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Leptin involvement in the survival of pancreatic adenocarcinoma patients with obesity and diabetes

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Abstract. – OBJECTIVE: Current molecular characterization of pancreatic ductal adenocarcinoma (PDAC) does not incorporate the host reaction to cancer cells and cannot predict the response to chemo- or immunotherapy. Leptin is an adipokine involved in regulating energy balance with a possible role in the development of obesity-associated cancers, but its relationship with other pathways in pancreatic carcinogenesis has not been established yet. The aim of this prospective study was to assess the involvement of leptin and phosphoinositide 3-kinase (PI3K) in the survival of overweight and/or diabetic patients with PDAC.

PATIENTS AND METHODS: A total of 112 patients were included, 56 diagnosed with PDAC and 56 age and sex-matched healthy controls, with a maximum follow-up of 24-months. The circulating leptin, interleukin 1-beta, tumor factor necrosis-alpha, and PI3K were measured by enzyme-linked immunosorbent assay (ELISA). A multivariate Cox regression model was used to determine the factors influencing survival.

RESULTS: The serum levels of leptin [38.5 (31.6-47.0) pg/ml] and other cytokines in PDAC patients were similar to controls, irrespective of the presence of diabetes. No significant correlation between the biomarkers was found. In obese and overweight patients, the leptin level and survival rate were lower than in non-obese patients.

CONCLUSIONS: The leptin level was not associated with the presence of PDAC, although it was lower in obese and overweight patients who had lower survival. No association with inflammatory biomarkers or PI3K was noted. Furthermore, leptin levels had no independent role in survival, suggesting that the prognostic role of obesity in PDAC is based on a different pathway.

Key Words:
Pancreatic cancer, Leptin, AKT pathway, Glucose metabolism, Obesity.

Introduction

Metabolic diseases, such as obesity and diabetes may contribute to pancreatic adenocarcinoma (PDAC) development *via* dysregulated metabolic pathways. Therefore, delineating key players in the oncogenic development in the pancreas, that are linked to metabolic disorders could be beneficial[1]. Fatty pancreas is associated with advanced age, high body mass index (BMI), and diabetes, which are also well-known risk factors for PDAC[2].

Leptin is an adipokine that regulates food intake and energy metabolism, and its circulating level has been proved to be significantly increased in obese individuals[3]. Leptin has also been identified as a potential biological link in the development of obesity-associated cancers, such as PDAC. A prognostic role was found for

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leptin in men with PDAC, but not in women[4]. An *in vitro* study[5] showed that leptin promotes cell proliferation and increases glucose uptake and lactate production in human PDAC and healthy pancreas cells, in a dose-dependent manner. Silencing the leptin receptor expression in PDAC cells leads to an inhibitory effect on protein kinase B(AKT) phosphorylation, by decreasing phosphatidylinositol 3-kinases (PI3K) activity *via* the PI3K/Akt/mTOR cell regulatory pathway[5].

Macrophages have a key role in PDAC initiation by promoting epithelial-mesenchymal transition, thereby enabling the ability of invasion and metastasis[6]. Hyperglycemia, common to both obesity and type 2 diabetes mellitus (T2DM) has been proved to sustain and promote macrophage-driven inflammation in adipose cells[7].

Despite valuable insight into metabolic pathways that promote pancreatic carcinogenesis, there are no established prognostic biomarkers for prediction of survival. Furthermore, studies exploring the association between leptin, PI3K/AKT/mTOR pathway and inflammatory biomarkers have not been yet conducted.

In this pilot, prospective study, we aimed to assess the involvement of circulating leptin and PI3K on pancreatic cancer survival in patients with obesity and T2DM.

Patients and Methods

Patients

Data from patients diagnosed with pancreatic cancer were prospectively collected, between January 2017 and June 2018, in a tertiary care hospital. This study was approved by the Ethics Committee of the hospital (No. 11387), and the report followed the Strengthening the Reporting of Observational studies in Epidemiology (STROBE) guidelines.

Subjects of the study group were at least 18 years old, with no previous history of cancer in the last 5 years. Written consent was given prior to inclusion in this study. Patients with PDAC were enrolled at the time of diagnosis before any therapeutic intervention. The final diagnosis was based on the histology report following tissue sampling by endoscopic ultrasonography fine needle aspiration (EUS-FNA) or surgery.

The exclusion criteria were the following: lack of adenocarcinoma histology, previous chemotherapy or pancreatic surgery, or refusal to participate in the study.

The control group included an age and sex-matched (+/- 5 years) cohort of healthy individuals of at least 18-years-old, with no previous history of cancer and/or chronic diseases. For the PDAC patients, age, sex, tumor stage, and location, BMI, smoking, and the presence of diabetes were recorded.

Body weight and height were measured at the time of inclusion. Diabetes was diagnosed if fasting glucose values met the ADA criteria[8], and the duration since the diabetes onset was recorded. All patients were followed up for 24 months and the date of death was noted.

Blood Sampling

Blood samples were collected at the time of diagnosis, after overnight fasting. Peripheral venous blood was collected into a tube containing Ethylenediaminetetraacetic Acid (EDTA) and was prepared by centrifugation at 5000 × g for 5 min. The serum samples were stored at -80°C until use. The selected protein was quantified from serum using ELISA analyses. Case and control specimens were handled in the same standard manner, and the laboratory was blinded to case-control status. Matched serum case and control samples were analyzed consecutively within batches, and blinded replicate quality control samples from each respective study were placed in each batch within each cohort and comprised 10% of each batch.

ELISA Methods

Serum levels of human leptin, IL-1beta, TNF-alpha and PI3K were determined by using sandwich enzyme-linked immunosorbent assays (ELISA). Individual serum samples were measured in duplicates using sandwich ELISA kits and following the manufacturer's instructions (Leptin: Cusabio catalog number CSB-E04649h, sensitivity <0.060 ng/mL, intra-assay precision CV < 8 % and inter-assay precision CV < 10%; IL-1 beta: BioVendor catalog number RD194559200R, sensitivity 0.4 pg/mL, intra-assay precision CV = 2.7-4.2 % and inter-assay precision CV =5.2-6.7%; TNF-alpha: BioVendor catalog number RAF128R, sensitivity 2.3 pg/mL, intra-assay precision CV = 6.0 % and inter-assay precision CV = 7.4%; PI3K: MyBiosource catalog number MBS268899 sensitivity 0.06 ng/mL, intra-assay precision CV ≤ 8 % and inter-assay precision CV ≤ 12%). For each kit, a standard calibration curve was generated using the protein standard provided.

Absorbance was measured with a ClarioStar multiplate reader (BMGLabtech) and data were acquired and processed using the integrated Mars software. For the quantification, a 4-parameter fit calibration curve was used, and final concentrations were calculated as mean of the two measurements.

Statistical Analysis

Categorical data were presented as counts and percentages. Quantitative data were presented as means and standard deviations (when data followed a normal distribution) or as medians and interquartile ranges (when data did not follow a normal distribution). Comparisons between two groups of categorical data were performed with chi-square and Fisher exact tests, whereas for quantitative data a *t*-test for independent samples (when data followed a normal distribution) or a Mann-Whitney U test (when data did not follow a normal distribution) were performed. ANOVA and Kruskal Wallis tests were used for comparing three groups regarding quantitative data, for normally and not normally distributed data respectively. Associations between continuous data were assessed with Spearman correlation coefficient. Survival data were analyzed with Cox proportional hazard models, initially univariate ones. Multivariate models were built for leptin expression adjusted T4, then for N1 and finally for T4 and N1. For all Cox models the proportional hazard assumption was checked, whereas for multivariate models, the multicollinearity assumption was checked. Predictors for metastases were assessed with univariate and multivariate logistic regression models. We checked the models for multicollinearity, misspecification, and the goodness of fit.

For all statistical tests, a two-tailed *p*-value was used, and the results were considered significant if $p<0.05$. The statistical analysis was performed using the R program for statistical computing and graphics, version 3.4.4.

Results

Patients' Characteristics

Baseline characteristics of the study participants are shown in Table I. Fifty-six patients with PDAC and 56 sex and age-matched controls were included in this study. Within the PDAC group, 28 patients had diabetes. No significant difference was observed for BMI, smoking status, tumor location, or TNM staging (Table I) between PDAC patients with and without diabetes and the control group.

Biomarkers Expression in PDAC With or Without Diabetes Compared to Controls

Half of the patients with PDAC had biomarkers serum levels higher than the median, similar to the control group (27/56 *vs.* 30/56 for leptin, 26/56 *vs.* 32/56 for IL1beta, 29/56 *vs.* 26/56 for TNF alpha and 29/56 *vs.* 26/56 for PIK3). The expression of the 4 molecules was not different between the control group and PDAC patients, with or without diabetes (Table II).

Table I. Baseline characteristics of study population.

	PDAC with diabetes (n = 28)	PDAC without diabetes (n = 28)	Controls (n = 56)	*p*-value
Age (years), mean (SD)	63.3 (9.8)	61.9 (10.3)	62.4(10)	0.98
Male, n (%)	19 (67.9)	14 (50)	29 (51.8)	0.4
BMI (kg/m^2), median (IQR)	24.7 (19.2-26.9)	26.3 (23.3-29.8)	1.6 (-5.0-0.3)	0.07
Underweight	3 (10.7)	9 (32.1)		
Normal	6 (21.4)	7 (25)		0.20
Overweight	12 (42.9)	8 (28.6)		
Obesity n (%)	7 (25)	4 (14.3)		
Location				
Cephalic, n (%)	22 (78.6)	20 (71.4)		0.28
Body, n (%)	9 (32.1)	12 (42.9)		0.41
Tail, n (%)	2 (7.14)	2 (7.14)		1
T4, n (%)	12 (42.86)	14 (50)		0.59
N1 stage	26 (92.9)	24 (85.7)		0.67
Metastasis	6 (21.4)	9 (32.1)		0.37

PC, pancreatic cancer; SD, standard deviation; IQR, interquartile range; BMI, body mass index; T, tumor; N, adenopathy.

Table II. Biomarkers levels according to diabetes status in the PDAC groups compared to controls.

	PDAC (n = 56)	Control (n = 56)	*p*-value	PDAC without DM (n = 28)	PDAC with DM (n = 28)	*p*-value
Leptin (pg/mL), median (IQR)	38.5 (31.6-47.0)	40.4 (33.6-53.1)	0.31	38.7 (28.6-45.7)	38.3 (35.0-49.3)	0.26
PI3K (ng/mL), median (IQR)	43.3 (39.9-49.2)	42.1 (39.3-46.2)	0.77	43.7 (40.0-49.3)	43.0 (39.9-48.44)	0.88
IL-β (pg/mL), median (IQR)	388.1 (363.0-409.0)	400.2 (372.3-410.3)	0.59	391.4 (368.69-419.49)	386.48 (361.38-405.55)	0.34
TNF-α (pg/mL), median (IQR)	1239.8 (1084.3-1551.7)	1227.0 (1133.2-1465.8)	0.93	1268.09 (1101.78-1553.71)	1235.46 (1084.3-1511.56)	1

PDAC, pancreatic ductal adenocarcinoma; IQR, interquartile range; PI3K, phosphatidylinositol 3-kinases; IL, interleukin; TNF alpha, tumor necrosis factor alpha.

The leptin serum levels were higher in overweight or obese patients, compared to the underweight or normal weight group (36.11 (29.41-42.44) pg/ml *vs.* 42.06 (34.62-53.79) pg/ml) (p=0.023). The other biomarkers serum levels did not correlate with the demographic and clinical patients' characteristics (**Supplementary Table I**).

The survival of PDAC patients was not associated with protein expression. The risk of metastasis at the time of diagnosis was independent of clinical or demographic factors, as well as biomarkers levels (Table III).

Forty-three percent of patients with PDAC did not survive beyond the 24 months follow-up. The overall median survival was 18 months for the PDAC group. Univariate and multi-variate analysis showed higher survival in younger patients; the biomarkers serum levels had no association with the survival. However, when adjusted for age, tumor stage or diabetes, obesity was associated with lower survival in PDAC patients (HR= 0.23, 95% CI: 0.0588 - 0.9698, p=0.045) (Table III).

Association Between Leptin, PI3K and Cytokines in PDAC Associated with Obesity or Diabetes

There was no association between the Leptin, PI3K, IL-1β or TNF-α levels in PDAC patients with diabetes. In obese/overweight PDAC group, we found a significant correlation between IL-1β and TNF-α (Spearman rho 0.41, p= 0.025) (Table IV).

Discussion

This prospective study showed that leptin serum level did not correlate with the PI3K/AKT pathway. There were no differences in leptin serum levels between PDAC patients compared to controls, irrespective of the presence of diabetes. Leptin level was higher in obese and overweight patients but did not influence the survival rate at 24 months follow-up.

Obesity is considered a strong risk factor for PDAC, up to 20% higher when compared to normal weight individuals[9]; however, the underlying mechanism of this association is not fully understood. A chronic state of adipose tissue inflammation could promote cancer growth *via* the secretion of proinflammatory cytokines, such as TNF-α, TGF-β, IL-6 and leptin[10]. Leptin in particular was shown to promote tumor vascularization, as well as the proliferation, migration, and invasion of tumor cells in rodent models[11].

A previous study[12], assessing prediagnostic circulating leptin levels in three cohorts of patients with pancreatic cancer followed-up for 22.8 years, 14.5 years and 7.4 years, reported no risk for developing PDAC during the earlier years of follow-up. However, a positive association with follow-up longer than 10 years was noted (OR=1.44), which increased after adjustment for BMI, but not for TGF-β1 or adiponectin. Furthermore, the role of leptin was explored in 7110 PDAC patients and 7264 controls, showing no correlation to cancer risk (OR = 0.39 [95% CI = 0.11-1.37]; p = 0.14)[13], which is in line with our results. In contrast, another report[14] showed that low leptin and high adiponectin levels were independently associated with PDAC risk[14]. These results were not confirmed by a meta-analysis of 93 studies on circulating adipokines and obesity-related cancers, including 5 studies studying the role of adipokines in PDAC; no association with leptin (R=0.92) or adipokine level (OR=0.98) were shown[15]. Our study reported low level of leptin in obese patients, but no association with survival was noted in these patients.

Leptin is a 16-KDa non-glycosylated protein encoded by LEP and secreted by adipocytes, in proportion to white adipose tissue mass[11]. Factors that alter leptin production include insulin, estrogen and inflammatory mediators such as IL1B, IL6 and TNFα. Leptin binds to transmembrane receptors on stomach and colon cancer cells, resulting in activation of the JAK-STAT, MAPK, PI3K-AKT, insulin receptor substrate and mTOR signaling pathways.

Leptin enhances tumor vascularization, promotes cellular proliferation, migration, invasion, and inhibits apoptosis of tumor cells[11]. Different pathways have been proposed as possible mechanisms, such as leptin STAT3 mediated signaling[1] or leptin-induced tumor growth *via* Notch receptors signaling[16] but none of these were confirmed in further studies. Cytokines secreted by inflammatory cells in the tumor tissue *via* STAT3 pathway, like IL-1 β or TNF-α, were not overexpressed in our group.

PI3K/Akt signaling is one of the most commonly dysregulated signaling pathway in pancreatic cancer and represents an important therapeutic target[17]. It has been shown that PI3K/Akt

Table III. Metastasis risk at the time of diagnosis and survival, in association with clinical, demographic and serologic parameters in PDAC patients.

	Metastasis risk at the time of diagnosis			Survival		
Univariate analysis	**OR unadjusted**	**95% CI**	***p*-value**	**HR unadjusted**	**95% CI**	***p*-value**
Age	1.07	(1-1.15)	0.08	1.05	(1-1.1)	0.047
Sex (male *vs*. female)	0.73	(0.22-2.46)	0.607	1.12	(0.49-2.57)	0.784
Obesity	0.55	(0.08-2.5)	0.477	0.76	(0.26-2.21)	0.61
Smoking	0.35	(0.1-1.18)	0.101	0.83	(0.37-1.84)	0.638
Diabetes	0.58	(0.17-1.89)	0.368	1.32	(0.59-2.95)	0.497
Tumor size ≥ 3 cm	2.5	(0.37-49.77)	0.418	1.37	(0.4-4.68)	0.613
T4	3.13	(0.93-11.65)	0.072	1.06	(0.48-2.37)	0.881
N1	0.32	(0.05-1.9)	0.191	0.32	(0.12-0.87)	0.025
M1	-	-		0.79	(0.32-2)	0.627
Leptin level (high vs low)	2.56	(0.77-9.47)	0.137	0.78	(0.35-1.75)	0.549
PI3K (high-expressed vs low-expressed)	0.83	(0.25-2.74)	0.763	1.13	(0.51-2.52)	0.764
IL-1β (high vs low)	1.2	(0.36-4.02)	0.763	1.57	(0.7-3.54)	0.275
TNF-α (high vs low)	1.74	(0.53-6.04)	0.368	0.67	(0.3-1.5)	0.327
Multivariate analysis	**OR adjusted**			**HR adjusted**		
Leptin level Adjusted T4	2.3	(0.66-8.75)	0.2	0.77	(0.34-1.74)	0.532
Leptin level Adjusted N1	2.65	(0.77-10.15)	0.132	0.85	(0.38-1.91)	0.692
Leptin level Adjusted T4, N1	2.36	(0.66-9.27)	0.195	0.85	(0.37-1.95)	0.705

OR, odds ratio; HR, hazard ratio; CI, confidence interval; T, tumor; N, adenopathy; M, metastasis; PI3K, phosphatidylinositol 3-kinases; IL, interleukin; TNF alpha, tumor necrosis factor alpha; high, values above or equal to the median; low, values below the median; The multivariate logistic regression models were adjusted for T4, then for N1, and finally for T4 and N1.

Table IV. Correlation between the biomarkers level in PDAC with or without diabetes and PDAC with or without obesity and/ or overweight.

Spearman rho (*p*-value)	PDAC with DM				PDAC without DM			
	Leptin (pg/mL)	PI3K (ng/mL)	IL-1 beta (pg/mL)	TNF-a (pg/mL)	Leptin (pg/mL)	PI3K (ng/mL)	IL-1 β (pg/mL)	TNF-α (pg/mL)
Age	1.07	(1-1.15)	0.08	1.05	(1-1.1)	0.047		
Leptin (pg/mL)	-	-0.26 (0.192)	-0.06 (0.776)	0.09 (0.669)	-	0.08 (0.679)	-0.03 (0.89)	0.04 (0.827)
PI3K (ng/mL)	-0.26 (0.192)	-	0.16 (0.417)	0.23 (0.249)	0.08 (0.679)	-	0.07 (0.724)	0.31 (0.11)
IL-1 beta (pg/mL)	-0.06 (0.776)	0.16 (0.417)	-	0.32 (0.101)	-0.03 (0.89)	0.07 (0.724)	-	0.13 (0.503)
TNF-a (pg/mL)	0.09 (0.669)	0.23 (0.249)	0.32 (0.101)	-	0.04 (0.827)	0.31 (0.11)	0.13 (0.503)	-
	PDAC with obesity/overweight				PDAC without obesity/overweight			
Leptin (pg/mL)	-	0.08 (0.676)	0.17 (0.366)	0.29 (0.119)	-	-0.2 (0.35)	-0.04 (0.835)	-0.25 (0.226)
PI3K (ng/mL)	0.08 (0.676)	-	0.17 (0.383)	0.33 (0.073)	-0.2 (0.35)	-	-0.15 (0.47)	0.23 (0.274)
IL-1 beta (pg/mL)	0.17 (0.366)	0.17 (0.383)	-	0.41 (0.025)	-0.04 (0.835)	-0.15 (0.47)	-	0.09 (0.679)
TNF-a (pg/mL)	0.29 (0.119)	0.33 (0.073)	0.41 (0.025)	-	-0.25 (0.226)	0.23 (0.274)	0.09 (0.679)	-

OR, odds ratio; HR, hazard ratio; CI, confidence interval; T, tumor; N, adenopathy; M, metastasis; PI3K, phosphatidylinositol 3-kinases; IL, interleukin; TNF alpha, tumor necrosis factor alpha; high, values above or equal to the median; low, values below the median; The multivariate logistic regression models were adjusted for T4, then for N1, and finally for T4 and N1.

pathway stimulates the phosphorylation of mTOR kinase *via* activation of cyclin D1 and VEGF and mediates the effect of Kras in pancreatic carcinogenesis[18]. *In vitro* studies showed that the involvement of PI3K/AKT is the main signaling cascade in glucose metabolism and cell growth regulation[5]. PI3Kγ activity in pancreatic β cells is required for normal insulin secretion in response to glucose; therefore, PI3Kγ ablation was shown not to promote diabetes in mice, but improve insulin sensitivity and reduce pancreatic β-cell apoptosis[19].

Insulin has an important role in adipogenesis, by stimulating glucose and free-fatty acid uptake, inhibiting lipolysis and stimulating de novo fatty acid synthesis in adipocytes[20]. In addition, insulin regulates adipose tissue growth and differentiation, stimulates the production and secretion of leptin, which in turn, suppresses insulin secretion by both central actions and direct actions on β cells[20]. In contrast with one previous *in vitro* study[5], we found no connection between leptin and AKT pathway in PDAC patients with or without diabetes, questioning the involvement of these pathways in PDAC patients. In the same way, previous studies[18,21,22] with mTOR inhibitors failed to prove any benefit for targeted therapy.

The strengths of this study are represented by its prospective design, the 2-year follow-up duration, and the assessment of these patients before any chemotherapy or surgery. However, our results may be limited by the small sample size and the observational nature of the study. This implies that confounding cannot be overruled, despite measures to adjust for confounders in multiple regressions.

Conclusions

In summary, our results showed that leptin level was not associated with the presence of PDAC, with or without diabetes, although it was lower in obese and overweighted patients who had lower survival. No association with inflammatory biomarkers or PI3K was noted. Furthermore, leptin had no independent role in survival, suggesting that the prognostic role of obesity in PDAC may be based on a different pathway. A better understanding of the underlying role of leptin and its receptor (LepR) on PDAC growth is crucial, particularly as research efforts aimed at building new therapeutic strategies based on targeting LepR/AKT pathway are being developed.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the hospital (No. 11387) and the reporting followed the STROBE criteria. Written informed consent was obtained from all subjects involved in the study.

Authors' Contribution

R.S Conceptualization, Methodology, Data curation, Investigation, Validation, and Writing the original draft, Writing - Review and Editing. T.M Conceptualization, Data curation, Investigation, Validation, Writing - Review and Editing L.L Visualization, Review and editing D.L Formal analysis M.I Formal analysis and Writing - Review and Editing C.I Validation, Review and Editing L.P Investigation A.S Conceptualization, Methodology Data curation, Validation, Supervision, Review and Editing.

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