Original Articles

Expression of Vascular Endothelial Growth Factor and Epidermal Growth Factor Receptor in Pancreatic Ductal Adenocarcinomas, Neuroendocrine Tumours and Chronic Pancreatitis

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ABSTRACT

Objective: Angiogenesis is a crucial event for pancreatic carcinogenesis, and it also plays an important role in chronic pancreatitis. The aim of our study was to evaluate the mRNA expression of vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) in chronic inflammatory or malignant pancreatic pathology in order to elucidate the differences in expression patterns and potential clinical implications.

Methods: Thirty-five patients who had undergone endoscopic ultrasonography followed by endoscipic ultrasound-guided fine needle aspiration (EUS-FNA) of focal pancreatic masses were included in the study. VEGF and EGFR mRNA expression levels in the samples collected by EUS-FNA were analyzed using quantitative real-time polymerase chain reaction (PCR).

Results: VEGF expression was detected in all chronic pancreatitis and adenocarcinoma samples and in only 62.5% of pancreatic neuroendocrine tumors. EGFR expression was detected in only 40% of the chronic pancreatitis cases, 76.9% of adenocarcinomas and in 50% of pancreatic neuroendocrine tumors. Both VEGF and EGFR mRNA levels were significantly higher in pancreatic ductal adenocarcinoma than those in normal tissue. VEGF expression inversely correlated with pancreatic ductal adenocarcinoma size, while EGFR expression was related to local invasiveness of adenocarcinoma.

Conclusion: Both VEGF and EGFR mRNA expression in EUS-FNA samples may be used as a diagnostic marker associated with invasiveness in patients with pancreatic adenocarcinoma.

Keywords: vascular endothelial growth factor; endoscopic ultrasound; pancreatic ductal adenocarcinoma; pancreatic neuroendocrine tumor; epidermal growth factor receptor

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INTRODUCTION

In pancreatic tumors, as in the majority of solid malignancies, angiogenesis is essential for the local growth, invasion and metastasizing potential. In the absence of neoangiogenesis, tumors can grow only to up 1-2 mm in diameter.[1] The expression of pro-angiogenic factors is a crucial event in the process of pancreatic carcinogenesis, and it is also present in various degrees in chronic pancreatitis.[2]

Vascular endothelial growth factor (VEGF) induces some important events in the process of tumor angiogenesis such as endothelial cell survival, proliferation, migration and microvascular permeability.[3,4] Thus, in the past years many studies have focused on relating pancreatic tumor angiogenesis to cancer invasion or survival, while inhibition of angiogenesis was considered part of the therapy.

Epidermal growth factor (EGF) is a potent mitogenic factor that plays an important role in the growth, proliferation and differentiation of numerous cell types. This protein acts

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by binding the high affinity cell surface receptor, epidermal growth factor receptor (EGFR). EGFR is a trans-membrane tyrosine kinase receptor belonging to the erbB family. Activation of EGFR initiates intracellular signal transduction leading to cell migration, growth, morphological alterations, but also angiogenesis through activation of VEGF.[5]

In this study we evaluated VEGF and EGFR mRNA expressions in patients with chronic inflammatory or malignant pancreatic pathology in order to assess the differences in expression patterns and potential clinical implications.

MATERIALS AND METHODS

Patients

Pancreatic tissue samples were collected from 35 patients who had undergone endoscopic ultrasonography (EUS) followed by EUS-guided fine needle aspiration (FNA) of focal pancreatic masses at the Research Center in Gastroenterology and Hepatology of Craiova, Romania, between 2009 and 2011. For EUS-FNA Olympus 22-G (Olympus Corporation, Japan) needles were used. The samples were collected in RNAlater solution (Ambion, Inc., Austin, Texas, US) and stored at -80°C. All the samples were examined through usual cytopathological techniques at the Department of Pathology, University of Medicine and Pharmacy of Craiova. The study was approved by the Ethical Committee of the University of Medicine and Pharmacy of Craiova, Romania and informed consent for EUS-FNA, followed by molecular studies was obtained from each of the patients.

RNA Isolation and Reverse-Transcription

SV Total RNA Isolation System (Promega, Madison, WI, USA) was used for the isolation and purification of total RNA from tissue samples. The RNA concentration and purity were measured spectrophotometrically (Eppendorf Biophotometer, Eppendorf, AG, Hamburg, Germany). The integrity of isolated RNA was further assessed using the Agilent 2010 Bioanalyzer (Agilent Technologies Inc., US). The reverse-transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, US). The reverse-transcription reactions were carried out in 20µl volume; the input amount of total RNA was 100 ng diluted to a volume of 10 µL in Nuclease Free Water.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The cDNA was diluted 1:10 in Nuclease Free Water prior to use in PCR reaction. At least one no template control reaction (NTC) was performed in each run as negative control. Quantitative real-time PCR was performed using TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA, US) with specific primers and TaqMan® probes for target genes and for endogenous control gene (VEGF - Hs00900054_m1, EGFR - Hs01076092_m1 and GAPDH - Hs99999905_m1). The amplifications were carried out in 20 µL volume, in triplicate, on a Rotor-Gene 6200 HRM (Corbett Life Science, Sydney, Australia). The cycling parameters were: 50 ℃ for 2 min, 95 ℃ for 10 min, followed by 50 cycles of PCR at 95℃ for 15 s and 60℃ for 1 min. The expression of the target genes was normalized to the GAPDH endogenous control gene and the results are shown as relative mRNA expression. We have considered a biological difference in gene expression when the relative mRNA levels for target genes varied at least 1.8 times (>1.8: over-expression, <0.55: under-expression, 0.55-1.8: no difference in gene expression).

Statistical analysis

Kolmogorov-Smirnov and Shapiro-Wilk tests were performed to determine whether the variables followed a Gaussian distribution. When the variables did not follow a normal distribution, non-parametric tests were used. Kruskal-Wallis tests were performed for statistical analysis of target genes expression between groups of samples. For assessing differences in genes expression according to clinicopathological parameters, Mann-Whitney tests were performed. Linear regression analysis was performed to assess correlations between genes expression. Two-tailed *P* values <0.05 were considered statistically significant. The data were analyzed using GraphPad Prism 5 and GraphPad InStat softwares (GraphPad Software, Inc, CA, US).

RESULTS

A total number of 35 patients with an imaging suspicion of focal pancreatic masses investigated by transabdominal ultrasound or computed tomography were investigated using EUS (including contrast enhancement and elastography) at the Research Center in Gastroenterology and Hepatology of Craiova. The gender distribution (male: female) was 9:4 in the PDAC (pancreatic ductal adenocarcinoma) group, 5:3 in the PNET (pancreatic neuroendocrine tumor) group, 9:1 in the CP (chronic pancreatitis) group and 3:1 in the control group.

The mean age of the patients was 61.23±8.55 (8 patients over and 5 patients under 60 years old) in the PDAC group, 57.25±8.89 (5 patients over and 3 patients under 60 years old) in the PNET group, 52.1±12.85 (3 patients over and 7 patients under 60 years old) in the chronic pancreatitis group and 58.3±8.51 (2 patients over and 2 patients under 60 years old) in the control group.

Based on EUS appearance and cytopathological examination, 13 patients were diagnosed with PDACs, 8 with PNETs and 10 with CP. Four samples, from patients with acute pancreatitis and a suspicion of focal pancreatic masses (fullness of the pancreatic head on other imaging tests) were found to include normal pancreatic tissue (NP) and were used as control samples.

Table 1. Correlation between VEGF relative expression and clinicopathological parameters in PDACs and PNETs

Patients	PDAC			PNET		
characteristics	Cases	Relative expression	*P* value	Cases	Relative expression	*P* value
Age (yr)						
⩾ 60	8	0.10 ± 0.050	>0.05	5	0.03 ± 0.016	>0.05
<60	5	0.23 ± 0.130		3	0.01 ± 0.010	
Gender						
Male	9	0.18 ± 0.080	>0.05	5	0.03 ± 0.017	>0.05
Female	4	0.07 ± 0.020		3	0.02 ± 0.009	
Tumor location						
Head	10	0.18 ± 0.070	>0.05	5	0.04 ± 0.014	<0.05
Body	3	0.06 ± 0.038		3	0	
Tumor size (cm)						
<3	3	0.38 ± 0.178	<0.05	2	0.02 ± 0.008	>0.05
⩾ 3	10	0.08 ± 0.037		6	0.04 ± 0.017	
Local invasiveness						
(-)	3	0.03 ± 0.017	>0.05	5	0.02 ± 0.006	>0.05
(+)	10	0.19 ± 0.070		3	0.03 ± 0.029	
Lymph node						
metastases						
(-)	4	0.27 ± 0.160	>0.05	4	0.01 ± 0.008	>0.05
(+)	9	0.10 ± 0.040		4	0.03 ± 0.020	
Liver metastases						
(-)	5	0.21 ± 0.138	>0.05	5	0.01 ± 0.006	>0.05
(+)	8	0.12 ± 0.045		3	0.04 ± 0.025	

Relative expression: mean ± SEM, Mann-Whitney test. PDAC: pancreatic ductal adenocarcinoma; PNET: pancreatic pancreatic neuroendocrine tumor.

Gene expression profiling

Total RNA was isolated from all the EUS-FNA pancreatic tissue samples. The RNA concentrations were relatively low in the studied samples (between 5.1 and 85.5 μg/mL) and the 260/280 and 260/230 ratios were within the recommended ranges for use in reverse-transcription reaction. The RNA Integrity Numbers (RINs) were lower than 5 in most of the samples. To investigate VEGF and EGFR mRNA expression profiles, relative mRNA levels (target gene/GAPDH) were assessed in all the samples. Relative mRNA levels for each of the lesions included were compared with the relative expression in normal pancreas.

VEGF was expressed in all the CP and PDAC samples and in 62.5% of PNETs. VEGF significantly over-expressed in PDACs when compared with that in normal pancreatic tissue ($P<0.01$, Kruskal-Wallis test). VEGF relative expression had a tendency of over-expression in PNETs compared with normal pancreas, but without reaching a statistically significant level (Fig. 1).

EGFR was expressed in only 40% of the CP cases, 76.9% of the PDAC cases and 50% of PNETs. EGFR mRNA expression was significantly higher in the PDAC tissue than that in normal tissue ($P < 0.05$, Kruskal-Wallis test). There were no significantly statistical differences in EGFR expression between PNETs and normal tissue or CP samples. In only 25% PNET cases, EGFR was over-expressed when compared with that in the normal tissue (Fig. 2).

VEGF and EGFR expression levels in PDACs and PNETs were analyzed by univariate analysis in comparison with several clinicopathological parameters: age, gender, tumor location, tumor size, local invasiveness, lymph nodes metastasis and liver metastasis (Tab. 1, 2). In the PDAC group, VEGF expression was higher in tumors smaller than 3 cm in size than that in tumors larger than 3 cm. An over-expression of EGFR in local invasive adenocarcinomas compared with non-invasive adenocarcinomas of the pancreas was also observed.

The linear regression analysis showed a strong positive correlation between VEGF and EGFR relative mRNAs expression in the studied samples ($F = 15.57$, $P = 0.03$).

DISCUSSION

In this study we evaluated the expression profiles of VEGF

Table 2. Correlation between EGFR relative expression and clinico-pathological parameters in PDACs and PNETs

Patients	PDAC			PNET		
characteristics	Cases	Relative expression	*P* value	Cases	Relative expression	*P* value
Age (yr)						
≥60	8	0.10 ± 0.050	>0.05	5	0.03 ± 0.016	>0.05
<60	5	0.23 ± 0.130		3	0.01 ± 0.010	
Gender						
Male	9	0.18 ± 0.080	>0.05	5	0.03 ± 0.017	>0.05
Female	4	0.07 ± 0.020		3	0.02 ± 0.009	
Tumor location						
Head	10	0.18 ± 0.070	>0.05	5	0.04 ± 0.014	<0.05
Body	3	0.06 ± 0.038		3	0	
Tumor size (cm)						
<3	3	0.38 ± 0.178	<0.05	2	0.02 ± 0.008	>0.05
≥3	10	0.08 ± 0.037		6	0.04 ± 0.017	
Local invasiveness						
(-)	3	0.03 ± 0.017	>0.05	5	0.02 ± 0.006	>0.05
(+)	10	0.19 ± 0.070		3	0.03 ± 0.029	
Lymph node metastases						
(-)	4	0.27 ± 0.160	>0.05	4	0.01 ± 0.008	>0.05
(+)	9	0.10 ± 0.040		4	0.03 ± 0.020	
Liver metastases						
(-)	5	0.21 ± 0.138	>0.05	5	0.01 ± 0.006	>0.05
(+)	8	0.12 ± 0.045		3	0.04 ± 0.025	

Relative expression: mean ± SEM, Mann-Whitney test. PDAC: pancreatic ductal adenocarcinoma; PNET: pancreatic pancreatic neuroendocrine tumor; EGFR: epidermal growth factor receptor.

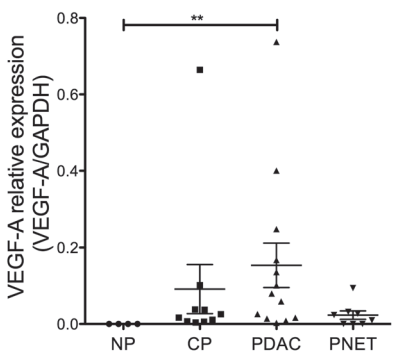

Figure 1. Comparative relative expression of VEGF mRNA in pancreatic ductal adenocarcinoma (PDAC) (0.15 ± 0.058), pancreatic neuroendocrine tumour (PNET) (0.023 ± 0.011), chronic pancreatitis (CP) (0.091 ± 0.064) and normal pancreas (NP) (0.00006±0.00003) (*n* = 35). Data are presented as relative mRNA expression of VEGF to GAPDH. Kruskal-Wallis test.

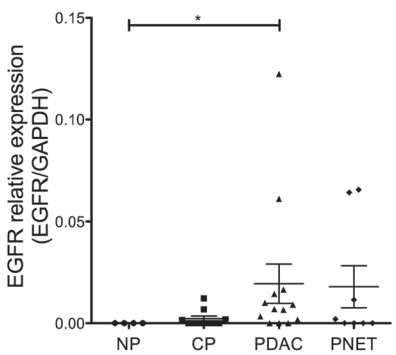

Figure 2. Comparative expression of EGFR mRNA in pancreatic ductal adenocarcinoma (PDAC) (0.019 ± 0.009), pancreatic neuroendocrine tumour (PNET) (0.018 ± 0.010), chronic pancreatitis (CP) (0.0023 ± 0.001) and normal pancreas (NP) (0) (*n* = 35). Data are presented as relative mRNA expression of EGFR to GAPDH. Kruskal-Wallis test.

and EGFR in EUS-FNA samples from CP and malignant tumors of the pancreas (PDACs and PNETs).

At least 200 ng of total RNA were isolated from each sample, with purities within the recommended ranges for reverse-transcription reaction. The RIN calculation, used for the evaluation of RNA integrity, revealed that the isolated RNA was partially degraded. This was not an impediment for the evaluation of gene by qRT-PCR, since it has been shown that PCR efficiency does not vary according to RIN when small amplicons are generated.[6] In our study the amplicon lengths were as follows: 60 bp for VEGF, 103 bp for EGFR

and 122 bp for GAPDH.

Several immunohistochemistry studies based on surgical samples have shown an over-expression of VEGF in both PDACs and PNETs, but the reported findings concerning the association between its expression and clinicopathological features and prognosis are still controversial. For PDAC patients it was found that VEGF expression was associated with poor prognosis,[7] whereas in PNETs there are no correlations between VEGF expression and tumor growth or spread.[8] Also, VEGF tissue levels measured by enzyme-linked immunosorbant assay (ELISA) were not related to clinicopathological features and poor prognosis in PNET patients.[9] Many studies have shown that VEGF expression is crucial for the development of PDAC, but the majority of the studies did not found a correlation between VEGF expression and tumor stage or patients' survival.[10,11] In other studies, high levels of serum VEGF were correlated with tumor size, lymph node metastases and distant metastases in PDAC patients.[12,13] In our study VEGF mRNA levels were not associated with tumor invasiveness, lymph nodes invasion or liver metastases neither in PNETs nor in PDACs. On the other hand, VEGF mRNA relative expression in adenocarcinomas was higher in small tumors (tumors with the maximum diameter lower than 3 cm) than that in large tumors (tumors with the maximum diameter higher than 3 cm), suggesting that new blood vessels formation is a key process in the early stages of tumor development and becomes less evident in advanced stages, probably due to necrosis or to the strong desmoplastic reaction that accompanies advanced PDACs. Thus, the defective angiogenesis in some of the advanced PDAC cases might be responsible for the lack of response to chemotherapy and/or antiangiogenic therapy, which does not reach the tumor cells due to decreased vessel formation and intense desmoplastic reaction at the level of tumor stroma. Although a correlation with contrast-enhanced patterns during computed tomography or EUS was not an objective of the current study, it has been already proven that most advanced PDAC cases were hypovascular as compared to surrounding normal pancreatic tissue.[14]

EGFR gene is over-expressed in PDACs when compared with normal pancreatic cells.[15] Over-expression of EGFR was associated with tumor stages[7] and it was suggested that EGFR plays a crucial role in the progression of PDACs, especially in the invasion and in the acquisition of aggressive clinical behavior.[16] In contrast, other authors have shown no significant correlation between expression of EGFR and tumor size or lymph nodes status.[17] A recent meta-analysis of previous studies based on surgical samples reported that EGFR expression is a poor prognosis factor for survival in patients with pancreatic cancer.[18] The immunoexpression of EGFR in PNETs has also been correlated with the grade of malignancy.[19]

EGFR seems to play an important role in pancreatic fibrosis in both CP and PDAC, characterized by stromal expansion and excessive deposition of extracellular matrix (ECM) that replaces pancreatic tissue. This eventually leads to dysregulation of ECM turnover, production of cytokines and restricted blood flow.[20] The restriction of blood flow may be a stimulus for VEGF over-production by tumor cells, supported by the association between VEGF and EGFR expression shown in our study.

Our study has several limitations, which certainly include the small number of patients, which might have influenced the statistical analysis. The results were not correlated with immunohistochemistry which is notoriously difficult to be performed in the small cell blocks extracted through EUS-FNA only in a small percentage of the lesions sampled. However, the evaluation of VEGF or EGFR expression levels in EUS-FNA samples might be viewed as a possible strength of the current study, because these markers cannot be assessed properly and quantified by immunocytochemistry or immunohistochemistry in these cases. Another limitation of our study was represented by the use of samples from patients with EUS findings of acute pancreatitis in the control group. However, these samples were used as control after cytological examination that confirmed the presence of normal pancreatic tissue. This also explains the small number of samples we have used in the control group.

In summary, we have shown that VEGF is over-expressed in CP, PDACs and PNETs when compared with normal tissue, whereas EGFR was over-expressed only in adenocarcinomas and less than 25% of PNETs. Furthermore, EGFR expression was related to adenocarcinoma invasiveness, whereas VEGF was inversely correlated with tumor size. In conclusion, EGFR expression in EUS-FNA samples may be used as a diagnostic marker associated with invasiveness in PDACs, although this small feasibility study has to be extended on a larger group of patients. Also, evaluation of EGFR or VEGF expression in EUS-FNA samples might be important to assess angiogenesis in PDACs or PNETs, in order to choose the best therapeutic regimen.

DISCLOSURE

The authors have no conflicts of interests to declare.

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