,2]. Although many therapies were used to treat pancreatic cancer, 5-year survival rate was less than 9% in patients with pancreatic cancer[3,4]. The prognosis of pancreatic cancer was closely associated with early detection and treatment. Now the main diagnostic method of pancreatic cancer was through imaging technology or pancreatic biopsy. Researchers had begun to discover prognostic blood markers, containing the neutrophil-lymphocyte rate(NLR)[5,6] and platelet-lymphocyte rate (PLR)[7].

CA19-9 and D-dimer played important roles in the survival of pancreatic cancer[8,9]. CA19-9 was a sialic acid containing glycan antigen found in both glycoproteins and glycolipids and used as a marker of pancreatic cancer or other tumors[10]. D-dimer was a soluble fibrin degradation product that resulted from ordered breakdown of thrombi by the fibrinolytic system. The results of this meta-analysis indicated

that plasma D-dimer levels can be used as an important reference for the early identification and staging of breast cancer[11]. D-dimer was reported as an independent prognostic marker for some cancer[12,13].

A20 was a cytoplasmic zinc-finger protein, which was found to be expressed in a variety of human cells, which associated with disease[14]. At present, A20 was reported to be associated with inflammatory responses by regulating canonical NF- κ B pathway, which played key roles in termination of the active canonical NF- κ B pathway[15]. The study suggested that miR-125a promoted chemo-resistance to gemcitabine in pancreatic cells through targeting A20[16] and A20 expression was reduced in pancreatic cancer tissues and significantly associated with the pancreatic cancer behavior[17].

Although CA19-9 is a marker of pancreatic tumor, It is easily interfered by other factors, such as jaundice[18]and inflammatory level[19]. D-dimer and A20 have also been studied in cancer[12,17]. As a stress response gene in endothelial cells (ECs), A20 has a protective effect from tumor necrosis factor (TNF)-mediated apoptosis and inhibits inflammationis[20,21]. D dimer level is associated with vascular endothelial cell injury[22]. Therefore, A20 may be associated with D- dimer and CA199. The prognostic value of CA19-9, D-dimer and A20 in patients with pancreatic ductal adenocarcinoma have not been thoroughly and extensively discussed. In this study, three indicators were selected to explore the relationship between evaluation and survival of pancreatic cancer in patients with pancreatic ductal adenocarcinoma.

Materials And Methods

Inclusion and exclusion criteria

The study was approved by the Ethics Committee in Northern Jiangsu People's Hospital affiliated to Yangzhou University. All patients had signed the wrirren informed consent. This study got carried out according to the guidance from Statement of Helsink.

Inclusion criteria included: 1)Patients was diagnosed as pancreatic ductal adenocarcinoma by pathological diagnosis; 2)Patients did not receive radiotherapy and/or adjuvant chemotherapy before the resection; 3)Patients were determined to require surgical treatment; 4)The patient's peripheral blood tests were performed within one week before operation.

Exclusion criteria included

1) Patients previously had malignant tumors or other primary tumors; 2) Patients suffered from certain diseases that affected peripheral blood cell count, such as infection; 3) Patients had accepted radiotherapy and/or adjuvant chemotherapy previously before operation; 4) Patients died within four weeks after the operation.

Patient characteristics and tissue specimens

148 patients who were diagnosed as pancreatic ductal adenocarcinoma and subjected to curative surgery during the Northern Jiangsu People's Hospital affiliated to Yangzhou University from January 2012 to December 2016, were studied. The patients that were being investigated in this study are all resectable. Information on the basic characteristics of the patient was collected (Table 1), including blood tests and pathological features. The progression of the pancreatic cancer patients were classified according to the eighth version of American joint committee on cancer staging system. The patient's peripheral blood tests were performed within one week before operation. Prognostic Value of NLR and PLR in Patients with pancreatic ductal adenocarcinoma were analyzed. The recommended cutoff values of CA19-9, D-dimer, PLR and NLR before operation were determined by ROC curve based on the Youden index (maximum (sensitivity + specificity-1)).

Table 1
Characteristics of patients in the study

Characteristics	Median(25th–75th percentile) or No. (%)
Gender	
Male	85(57.4)
Female	63(42.6)
Differentiation grade	
Low	50(33.8)
Moderate	78(52.7)
High	20(13.5)
Tumor volume(cm ³)	36.66 (13.34–66.64)
Neutrophil count	5.30 (4.60–5.80) × 10 ⁹ /l
Platelet count	182.50 (104.25–255.75) × 10 ⁹ /l
Lymphocyte count	2.50 (2.1–2.90) × 10 ⁹ /l
Smoking	
Yes	72(48.6)
No	76(51.4)
Alcohol consumption	
Yes	57(38.5)
No	91(61.5)
Diabetes	
Yes	56(37.8)
No	92(62.2)
High blood pressure	
Yes	26(17.6)
No	122(82.4)
BMI	
18.5 ≤ BMI < 24	105(70.9)
BMI < 18.5, 24 ≤ BMI	43(29.1)

Characteristics	Median(25th–75th percentile) or No. (%)
Lymph node metastasis	
N1	104(70.3)
N0	44(29.7)
TNM stage	
I	31(20.9)
I+II	117(79.1)
Tumor size	
> 5 cm	39(26.4)
≤ 5 cm	109(73.6)
Age(year)	
< 60	47(31.8)
≥ 60	101(68.2)
TNFAIP3(A20)	
Positive	37(25.0)
Negative	111(75.0)
CA19-9 U/ml	271.54(81.08-453.34)
D-dimer mg/L	3.17(0.98–5.41)

Immunohistochemical analysis

According to standard procedures of immunohistochemical procedures, the tissues of 148 patients with pathologically diagnosed pancreatic ductal adenocarcinoma were fixed in formalin and the tissues were embedded in paraffin. The paraffin tissue was cut into 4 μm thick sections, and the sections were dewaxed and hydrated. Rinse sections with 10 μmol/L citrate buffer and heat under the microwave for 10 minutes. The sections were then immersed in 3% hydrogen peroxide and anhydrous methanol. Sections were treated with 10% goat serum albumin for 20 minutes. Add a primary antibody (rabbit polyclonal antibody, Abcam, USA) to A20 protein diluted 1: 100 to the section, store it at 4 °C overnight, and then add a secondary antibody at room temperature, incubating for 1 hour. The sections were incubated with horseradish peroxidase-conjugated streptavidin for 1 hour, and the sections were developed with diaminobenzidine (DAB). Finally, the sections were counterstained with Mayer's hematoxylin.

The expression of A20 was evaluated by the following method. Each section was randomly graded based on the average percentage of positive cells and the intensity of staining in five areas (per x 100 fields of

view). Based on the average percentage of positive cells (P), the percentage score was: 0 (< 5%); 1 (5% -24%); 2 (25% -49%); 3 (< 50% -75%); 4 (> 75%). Intensity (S) was classified as: 0, no staining; +1, weak; +2, medium; +3, strong. The final score was obtained by the following algorithm: SCORE = Σ (P \times S). The grading standards are as follows: 0 (-); 1-3 (+); 4-7 (++); 8-12 (+++).

Follow-up and treatment

Our study collected information of 162 patients with pancreatic ductal adenocarcinoma. After operation, some patients with poor health were given supportive therapy, and some patients received chemotherapy or targeted treatment. Their follow-up data were obtained by telephone and outpatient service. The registered patients were followed up every month. All the patients were followed up, eight people lost contact and six patients died from non-cancer diseases, who were excluded from the study. 148 patients with pancreatic ductal adenocarcinoma were included in this study. The follow-up period of our study ended in December 2018.

Statistical explanation

Statistical analysis was analyzed by using SPSS 23.0 software (SPSS Inc., Chicago, IL). The cutoff values of indexes was performed according to the ROC curve based on the Youden finger (maximum (sensitivity + specificity-1)). Categorical variables were analyzed by Pearson χ^2 test or Fisher's exact test. For overall surviving, Kaplan Meier curves got established using the log-rank test. the Cox appropriate hazard pattern was used for survival analysis. $P < 0.05$ was defined as statistically significant.

Results

Patient characteristics

Overall, 85 (57.4%) patients were males and 63 (42.6%) turned out to be females. The median age of the patients included in the study was 63 years old (range, 34-81 years). The median follow-up period was 12 months (range, 2-28 months). Tumors larger than 5 cm were found in 39 (26.4%) patients, the pathological stage of I was observed in 31 (20.9%) patients and stage of II-III were observed in 117 (79.1%) patients. The low differentiation level was 50 cases, the moderate differentiation level was 78 cases, and the high differentiation level was 20 cases. (Table 1)

The ROC curve predicted cutoff values of prognostic factors

On the basis of the Youden finger, the recommended cutoff values of NLR, PLR, CA19-9 and D-dimer were 2.04 (sensitivity, 0.59; specificity, 0.9; AUC, 0.749; $P < 0.001$), 52.94 (sensitivity, 0.73; specificity, 0.95; AUC, 0.829; $P < 0.001$), 176.66 U/ml (sensitivity, 0.7; specificity, 0.9; AUC, 0.794; $P < 0.001$) and 1.18 mg/L (sensitivity, 0.82; specificity, 0.9; AUC, 0.845; $P < 0.001$) respectively (Fig. 1).

Relationship between cutoff values of CA19-9, D-dimer and A20 with clinicopathologic characteristics

On the basis of the Youden finger, the recommended cutoff values of CA19-9 and D-dimer were determined. Cutoff values of CA19-9 had no significant relationship with age, gender, tumor size, differentiation grade, smoking, alcohol consumption, diabetes, high blood pressure and BMI. However, the association between CA19-9 and lymph node metastasis ($P < 0.001$), TNM stage ($P < 0.001$), survival ratio ($P < 0.001$) were statistical significance. Cutoff values of D-dimer was statistical differences with differentiation grade ($P = 0.014$), tumor size ($P = 0.045$), TNM stage ($P = 0.006$) and survival ratio ($P < 0.001$), and no statistical differences with gender, age, smoking, alcohol consumption, diabetes, high blood pressure, BMI and lymph node metastasis. The expression of A20 (37/148, 25.0%) was reduced in pancreatic ductal adenocarcinoma tissues (Fig. 2) and significantly connected with differentiation grade ($P < 0.001$), BMI ($P < 0.001$), TNM stage ($P = 0.024$) and survival ratio ($P < 0.001$). There were no relationship between A20 with gender, age, smoking, alcohol consumption, diabetes, high blood pressure and lymph node metastasis. (Table 2)

Table 2

Relationship between cutoff values of CA19-9, D-dimer and TNFAIP3(A20) with clinicopathologic characteristics

Patient related factors	CA19-9 (U/ml)		D-dimer (mg/L)		TNFAIP3(A20)		P value		
	< 176.66 (n = 57)	≥ 176.66 (n = 91)	< 1.18 (n = 41)	≥ 1.18 (n = 107)	Positive (n = 37)	Negative (n = 111)			
Gender							0.265	0.589	0.502
Male	36	49	25	60	23	62			
Female	21	42	16	47	14	49			
Age (years)							0.137	0.425	0.126
< 60	14	33	11	36	8	39			
≥ 60	43	58	30	71	29	72			
Tumor size							0.439	0.045	0.914
< 5cm	44	65	35	74	27	82			
≥ 5cm	13	26	6	33	10	29			
Differentiation grade							0.141	0.014	< 0.001
Low	14	36	8	42	1	49			
Moderate	33	45	23	55	25	53			
High	10	10	10	10	11	9			
Survival ratio							< 0.001	< 0.001	< 0.001
Yes	18	2	18	2	13	7			
No	39	89	23	105	24	104			
Smoking							0.805	0.279	0.255

Patient related factors	CA19-9 (U/ml)		D-dimer (mg/L)		TNFAIP3(A20)	
	< 176.66 (n = 57)	≥ 176.66 (n = 91)	< 1.18 (n = 41)	≥ 1.18 (n = 107)	Positive (n = 37)	Negative (n = 111)
Yes	27	45	17	55	21	51
No	30	46	24	52	16	60
Alcohol consumption	0.477		0.226		0.380	
Yes	24	33	19	38	12	45
No	33	58	22	69	25	66
Diabetes	0.585		0.341		0.117	
Yes	20	36	13	43	18	38
No	37	55	28	64	19	73
High blood pressure	0.077		0.386		0.803	
Yes	14	12	9	17	6	20
No	43	79	32	90	31	91
BMI	0.364		0.439		< 0.001	
18.5 ≤ BMI < 24	38	67	31	74	35	70
BMI < 18.5, BMI ≥ 24	19	24	10	33	2	41
Lymph node metastasis	0.010		0.939		0.213	
N1	47	57	29	75	29	75

Patient related factors	CA19-9 (U/ml)		D-dimer (mg/L)		TNFAIP3(A20)	
	< 176.66 (n = 57)	≥ 176.66 (n = 91)	< 1.18 (n = 41)	≥ 1.18 (n = 107)	Positive (n = 37)	Negative (n = 111)
NO	10	34	12	32	8	36
TNM stage	< 0.001		< 0.001		0.014	
0	21	10	19	12	13	18
0+0	36	81	22	95	24	93

Relationship of CA19-9, D-dimer and A20 with prognosis

The Kaplan-Meier survival curves for PDAC patients in high expression of CA19-9 and low expression of CA19-9 group (Fig. 3), high expression of D-dimer and low expression of D-dimer (Fig. 4), high expression of A20 group and low expression of A20 group (Fig. 5) were showed. The Overall survival time of patients with CA19-9 < 176.66 U/ml ($P < 0.001$), D-dimer < 1.18 mg/L ($P < 0.001$) and high expression of A20 group ($P < 0.001$) was longer. Cox multivariate and univariate analysis showed overall survival risk factors. Cox multivariate analysis showed that CA19-9 ($P = 0.005$), D-dimer ($P = 0.005$), A20 ($P < 0.001$), TNM stage ($P = 0.007$) and differentiation grade ($P < 0.001$) were independent risk factors.(Table 3)

Table 3
Proportional risk model for predicting overall survival

Characteristics	Univariate analysis		Multivariate analysis	
	HR value (95% CI)	P value	HR value (95% CI)	P value
Gender	1.208(0.852–1.714)	0.289		
Age (years)	0.821(0.565–1.193)	0.302		
Tumor size	1.306(0.890–1.916)	0.172		
Differentiation grade	0.403(0.3-0.541)	< 0.001	0.573(0.419–0.783)	< 0.001
Lymph node metastasis	1.535(1.053–2.237)	0.026	1.266(0.851–1.884)	0.244
Tumor volume	1.0039(0.989–1.018)	0.646		
Smoking	0.957(0.674–1.359)	0.804		
Alcohol consumption	0.912(0.635–1.310)	0.617		
Diabetes	1.084(0.761–1.544)	0.656		
High blood pressure	1.379(0.869–2.188)	0.172		
BMI	1.30(0.883–1.914)	0.184		
TNM stage	4.454(2.699–7.352)	< 0.001	2.228(1.240–4.003)	0.007
NLR	1.420(1.107–1.820)	0.006	1.024(0.722–1.453)	0.892
PLR	1.007(1.004–1.010)	< 0.001	1.004(0.999–1.009)	0.131
TNFAIP3(A20)	0.227(0.141–0.365)	< 0.001	0.357(0.209–0.604)	< 0.001
CA199 ≥ 176.66 U/ml	2.530(1.722–3.716)	< 0.001	1.827(1.205–2.770)	0.005

Characteristics	Univariate analysis		Multivariate analysis	
	HR value (95% CI)	P value	HR value (95% CI)	P value
D-dimer \geq 1.18 mg/L	3.964(2.436–6.449)	< 0.001	2.120(1.252–3.588)	0.005

Discussion

It is known that pancreatic ductal adenocarcinoma is one of the high malignant degree of cancer. At present, several biomarkers for pancreatic cancer have been proposed for earlier diagnosis, there is no effective diagnostic method to find early pancreatic in clinical application cancer, surgical resection offers the chance to cure PDAC. But Most of PDAC patients have lost the opportunity of operation when pancreatic cancer was found. To found biomarkers for early diagnosis of pancreatic cancer propose future research directions[23–25].

Surgery is one of the most important therapy for pancreatic ductal adenocarcinoma. However, due to the limitation of diagnostic techniques, it is usually difficult to detect pancreatic cancer early, which leads to poor prognosis[24]. Some scholars had shown that some markers may be related to the prognosis of PDAC patients, for examples, differentiation grade and TNM stage[1,8]. These prognostic factors were difficult to judge preoperatively, so the study of preoperative serum markers had become a research hot spot[12,26]. As an independent prognostic indicator, CA19-9 had attracted more and more attention[26]. Level D-dimer was found to be associated with prognosis of digestive tumors[12,13]. But CA19-9 level may be affected by jaundice[18]and inflammatory[19], many pancreatic cancer patients complicated with jaundice.

In our study, patients with preoperative CA19-9 \geq 176.66 U/ml had a high degree of malignancy and a short overall survival period, but the mechanism of CA19-9 and tumor progression was still unclear. CA19-9 was positively correlated with lymph node metastasis ($P= 0.010$), TNM stage ($P< 0.001$), survival ratio ($P< 0.001$). D-dimer degraded by plasmin during fibrinolysis was a degradation product of the cross-linked fibrinpolymer. Previous studies had showed that plasma level of D-dimer can predict poor prognosis in several types of malignant tumors, including ovarian[27], breast[28] and colorectal[29]. The D-dimer was positively correlated with differentiation grade ($P= 0.014$), tumor size ($P= 0.045$), TNM stage ($P= 0.006$) and survival ratio ($P< 0.001$). Apart from being a diagnostic marker for activation of coagulation and fibrinolysis, D-dimer also played an important role in cancer progression, invasion, and prognosis[29]. Recent research showed that activation of the coagulation system was associated with tumorigenesis development, dissemination and transfer[31,32]. Fibrinolytic enzymes in fibrinolytic systems played an important role in tumor invasion and penetration into the blood circulation. Studies had also shown that tumors were encapsulated in the network structure, as the disease developed and the rate of metastasis accelerated, the release of D-dimer increased after the destruction of the network structure, and thrombotic diseases were prone to occur[12,33,34].

A20 was significantly correlated with differentiation grade ($P < 0.001$), BMI ($P < 0.001$), TNM stage ($P = 0.024$) and survival ratio ($P < 0.001$). As a cytoplasmic zinc-finger protein, A20 was reported to be expressed in a variety of human cells, such as T lymphocytes and B lymphocytes[35,36], which had been reported to regulate dynamic immune responses by negatively mediating the activity of the transcription factor NF- κ B and the proinflammatory gene expression[14,37,38]. NF- κ B signaling pathways involved in regulating tumor proliferation and apoptosis. A20 participates in endothelial cell stress response and inflammatory response[15,21], which may be associated with D-dimer and CA19-9. Their mechanism is still unclear, which is also our next research direction.

A20 in PDAC was low expression, which found to be linked with TMN stage ($P < 0.05$). Other researchers suggested that expression of A20 was reduced in pancreatic cancer tissues, but not deeply associated with TNM stages[17]. The overall survival time of patients with CA19-9 < 176.66 U/ml ($P < 0.001$), D-dimer < 1.18 mg/L ($P < 0.001$) and high expression of A20 group ($P < 0.001$) was longer by Kaplan-Meier curves. The COX multivariate analyses showed that TNM stage ($P = 0.007$), differentiation grade ($P < 0.001$), CA19-9 ($P = 0.005$), D-dimer ($P = 0.005$) and A20 ($P < 0.001$) were independent prognostic markers for patients suffering from pancreatic ductal adenocarcinoma, which may be useful in evaluating the prognosis and progress of PDAC patients.

TNM stage and differentiation grade were not easy to be evaluated by imaging examination before operation, however, the levels of preoperative CA199 and D-dimer was easier to be detected, which may be helpful to evaluate the prognosis of pancreatic ductal adenocarcinoma patients. For patients with high levels of CA19-9 and D-dimer before operation, early intervention can be considered. We can not only use the CA19-9 and D-dimer level to judge the prognosis, but also A20, TNM stage and differentiation grade should also be considered comprehensively after the surgery.

We should make sufficient evaluation and preparation for the patients with PDAC before operation, and make the best individualized treatment plan for the patients. Follow-up should be carried out after operation to find early recurrence or metastasis to prolong survival time of patients. Therefore, by evaluation of relevant indexes before and after surgery, the optimal comprehensive treatment plan for PDAC patients was formulated, which may improve the prognosis of patients, which required a lot of further clinical and basic research.

Conclusions

CA19-9, D-dimer and A20 were independent prognostic markers for pancreatic ductal adenocarcinoma patients.

List Of Abbreviations

TNFAIP3 (A20); pancreatic ductal adenocarcinoma(PDAC); Receiver operating characteristic curve (ROC curve); Neutrophil-lymphocyte rate (NLR); Platelet-lymphocyte rate (PLR); Area under the ROC curve (AUC);

Endothelial cells (ECs); Diaminobenzidine (DAB)

Declarations

Authors' contributions

Peng Xu, Yao Jie and A-Ma Xu carried out the main work and contributed equally. They designed this study, drafted this manuscript and revised this work. XiaoDong Wang, ZhengNan Li and JianJun Qian performed the study and participated in this work. All authors read and approved the final manuscript.

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Consent for publication

Not applicable.

Data availability

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

This study was approved by the Institute Research Ethics Committee of Northern Jiangsu People's Hospital affiliated to Yang Zhou University, Yang Zhou, China. Each patients signed the informed consent.

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Competing interests

There are no potential conflicts of interest to disclose.

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Figures

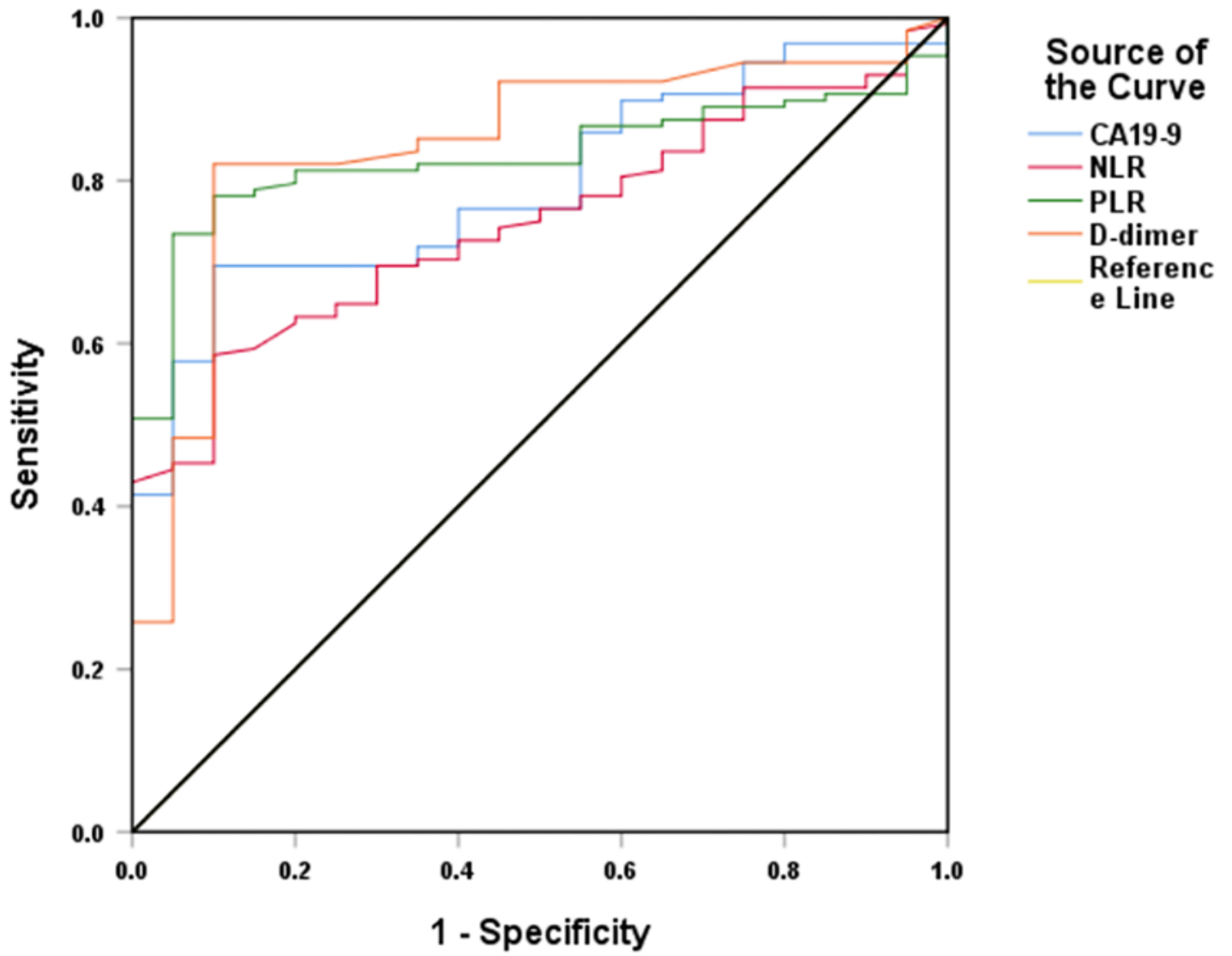


Figure 1

ROC curve of marker in patients with pancreatic ductal adenocarcinoma

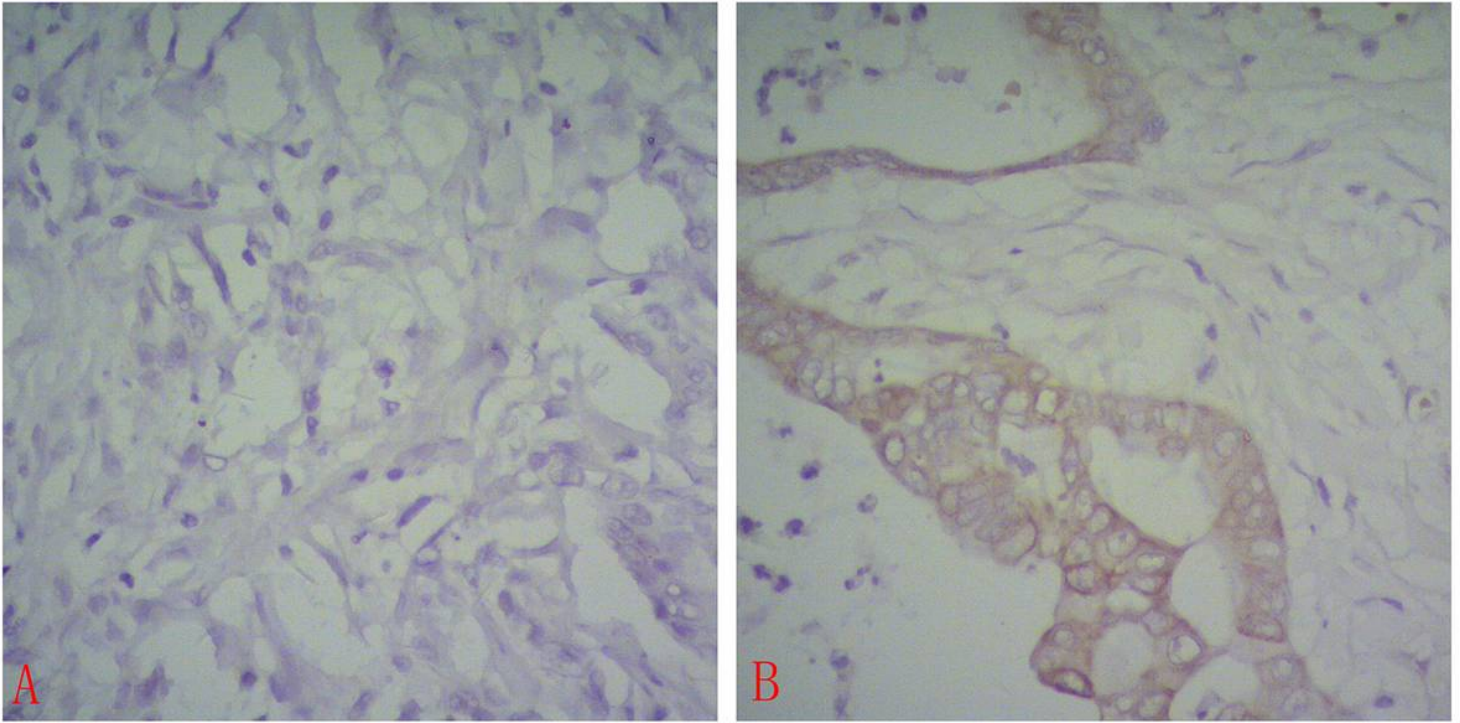


Figure 2

Expression of TNFAIP(A20) in pancreatic ductal adenocarcinoma A. Negative expression of TNFAIP(A20) in pancreatic ductal adenocarcinoma(X400). B. Positive expression of TNFAIP(A20) in pancreatic ductal adenocarcinoma(X400).

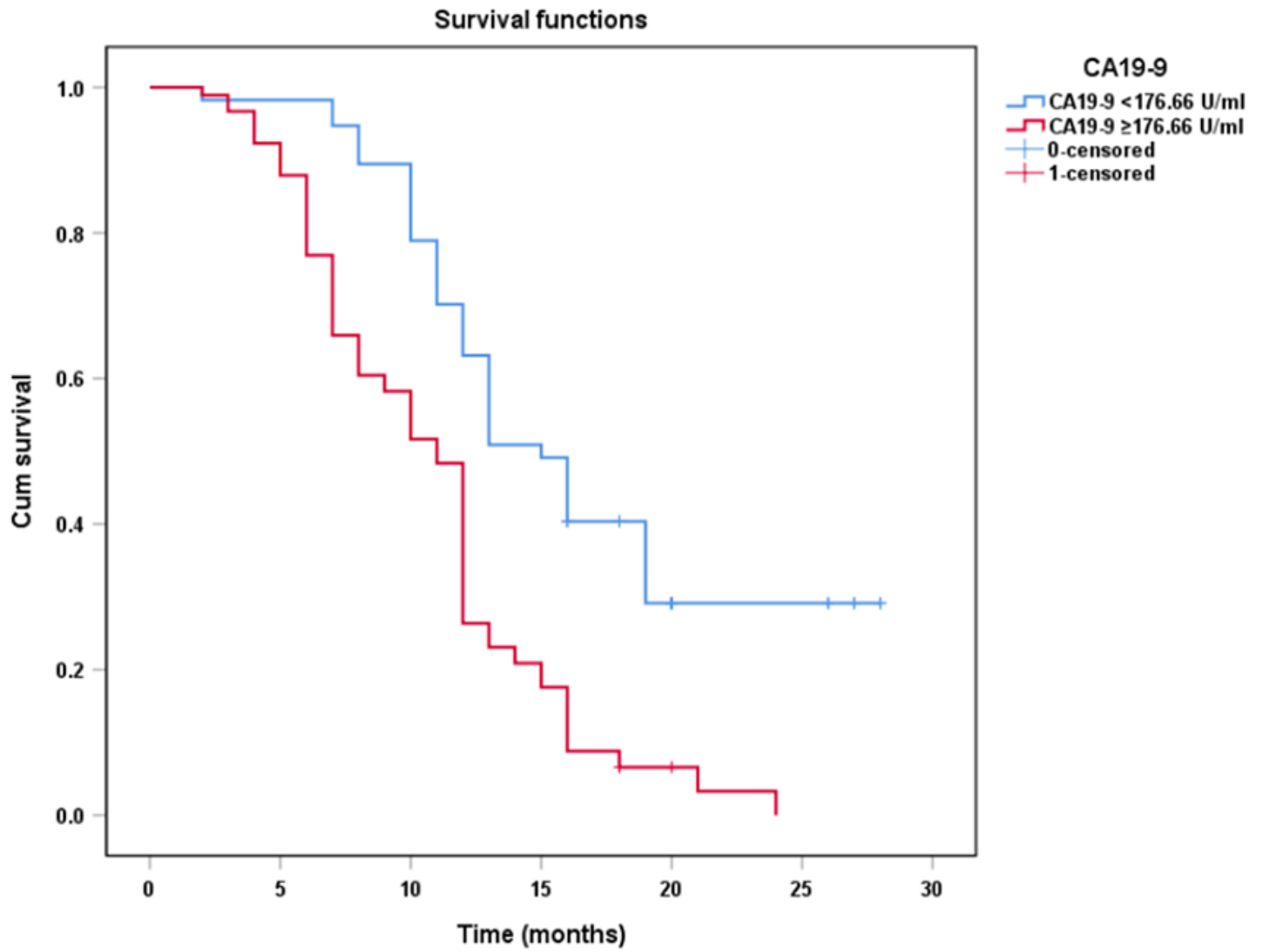


Figure 3

Kaplan-Meier curves of survival patients with CA19-9 expression

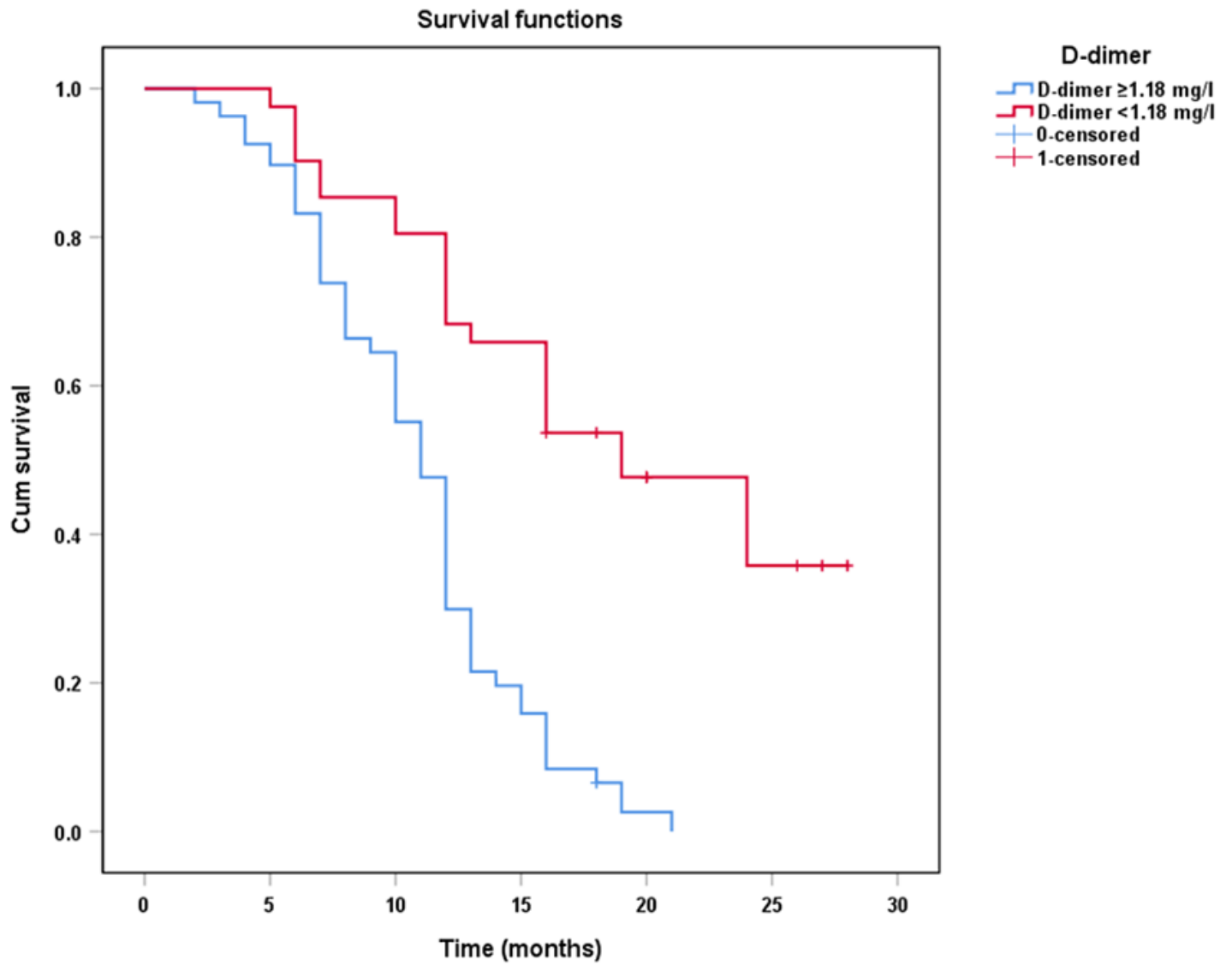


Figure 4

Kaplan-Meier curves of survival patients with D-dimer expression

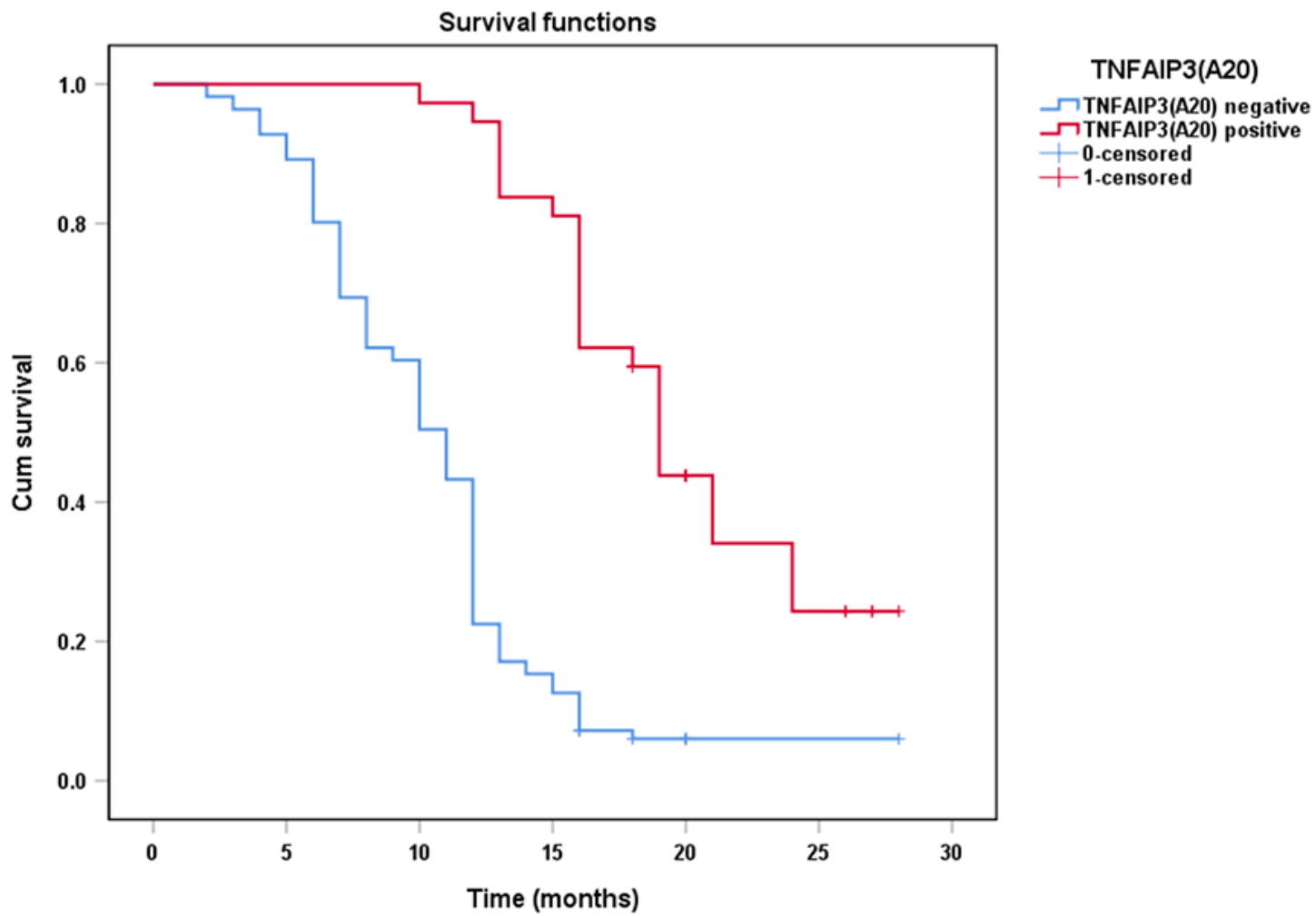


Figure 5

Kaplan-Meier curves of survival patients with TNFAIP(A20) expression