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Prognostic role of IRS-4 in the survival of patients with pancreatic cancer

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Summary. Pancreatic cancer is a malignancy of rising incidence, especially in developed countries due to causes such as sedentary lifestyles, tobacco smoking and ultraprocessed high fat and high sugar diets, amongst others. It is in fact the 7th cause of cancer-related deaths worldwide, and, in the following years, it is expected to climb upwards to 2nd position, after lung cancer. This is because it may have an asymptomatic course, and when it becomes evident it is in advanced stages, accompanied by metastasis generally. For this reason, survival rates are so low and, even in the few successful cases there is a high possibility of recurrence. Identifying new molecular biomarkers is arising as a highly useful tool for pancreatic cancer clinical management, although much research and work remain to be done in this field. Thus, the present study aims to analyze a series of molecules (IRS-4, Rb1, Ki-67 y COX-2) as candidates for prognosis and survival by immunohistochemistry techniques. Additionally, a 60-month longitudinal surveillance program was conducted, associated with diverse clinical parameters. Kaplan-Meier curves estimating the time of survival according to tumoral expression of those molecules denoted a low cumulative survival rate. Importantly, we observed that high levels of IRS-4 were significantly associated with a bad prognosis of the disease, increasing 160 times the mortality risk. In this way, our research showed a relevant value of these biomarkers in pancreatic cancer patients' survival, opening a pathway for future research areas designed to inhibit these components.

Key words: Pancreatic cancer, IRS-4, Prognostic biomarkers, 5-year survival rate

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Introduction

Pancreatic cancer is a neoplasia with a high impact on our society. According to the 2020 updated statistics from the International Agency for research on cancer GLOBOCAN, pancreatic cancer entailed 495,773 new cases worldwide, reaching 12th position in incidence list of cancers. In contrast, the mortality rate has ranked 7th position, after esophageal cancer, counting 466,003 deaths last year (Sung et al., 2021). Over the last 10 years, the incidence has increased, even more in developed countries, the main cause being the rising

prevalence of chronic diseases such as obesity and diabetes, or other risk factors such as tobacco smoking, alcoholism, sedentarism and ultraprocessed high sugar and high fat diets consumption (Luo et al., 2020). For these reasons, the number of new cases worldwide is expected to be 801,634 by 2040, being 81.5% of incipient cases in Asia and 27.4% in Europe (GLOBOCAN, 2021). From the mid-1980s, the tendency in Spain has also undergone an increase in mortality of 1.1% in men and 1.4% in women every year (Seoane-Mato et al., 2018). It is of note that, with the current panorama, pancreatic cancer might surpass breast, prostate and colorectal cancers, ranking the 2nd leading cause of cancer-related deaths by 2030, just after lung cancer (Rahib et al., 2014). The elevated mortality is due in great part to a late diagnostic since patients do not usually manifest symptoms until advanced stages of the disease (Rawla et al., 2019). Given that the pancreas is an organ localized deep under the abdomen, behind the stomach, pancreatic tumors are difficult to detect in time and are only visible using imaging techniques. It may pass asymptomatically, but some symptoms that usually become evident are jaundice, abdominal pain, back pain, loss of appetite, pancreatitis or unintended weight loss (Moore and Donahue, 2019). Most types of pancreatic cancer are exocrine ductal adenocarcinomas (90%) which originate in the head of the pancreas and invade the rest of the organ from there, with the possible occurrence of obstruction of several structures, like biliary vesicles, in the peritoneal cavity, implying the mentioned clinical manifestations (Haeberle and Esposito, 2019). Nowadays there are no screenings for early detection, so primary prevention is considered of utmost importance. Management of this type of cancer is yet being studied with the aim of optimizing diagnosis techniques and identifying high-risk patients that could benefit from screening for detecting pre-tumoral conditions such as pancreatic intraepithelial neoplasia, intraductal papillary mucinous neoplasms and mucinous cystic neoplasms; or also for those who present risk factors such as new-onset diabetes, a history of pancreatitis or germline mutations (Kunovsky et al., 2018; McGuigan et al., 2018; Singhi et al., 2019). Thus, it is necessary to conduct different kinds of research related to this cancer to allow a more effective clinical management.

The vast majority of patients, within 80-85%, are diagnosed with a quite advanced local tumor or metastasis. 5-year survival rate is approximately less than 5%, although in some cases, radical surgery may significantly improve that rate (Bengtsson et al., 2020). Currently, surgery is the only potentially curative treatment. However, only 15-20% of patients are candidates to undergo this procedure (Fogel et al., 2017). The rest of the patients should undergo other alternatives such as chemotherapy, radiotherapy or immunotherapy, mainly with palliative aim (Roth et al., 2020). The boom of molecular techniques and major knowledge of tumoral biology is opening relevant pathways in pancreatic cancer translational research. In fact, the use of molecular biomarkers in these patients may suppose the development of specific therapies targeting these components, allowing at the same time more precise prognosis or even the prediction of therapeutic success of available treatments (Brunner et al., 2019). Then, searching for new markers of clinical relevance seems necessary to favor a more adequate clinical management of these patients.

Insulin receptor substrate 4 (IRS4) is a member of the IRS family, adaptor proteins which are key in cellular signaling mediated by insulin receptor and insulin-like growth factor (IR/IGF-1R), with important effects at physiological and metabolic level (Shaw, 2011). Previous research has shown the importance of IRS either in pancreatic cells (Hügl and Merger, 2007) or in the carcinogenesis process origin (Gorgisen et al., 2017), which is responsible for the activation of different signaling pathways, regulating the cellular destiny and other processes (Machado-Neto et al., 2018). In this line, an association between certain polymorphisms of IRS and a higher risk of developing pancreatic cancer has been found (Dong et al., 2012). In the same manner, Huang et al. (2018) described the role of IRS1 in proliferation, invasivity and metastasis in pancreatic tumor cells, showing the relevance of these proteins in the onset of the disease. On the other hand, the role of IRS4 in pancreatic cancer has not been completely elucidated. Nevertheless, other studies have denoted the implication of IRS4 in diverse kinds of cancer (Qiu et al., 2005; Karrman et al., 2011; Hao et al., 2021), exhibiting the possible value of inspecting this biomarker in pancreatic cancer.

Ki-67 is a biomarker widely utilized as an indicator of cellular proliferation, although its functions are extensive, acting an changingits localization during different phases of the cell cycle (Sun and Kaufman, 2018). Alterations in the expression of this component are frequent, showing important prognostic, predictive and diagnostic value in cancer (Li et al., 2015). In the same manner, retinoblastoma protein 1 (Rb1) is a tumor suppressor gene, frequently mutated in multiple neoplasias, presenting numerous translational applications (Indovina et al., 2019). Finally, cyclooxygenase 2 (COX-2) has also emerged as a potential biomarker for some types of cancer, promoting the alteration of diverse cellular mechanisms (Tudor et al., 2020).

In this manner, the aim of the present study resides in the analysis of the expression of a group of markers and to assess the prognostic impact (IRS4, Ki-67, Rb1 and COX-2) in pancreatic cancer patients. Establishing this objective, samples from patients with pancreatic tumor have been collected and immunohistochemical and histopathological studies have been performed in these tissues. Finally, several clinical parameters were gathered to monitor these patients for 5 years, to observe the impact of these studies on these individuals.

Materials and methods

Samples

In this study we used paraffin-embedded sections of pancreatic tissue obtained from 41 patients with ductal adenocarcinoma who underwent surgery (curative resection of pancreatoduodenectomy), with a 60-month follow-up of the patients. Clinical diagnosis was conducted following the principles of Esposito et al. (2014). The present study was designed as an observational, analytical, retrospective cohort study with longitudinal follow-up. Paraffin blocks and all the details including extensive clinical information about the patients and the follow-up data were retrospectively reviewed.

This study was carried out in accordance with the basic ethical principles of autonomy, beneficence, non-maleficence and distributive justice, following the statements of Good Clinical Practice, the principles contained in the most recent Declaration of Helsinki (2013) and the Oviedo Convention (1997). The data and information collected complied with current legislation on data protection (Organic Law 3/2018 of December 5, Protection of Personal Data and Guarantee of Digital Rights and Regulation (EU) 2016/679).

Histopathological and immunohistochemical studies

Immunohistochemical studies were conducted in paraffin-embedded samples of pancreatic tissue. The step of antibody recovery is described in Table 1. Antigen/Antibody reactions were detected by the avidin-biotin complex (ABC) with avidin-peroxidase, according to the established protocols of Ortega et al. (2021). After incubation with the primary antibody (1 h 30 min), samples were incubated with 3% BSA blocker (Catalog #37525; Thermo Fisher Scientific, Inc.) and PBS overnight at 4°C. Then, the samples were incubated with biotin-conjugated secondary antibody, diluted in PBS for 90 min at room temperature (RT; Rabbit IgG (RG-96, 1: 1000, Sigma-Aldrich/Mouse IgG (F2012/045K6072) 1:300, Sigma-Aldrich). The avidin-peroxidase conjugate ExtrAvidin®-Peroxidase (Sigma-Aldrich; Merck KGaA) was used for 60 min at RT (1: 200 dilution with PBS). Later, protein expression was determined using a Chromogenic Diaminobenzidine (DAB) Substrate Kit (cat. no. SK-4100; Maravai LifeSciences), prepared immediately prior to exposure (5 ml of distilled water, two drops of buffer, four drops of DAB, and two drops of hydrogen peroxide). The signal was produced with the chromogenic peroxidase substrate for 15 min at RT. This technique allows the detection of a brown stain. For the detection of each protein, sections of the same tissue were assigned as negative controls, substituting incubation with the primary antibody for a blocking solution (PBS). In all tissues, the contrast was performed with Carazzi hematoxylin for 15 min at RT.

Table 1. Primary antibodies used, together with the dilutions and protocol specifications.

Antigen	Dilution	Provider	Protocol specifications
IRS-4	1:500	Thermo Fisher Scientific – PA5-117329	Preincubation with Tris-EDTA Buffer pH9 and incubation with 0.1% TTX (Triton x100 in TBS) for 5 min
Ki-67	1:1000	Vitro, MAD-000310QD-3/V	---
Rb1	1:750	Vitro, MAD-000900QD-3/V	---
COX-2	1:1000	Vitro, MAD-000335QD-3/V	---

Histopathological assessment

Tissue sections were observed using a Zeiss Axiophot light microscope (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam HRc digital camera (Carl Zeiss, Oberkochen, Germany). Given the important role of the proteins studied, the evaluation of the histological results was conducted according to the intensity of expression for the immunohistochemical staining with Score. Therefore, histological samples from patients diagnosed with pancreatic cancer were classified as negative (0) or low/medium (1/2) and high (3) expression using the IRS-Score method (Sanmartín-Salinas et al., 2018a,b). For each established group of subjects, seven randomly selected microscopy fields were examined in the five sections of tissue. Subjects were classified as positive when the mean proportion of the labeled sample was greater than or equal to 5% of the total sample. This was done by calculating the total percentage of marked tissue in each microscopy field to obtain an average of the study sample as described (Ortega et al., 2019). The observation and quantification of the samples was achieved independently by two researchers.

Statistical analysis

Normality testing of markers was carried out (Kolmogorov-Smirnoff, all $p<0.001$). Thus, as they did not follow a normal distribution, it was necessary to describe the results with medians and interquartile ranges and perform non-parametric tests. Mann–Whitney U test was done. To evaluate the association between clinicopathological and immunohistochemical parameters and variables, a logarithmic rank test and Kaplan-Meier curves were performed for survival comparisons. To explore the correlation of the immunohistochemical parameters studied and the established prognosis of the variables, a univariate analysis and Cox proportional hazards regression analysis were executed. All statistical analyses were done using SPSS 22.0 software (SPSS Inc. Chicago, IL, USA). Values of $p<0.05$ were considered significant.

Results

Clinical and sociodemographic characteristics of the studied population.

The present study was designed as an observational, analytical, retrospective cohort study with longitudinal follow-up. A total of 41 patients were analyzed, with a median age of 72.00 [45.00-88.00] years. 65.85% were men (n=27) and 34.15% were women (n=14). Clinical and sociodemographic characteristics are collected in Table 2. Tumor Stage are 28 patients for <IV and 13 patients for IV. Patients showed a median expression for Ca 19.9 of 102.10 [44.91-805.00] U/ml. In the case of CEA it was 5.43 [2.71-11.31] ng/ml, and for AFP it was 2.32 [1.46-4.39] ng/ml (Table 3).

Overall, the survival of patients diagnosed with pancreatic cancer was 8.00 [2.98-13.02] months.

Patients with greater expresión of IRS-4, Ki-67, Rb1 y COX-2 report reduced survival to pancreatic cancer

Immunohistochemical studies showed that only 14.64% of patients with pancreatic cancer did not show tissue expression of the tumor for IRS-4, whereas the percentage of patients with low/moderate expression was 36.58%. In contrast, 48.78% showed high levels of IRS-4. In total, 85.36% of the patients showed IRS-4 expression (Table 4, Fig. 1A,B).

Median survival for patients with pancreatic cancer and negative tissue expression of IRS-4 was 26.00 [22.61-29.39] months. However, in the case of patients with low /moderate expression it was 15.00 [14.11-5.89] months, falling to 4.00 [2.90-5.09] months in the case of patients with high expression (Fig. 2A). The global comparisons showed how the significance value was p<0.001

Regarding Ki-67, immunohistochemical studies showed that 19.51% of patients with pancreatic cancer did not show tissue expression of this component, while 34.15% of patients showed low/moderate expression of Ki-67. Conversely, 46.34% of patients presented high levels of Ki-67. In total, 80.49% of the patients exhibited significant Ki-67 expression (Table 4, Fig. 1C,D).

Median survival for patients with pancreatic cancer and negative tissue expression of Ki-67 was 22.00 [14.61-29.40] months. However, in the case of patients with low-medium expression it was 13.00 [10.25-15.75] months, decreasing to 4.00 [2.93-5.01] months in the case of patients with high expression (Fig. 2B). The global comparisons displayed how the significance value was p <0.001.

Then, immunohistochemical studies showed that 7.32% of patients with pancreatic cancer did not show tissue expression for Rb1, while 41.46% of patients showed low/moderate expression of this component. In contrast, 51.22% of individuals evidenced high levels of Rb1. In total, 92.68% of the patients showed Rb1 expression (Table 5, Fig. 3A,B).

Median survival for patients with pancreatic cancer and negative Rb1 expression was 26.00 [19.59-32.40] months. However, in the case of patients with low-moderate expression it was 15.00 [13.01-16.98] months, falling to 5.00 [3.91-6.09] months in those with high expression (Fig. 4A). The global comparisons showed how the significance value was p<0.001

Finally, regarding COX-2, immunohistochemical studies showed that 2.44% of patients with pancreatic cancer did not show tissue expression of the tumor for COX-2, while 36.58% displayed low/moderate expression of this component. In contrast, 60.98% showed high levels of COX-2. In total, 97.56% of the patients showed COX-2 expression (Table 5, Fig. 3C,D).

Table 2. Description of the clinical and sociodemographic characteristics of the patients diagnosed with pancreatic cancer included in the study.

Age (Median [IQR])	72.00 [45.00-88.00]
Sex (Ratio% (n))	
Men	65.85 (27)
Women	34.15 (14)
Smoking habits	43.90 (18)
Drinking habits	26.83 (11)
Obesity	4.88 (2)
Type 2 diabetes	55.56 (15)
Chronic pathologies	9.76 (4)
Prior malignancies	26.83 (11)

IQR: Interquartile range, n: number of patients.

Table 3. Plasma levels of the main carcinogenic markers collected routinely. Data are expressed as a median and interquartile range.

Ca 19.9 U/ml (0-37)	102.10 [44.91-805.00]
CEA ng/ml (0-5)	5.43 [2.71-11.31]
AFP ng/ml (0-13.4)	2.32 [1.46-4.39]

Table 4. Percentage of positive expression for IRS-4 and Ki-67 in pancreatic cancer, classified according to tissue expression levels.

Expression	IRS-4 Ratio% (n)	Ki-67 Ratio% (n)
Negative	14.64 (6)	19.51 (8)
Low/Moderate	36.58 (15)	34.15 (14)
High	48.78 (20)	46.34 (19)

Table 5. Percentage of positive expression for Rb1 and COX-2 in pancreatic cancer, classified according to tissue expression levels.

Expression	Rb1 Ratio% (n)	COX-2 Ratio% (n)
Negative	7.32 (3)	2.44 (1)
Low/moderate	41.46 (17)	36.58 (15)
High	51.22 (21)	60.98 (25)

The median survival for patients with pancreatic cancer and negative tissue expression of COX-2 was 60 months. However, in the case of patients with low-medium expression it was 15.00 [4.90-25.09] months, going down to 5.00 [2.55-7.45] months in the case of patients with high expression (Fig. 4B). The global comparisons showed how the significance value was p <0.001.

IRS-4 is the central marker associated with pancreatic cancer mortality

After doing a backward stepwise multivariate Cox gression, in which all the clinical and sociodemographic variables described in section 3.1 were introduced, with an entry probability of 0.05 and an exit probability of 0.10, the only variable that remained in the model is the tissue expression by immunohistochemistry of IRS-4, with a p<0.001.

Patients with low level of IRS-4 expression have an OR of 3.32 [1.06-10.40], while in patients with high levels of expression OR is 163.83 [16.22-1614.69].

Discussion

Pancreatic cancer is a neoplasm of such a great prevalence and mortality in Western countries, mainly due to its diagnostic difficulties, the reduced efficacy of the treatments available, as well as the lack of an accurate stratification of each case of pancreatic cancer. In this sense, the identification of novel biomarkers is providing important support in the clinical management of patients affected by this cancer, especially as diagnostic, prognostic and predictive tools (Giannis et al., 2021). Our research evidences the use of IRS-4/Ki-67/Rb1/COX-2 as relevant markers in the study of pancreatic cancer, showing a direct association between the expression levels of these components and the

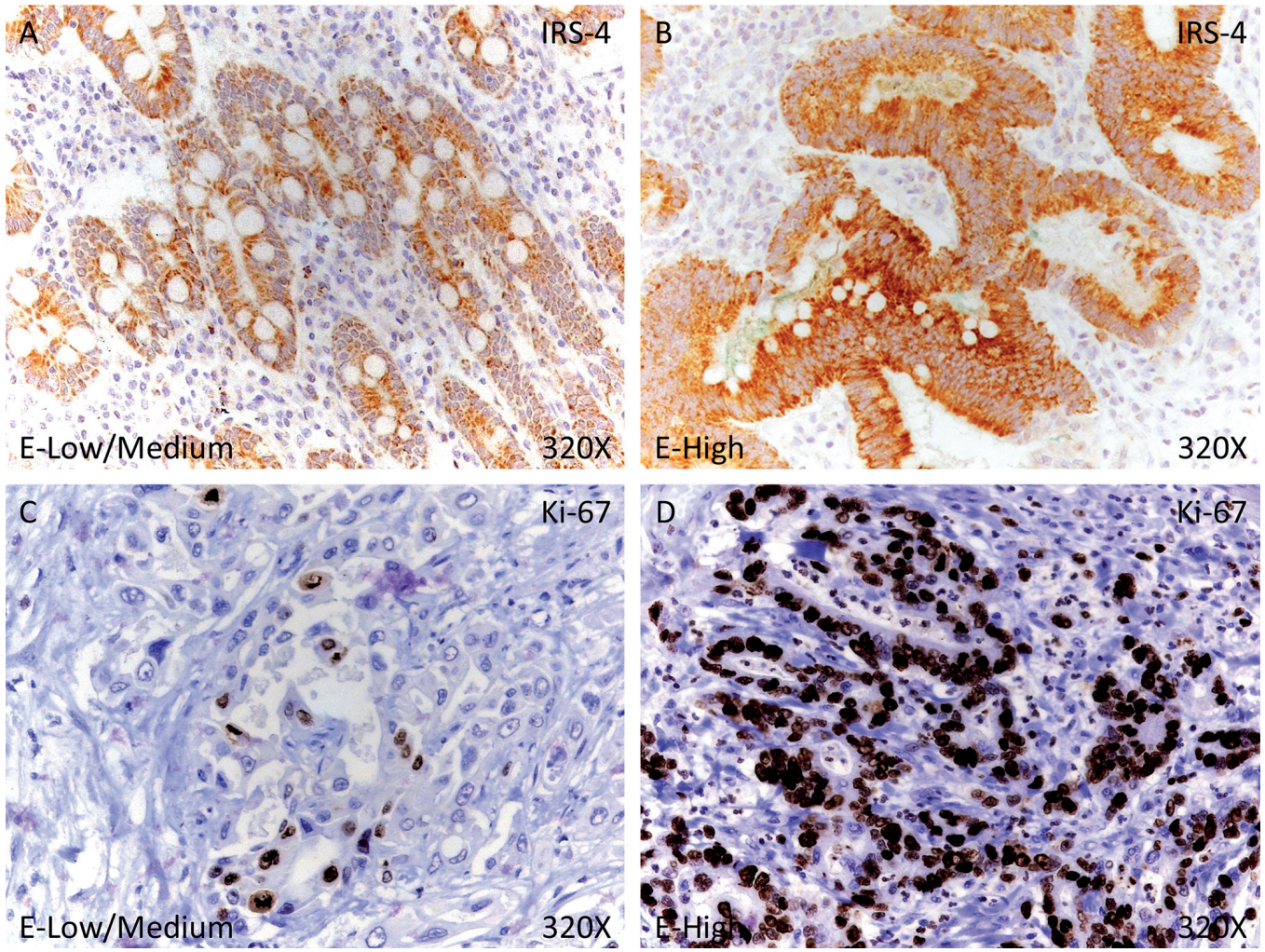

Fig. 1. Images showing the protein expression of IRS-4 **(A, B)** and Ki-67 **(C, D)** in patients diagnosed with pancreatic cancer.

survival rate of these patients.

IRS-4 is a central marker in our study. In fact, according to our multivariate analysis, a high expression of this molecule increases the risk of mortality more than 160 times, compared to those that show a low/moderate or no expression. Consistent with our results, Sanmartín-Salinas et al. (2018a,b) reported an augmented expression of IRS-4 in patients with advanced stages of colorectal cancer, thus denoting the usefulness of this marker in the progression and stratification of this type of cancer. IRS-4 is an adaptor protein involved in the activation of various cell signalling pathways (Dörpholz et al., 2017). It is of note that IRS-4 is responsible for PI3K/Akt hyperactivation, whose clinical significance in cancer has been widely described (Ortega et al., 2020). This route is involved in a plethora of biological processes in pancreatic cancer, leading to metabolic changes, cell cycle progression and survival, preventing apoptosis, and promoting protein synthesis, and genomic instability. Hence, PI3K/Akt inhibition has been proposed as a potential therapy of this type of cancer (Ebrahimi et al., 2017). Moreover, previous studies have found that the constitutive activation of IRS-4 and PI3K/Akt are related to an increased tumour resistance to the therapies received, collaborating with different oncogenes and molecular pathways (Ikink et al., 2016; Ikink and Hilkens, 2017; Hao et al., 2021). This could explain, in part, the association of IRS-4 with the mortality reported in our study, as it might be implicated in the progression of cancer while interfering with the therapeutic success of the treatments. In this line, IRS4 inhibition could be an interesting approach in patients with pancreatic cancer by limiting PI3K/Akt activation. Some studies have reported somatic mutations in the IRS-4 gene, which may be responsible for the increased expression of this component and PI3K/Akt (Karrman et al., 2011; Shull et al., 2012). On the other hand, epigenetic remodelling of the chromatin, non-coding

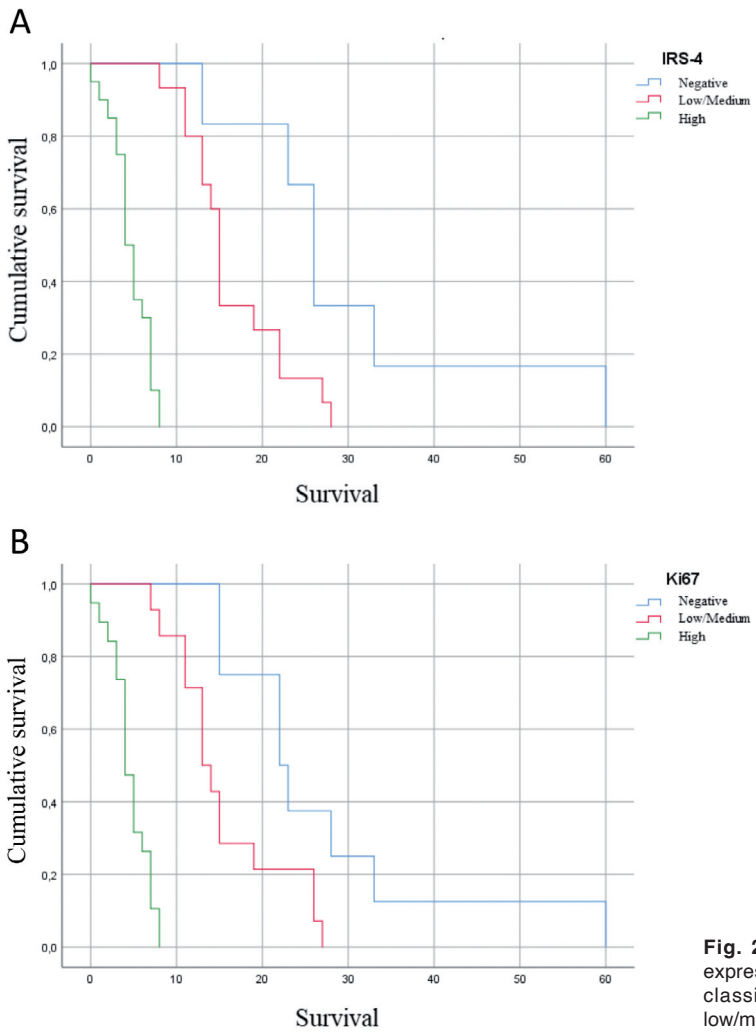

Fig. 2. Kaplan-Meier curves for survival time according to tumor expression of IRS-4 **(A)** and Ki-67 **(B)**. Blue curve: tissue expression classified as negative, red curve: tissue expression classified as low/medium, green curve: tissue expression classified as high.

RNAs and even posttranscriptional mechanisms, could be responsible for increased IRS-4 expression (Li et al., 2021; Weischenfeldt et al., 2017; Li et al., 2018). In the case of pancreatic cancer however, the concrete mechanisms of IRS-4 dysregulation remain undescribed, and future studies could be destinated to elucidate possible causes of IRS-4 up-regulation.

Recently, Guijarro et al. (2021) studied the importance of IRS-4 in the development of hepatocellular carcinoma. They observed that, increased levels of this component correspond with other proliferation and cell cycle markers like Ki-67 and PCNA. Similarly, we show increased levels of Ki-67 in patients with high IRS-4 levels, also reporting reduced survival. In the same line, prior evidence emphasized the value of Ki-67 as an indicator of poor prognosis in patients with pancreatic cancer, directly associated with the pathological and clinical grade of the tumour (Hu et al., 2012), along with an increased mortality while predicting risk of disease recurrence (Hamilton et al., 2012). Likewise, we observed a high Rb1 expression in patients with reduced survival. The dysfunction of this protein appears to be implicated in the promotion of the tumour growing factor β1 (TGF-β1), stimulating cellular proliferation, invasiveness and survival (Jesse Gore et al., 2014). Previously, it was shown that IRS-4 induced Rb1 activation in colorectal tumours (Sanmartín-Salinas et al., 2018a,b). It is possible that IRS-4 could be responsible for the Rb1 activation in pancreatic tumours, therefore explaining its association with poor prognosis in these patients. In this context, IRS-4 inhibition could be of use in Rb1 targeting in pancreatic cancer, where some difficulties around its inhibition have been reported (Huang and Zhang, 2020). A novel way of research towards particular subtypes of PDAC (namely anaplastic and/or undifferentiated giant cell rich

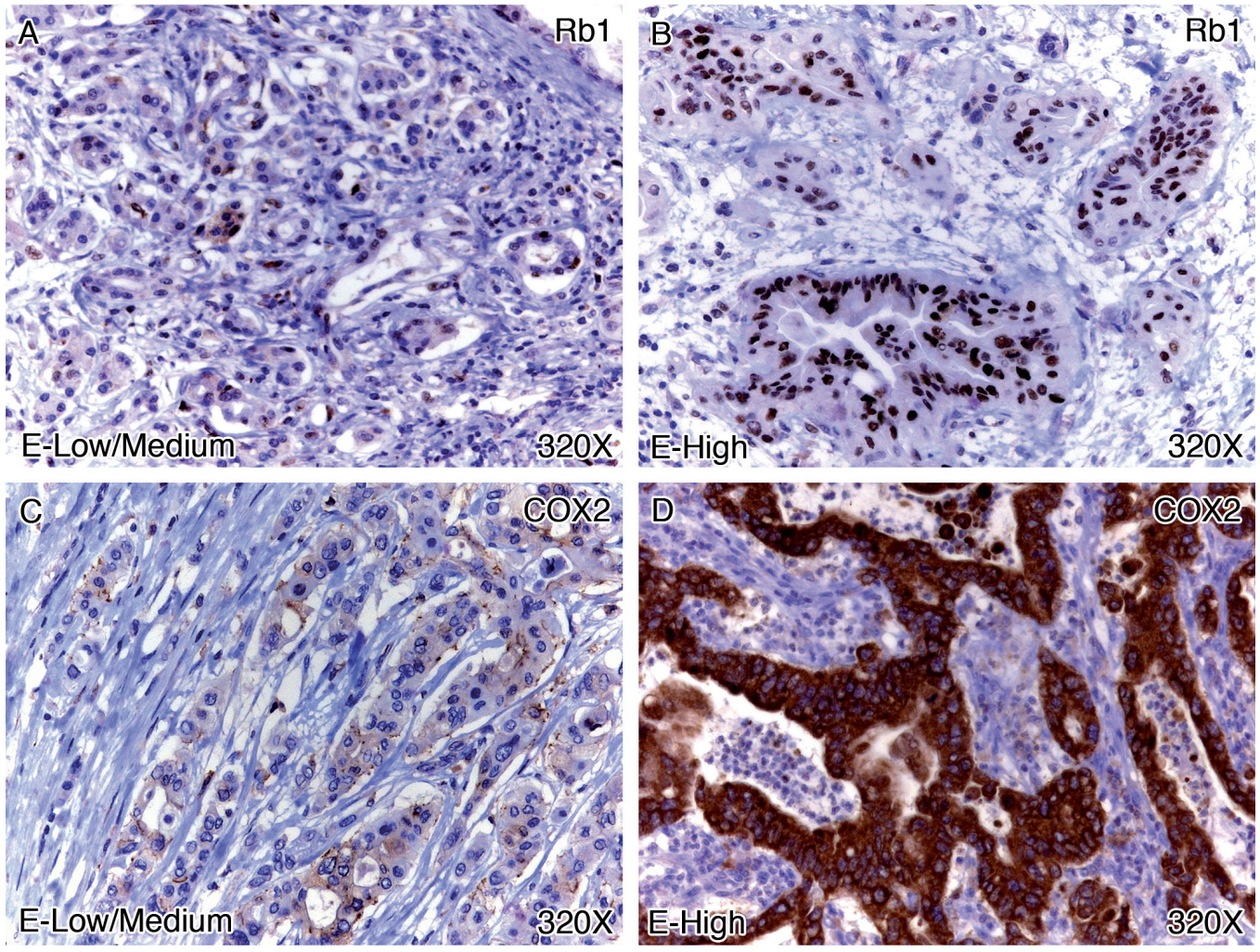

Fig. 3. Images showing the protein expression of Rb1 **(A, B)** and COX-2 **(C, D)** in patients diagnosed with pancreatic cancer.

carcinoma) which frequently express PD-L1 is studying for the treatment of patients may benefit from immune checkpoint treatment (nowadays, only in single cases) (Luchini et al., 2018; Hrudka et al., 2020; Obayashi et al., 2020).

Finally, we appreciated high levels of COX-2 in patients with the lowest survival. COX-2 is another central modulator of tumour biology. This molecule is broadly released by tumoral cells, fibroblasts and macrophages into the tumour microenvironment, essentially producing prostaglandin E1 (PGE1), exerting diverse functions (Hashemi Goradel et al., 2019). COX-2 is frequently augmented in pancreatic cancer, also responsible for PI3K/Akt hyperactivation (Hill et al., 2012). Thus, high COX-2 levels are generally a marker of poor prognosis in pancreatic cancer. Park et al. (2012) Moreover, patients with breast cancer with increased COX-2 expression were associated with a reduced survival and different adverse factors when the tumoral cells were under a proliferative state (Expressing Ki-67). Similar results were obtained in various types of gastrointestinal cancer (Wang et al., 2014), including colorectal (Sato et al., 2003), liver (Tai et al., 2019) and lung cancer cancer (Tsubochi et al., 2006), hence supporting the prognostic value of this marker in our study. Interestingly, IRS-4 might beimplicated COX-2 hyperactivation. IRS-4 may induce the function of procaspase 3, which in turn might promote phospholipase A2 activity, leading to the production of arachidonic acid, a COX-2 substrate, eventually resulting in the synthesis of PGE2 (Sanmartín-Salinas and Guijarro, 2018).

Overall, the multiple effects of IRS-4 in the different cell events, together with its noteworthy association with

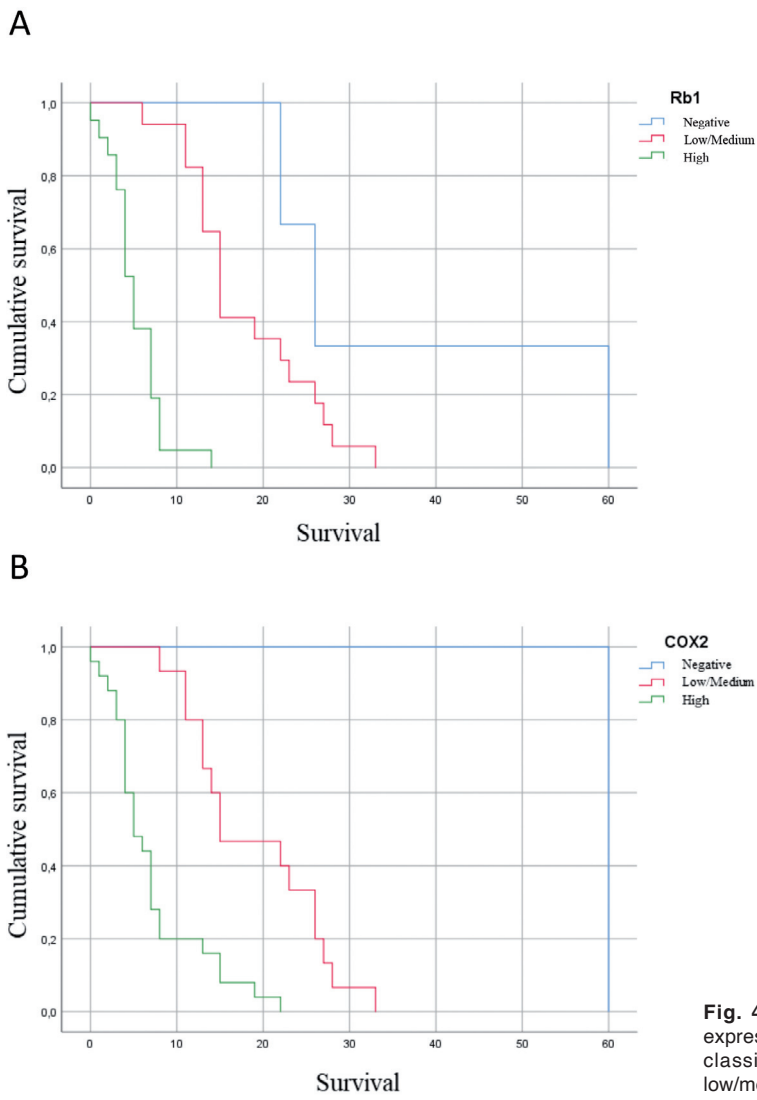

Fig. 4. Kaplan-Meier curves for survival time according to tumor expression of Rb1 **(A)** and COX-2 **(B)**. Blue curve: tissue expression classified as negative, red curve: tissue expression classified as low/medium, green curve: tissue expression classified as high.

increased mortality in patients with IRS-4 overexpression suggest the possible and important role that IRS-4 may have in pancreatic cancer, and further research should be intended to expand the basic and translational knowledge of this marker. One of the important limitations of this type of study is the sample size; subsequent studies should try to achieve larger sample sizes for a greater effective translation into clinical practice.

Conclusions

Our study demonstrates the importance of IRS-4, Ki-67, Rb1 and COX-2 in pancreatic cancer, especially as worse prognostic markers in patients that overexpress these components. Among those, IRS-4 denotes more value, so significantly augmenting mortality (even more than 160 times) in patients with pancreatic cancer.

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Conflicts of Interest. The authors declare no conflict of interest.

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