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Abstract: Pancreatic cancer is a malignant neoplasm that, despite its low frequency, has a 5-year survival rate of less than 10%. The study of different histopathological markers has allowed a better understanding of the onset and development of this type of tumor as well as facilitating an approach to clinical variables based on their diagnostic, prognostic, and predictive value. In this sense, the NLRP3 protein of the inflammasome has been shown to be a component of great relevance in the initiation and progression of pancreatic cancer, although the value of this biomarker in patients has not yet been clarified. In this study, we selected 41 patients with pancreatic cancer and followed them for 60 months (5 years), evaluating their NLRP3 expression using immunohistochemical techniques. Furthermore, by performing Kaplan–Meier curves, we evaluated the survival of these patients in relation to their NLRP3 expression. Our results show that a significant percentage of our cohort had high expression of this component (90.74%) and that there is an inverse relationship between the expression of NLRP3 and patient survival. High levels of NLRP3 expression are related to lower survival and worse prognosis in these patients, possibly due to an ineffective immune system response and increased tumor-promoted inflammation. Future studies should be aimed at confirming these results in larger groups and evaluating various clinical strategies based on this knowledge.

Keywords: pancreatic cancer; inflammasome; NLRP3; prognostic biomarker

Introduction

Pancreatic cancer is a malignant neoplasm with high mortality, with a 5-year survival rate of less than 10% (Mizrahi et al., 2020). Although it is a relatively rare cancer, its incidence is increasing from 0.5 to 1% every year, and it is projected that by 2030, it will become the second deadliest type of tumor after lung cancer (Park et al., 2021). Among the most important risk factors for pancreatic cancer are non-modifiable (inherited) and modifiable factors such as alcohol consumption, obesity, diabetes mellitus, pancreatitis, and, most importantly, smoking (Capasso et al., 2018). Pancreatic adenocarcinoma and its subtypes represent more than 90% of pancreatic tumors, involving a set of clinical difficulties
in early diagnosis, prognosis, and treatment and posing an authentic challenge for modern medicine (McGuigan et al., 2018; Gupta and Yelamanchi, 2021). The histopathological study of different molecular markers has allowed greater approximation for clinical translation to pancreatic cancer. In this sense, a wide variety of tissue biomarkers with diagnostic, prognostic, and predictive utility have recently been described (Giannis et al., 2021; Pekarek et al., 2021; Ortega et al., 2022a, 2022b). Furthermore, the identification of some molecular components has opened up new therapeutic possibilities for these patients, although there is still a long way to go in this line (Grapa et al., 2021; Sturm et al., 2022).

Inflammation is one of the so-called hallmarks of cancer and is involved in promoting tumor growth and spread, among other mechanisms (Hanahan and Weinberg, 2011). Inflammation mediated by tumor cells implies very close interplay with the immune system: they must activate inflammatory mechanisms that favor tumor progression and, simultaneously, modulate and prevent the immune response (Amedei et al., 2014). The Inflammasome is a fundamental mechanism composed of oligomeric protein complexes that are activated in response to various patterns of damage, concluding with the release of interleukin 1β (IL-1β) and IL-18 (He et al., 2018). The best-known inflammasome is nucleotide-binding domain leucine-rich pyrin-containing 3 (NLRP3), which is made up of this protein. Previous studies have shown the usefulness of this component as a prognostic biomarker in various types of cancer (Ju et al., 2021; Saponaro et al., 2021). However, the value of NLRP3 as a prognostic biomarker in pancreatic cancer has not yet been fully elucidated.

For all of these reasons, the aim of the present study was to analyze the histopathological expression of NLRP3 in patients with pancreatic cancer by performing immunohistochemistry (IHC) techniques. At the same time, we also tried to determine whether there is any relationship between the level of NLRP3 expression and patient survival in order to examine the possible value of this component as a prognostic biomarker.
Patients and methods

Study design

The present study was designed as an observational, analytical, retrospective cohort study with longitudinal follow-up. This study was performed according to the basic ethical principles of autonomy, beneficence, non-maleficence, and distributive justice, and its development was adapted to the standards of Good Clinical Practice and the principles given in the most recent Declaration of Helsinki (2013) and the Oviedo Convention (1997). Results 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).

Sample collection

Tissue samples and paraffin blocks were obtained from 41 patients with a clinical diagnosis of ductal adenocarcinoma after curative resection of pancreaticoduodenectomy. Then, these patients were then followed for 60 months. Paraffin blocks and certain details were retrospectively reviewed with extensive patient clinical information and follow-up data. The clinical diagnosis of the patients followed the principles reported by Esposito et al. (2014).

Histopathological and immunohistochemical studies

Immunohistochemistry was performed on paraffin-embedded pancreatic tissue blocks. Primary antibody details and protocol specifications are described in Table 1. We used the avidin–biotin complex (ABC) method with avidin–peroxidase to perform the antigen/antibody reactions according to previous specifications (Ortega et al., 2021a). Primary antibody was incubated during an hour and a half and then samples were incubated with 3% BSA blocker (catalog #37525; Thermo Fisher Scientific) and PBS overnight at 4 °C. Next day, samples were incubated with a biotin-conjugated secondary antibody diluted in PBS for 1 hour 30 min at room temperature (rabbit IgG, diluted 1/300 (RG-96; Sigma-Aldrich); goat IgG, diluted 1/300); 100 (GT-34/B3148; Sigma-Aldrich); and mouse IgG,
diluted 1/300 (F2012/045K6072; Sigma-Aldrich). ExtrAvidin®–Peroxidase (Sigma-Aldrich; Merck KGaA) was used for 60 min at room temperature (diluted 1:200 with PBS). Subsequently, the amount of protein expression was studied using chromogenic diaminobenzidine (DAB) substrate, an avidin–peroxidase conjugate (cat. no. SK-4100; Maravai LifeSciences), prepared just before exposure (composition: 5 mL distilled water, 2 drops of buffer, 4 drops of DAB, and 2 drops of peroxide hydrogen). The signal was developed with the chromogenic peroxidase substrate for 15 min at room temperature; allowing the detection with a brown stain. All tissues were contrasted with Carazzi's hematoxylin for 15 min at room temperature. Negative controls were assigned in the samples studied, with the primary antibody substituted with a blocking solution (PBS) during incubation.

Histopathological assessment

We visualized tissue samples by using a Zeiss AxioPhot light microscope (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam HRc digital camera (Carl Zeiss, Oberkochen, Germany) The observation and quantification of the tissue samples were performed independently by two researchers. Histological evaluation was conducted according to the intensity of expression for immunohistochemical staining with the IRS score method, as detailed in previous works (Cristóbal et al., 2018; Ortega et al., 2021b). Following this evaluation, histological samples were classified according to protein expression as negative (0), low/medium (1/2), or high (3). For each sample, 7 randomly selected microscopic fields were studied. Individual samples were classified as positive when the mean proportion of the marked sample was greater than or equal to 5% of the total sample. This was done by calculating the total percentage of labeled tissue in each microscopy field to obtain an average of the study sample, as described in a prior research (Ortega et al., 2022c).

Statistical analysis

First, Kolmogorov–Smirnoff test was performed as a normality check of the studied marker (all p < 0.001). As we did not observe that this marker has a normal distribution, a nonparametric test (Mann–Whitney U) was used to describe the results with medians and
interquartile ranges. To study the relationship between immunohistochemical and clinicopathological parameters, a log-rank test was performed, while Kaplan–Meier curves were made for survival comparisons. Univariate analysis and Cox proportional hazards regression analysis were performed in order to explore the correlation between the established prognosis and the studied immunohistochemical parameter. All statistical analyses were performed with Stata 16.1 software (StataCorp, College Station, TX, USA), considering p values < 0.05 as significants.

**Results**

*Clinical and sociodemographic characteristics of the study population*

In our study, we included 41 patients (14 women and 27 men) with a median age of 72 (45–88) years. Of them, 28 patients had tumors between stages I and III, while 13 patients had stage IV tumors. Median expression of Ca19.9 was 102.10 (44.91–805.00) U/mL, CEA was 5.43 (2.71–11.31) ng/mL, and AFP was 2.32 (1.46–4.39) ng/mL. Collectively, survival for these patients was 8.00 (2.98–13.02) months.

*A high percentage of pancreatic cancer patients express NLRP3 inflammasome marker*

In our study we were able to detect that 90.24% of patients with pancreatic cancer expressed the NLRP3 marker, while the remaining 9.76% did not. Among those who expressed NLRP3, 12 patients (29.27%) showed expression classified as low/moderate, while 60.97% showed high expression.

*Higher expression of NLRP3 is associated with poor prognosis and shorter survival among pancreatic cancer patients*

We observed that there was a direct correlation between NLRP3 expression and survival time for pancreatic cancer patients. Patients without NLRP3 expression had a median survival of 33 (30–60) months, while those with low/moderate expression had a median survival of 16 (13–22) months, and those with high expression had a median survival of 6
(4–8) months. Likewise, according to our statistical association, patients with high NLRP3 expression had a risk ratio that was 2,726 times higher than those with low/moderate expression. The global comparison of our study shows significance of p < 0.001.

Discussion

The inflammasome is a key element in the initiation and development of various types of neoplasms, including pancreatic cancer (Kolb et al., 2014; Jang et al., 2021; Missiroli et al., 2021). Our results show that a high percentage of our patients express NLRP3, thus demonstrating the impact that this marker has on pancreatic cancer. In addition, we observed that patients who express this component have lower survival rates, thus suggesting it is possible use as a prognostic biomarker in our cohort.

The NLRP3 inflammasome is a key mediator of the immune system response from the release of IL-1β and IL-18. Evidence seems to indicate that this inflammasome is activated by a wide variety of signals, including mitochondrial dysfunction, calcium-mediated signaling, lysosome breakdown, and potassium influx into the cell (He et al., 2016). Similarly, NLRP3 can also be activated by the presence of pathogen-associated molecular patterns (PAMPs), including bacteria and viruses and some of their components (Jo et al., 2016). Tumor cells present a significant loss of multiple homeostatic mechanisms, which leads to both malignancy and activation of the NLRP3 inflammasome, with important effects on tumor biology (Lin et al., 2021). However, other studies have shown that activation of the inflammasome can limit the growth and development of some types of cancer (Sharma and Kanneganti, 2021), demonstrating the complexity of this component in different oncogenic processes. In pancreatic cancer, two polymorphic variants of the NLRP3 inflammasome have been identified, Q705K and F359L, which increase the risk of chronic pancreatitis and pancreatic cancer due to exacerbated activation of the inflammatory response in this organ (Miskiewicz et al., 2015). Similarly, increased inflammasome activation may be indicative of a differential immune response in some pancreatic cancer patients, which in turn leads to decreased survival in these patients. Previous works have demonstrated that NLRP3 inflammasome promotes the expansion of suppressor macrophages, which leads to increased T helper type 2 (Th2), Th17, and regulatory T lymphocytes (Tregs) along with decreased Th1 and cytotoxic T lymphocytes, which favors cancer development and immune system
evasion (Daley et al., 2017). Thus, the combined use of NLRP3 inhibitors and immune system promoters has been proposed as a potential strategy for the treatment of these patients, which can reduce inflammation and improve the immune response against tumors (Liu et al., 2020). On the other hand, in vitro models have shown activation of the NLRP3 inflammasome from lipopolysaccharides (LPSs) of bacterial origin in pancreatic cancer cell lines, promoting further tumor proliferation and survival (Yaw et al., 2020). These LPSs come from the intestinal microbiota in an abnormal state (dysbiosis), which is related to increased intestinal permeability and chronic low-grade inflammation, which may contribute to the initiation and development of pancreatic cancer (Li et al., 2020a). In addition, it has been possible to demonstrate the direct influence of the intestinal microbiota on the activation of the NLRP3 inflammasome in episodes of acute pancreatitis (Li et al., 2020b; Sendler et al., 2020), which can also promote the subsequent development of pancreatic cancer (Kirkegård et al., 2018). In this sense, different strategies are being studied to modulate the composition and action of the intestinal microbiota in patients with pancreatic cancer, also considering the effect of current therapies on the microbial ecosystem (Chen et al., 2022). However, despite the potential utility of targeting the gut microbiota to influence inflammasome activation, further efforts are still needed in this field, along with finding effective therapeutic agents directed at this component.

It is broadly accepted that tumor development and survival is the result of a complex interplay between cancer cells, normal stromal cells and host defense mechanisms (Vinay et al. 2015). In the case of pancreatic cancer, a growing body of evidence supports that the immune system switches from attacking tumor cells to supporting and facilitating tumor growth, in a process mediated by regulatory T cells, myeloid derived suppressor cells, tumor-associated macrophages and fibroblasts, all of which leads to CD8 T and NK cells suppression (Sideras et al. 2014). In parallel, this immune evasion occurs through the expression of tolerance-inducing surface molecules such as the programmed death ligand-1 (PDL-1), different anti-inflammatory cytokines like IL-10 and TGF-β, and potentially by other components of the immunoinflammatory system like NLRP3, as previously shown. Besides, previous works have demonstrated that these and other changes in the immune system are strongly related with cancer prognosis (Bruni et al. 2020). In this sense, the prognostic value of NLRP3 has been proven in a broad spectrum of tumors. For instance, Saponaro et al. (2021) demonstrated that high histopathological expression of NLRP3 in
primary invasive breast carcinomas was directly correlated to worse 5-year disease-free survival than those with lower NLRP3 expression. Similar conclusions were drawn by Lin et al. (2018) and Shi et al. (2021), who observed that high histopathological expression of this marker was correlated with a poor prognosis in colorectal cancer patients. Wu et al. (2021) equally observed the same correlation between high NLRP3 expression and worse prognosis in patients with skin cutaneous melanoma, whereas Xue et al. (2019) report that patients with laryngeal squamous cell carcinoma and high NLRP3 expression present decreased 5 years-survival after surgery. Our study also draws similar conclusions from patients with pancreatic cancer, supporting again the relevance and extensive use of NLRP3 as a potential prognostic biomarker in different types of neoplasms.

Conclusions

In this work, we show that there is a significant percentage of patients who express the histopathological marker NLRP3. In addition, higher levels of expression of this component are associated with worse prognosis and shorter survival for these patients, possibly due to an ineffective response by the immune system and increased inflammation promoted by tumors. Future studies should be aimed at confirming the results of this work and to evaluating the efficacy of this component as a therapeutic target by considering comprehensive and combined treatments that target the intestinal microbiota and the cells of the immune system.

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Institutional Review Board Statement: The study was approved by the Hospital Universitario Principe de Asturias (LIB26/2020)-UAH Madrid.

Informed Consent Statement: Not applicable.
**Data Availability Statement:** The datasets used and/or analyzed during the present study are available in the manuscript.

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

**References**


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Wu S., Zang Q. and Dai B. (2021). The role of NLRP3 in the prognosis and immune


**Figure legends**

**Figure 1.** Images showing protein expression of NLRP3 in patients diagnosed with pancreatic cancer.

**Figure 2.** Kaplan-Meier curves for survival time according to tumor expression of NLRP3. Blue curve: negative tissue expression; red curve: low/medium expression; green curve: high expression.

**Table 1.** Primary antibodies used, along with dilutions and protocol specifications.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species</th>
<th>Clone</th>
<th>Dilution</th>
<th>Provider</th>
<th>Sodium citrate 10 mM pH = 6 before incubation with blocking solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLRP3</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>1:250</td>
<td>Abcam (ab214185)</td>
<td></td>
</tr>
</tbody>
</table>
Kaplan-Meier survival estimates

- NLRP3 = 0
- NLRP3 = 1
- NLRP3 = 2