Original Article

The occurrence of prion protein in surgically resected pancreatic adenocarcinoma

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Background: Among the several new targets for the comprehension of the biology of pancreatic ductal adenocarcinoma (PDAC), Prion proteins (PrPc) deserve particular mention, since they share a marked neurotropism. Actually, PrPc could have also a role in tumorigenesis, as recently demonstrated. However, only few in vitro studies in cell cultures showed the occurrence of PrPc in PDAC cells. We aim to evaluate the presence of PrPc in vivo in PDAC tissues as a potential new biomarker.

Methods: Samples from tumors of 23 patients undergone pancreatic resections from July 2018 to May 2020 at our institution were collected and analyzed. Immunohistochemistry and western blotting of PDAC tissues were compared with control tissues. Immunohistochemistry was used also to evaluate the localization of PrPc and of CD155, a tumoral stem-cell marker.

Results: All cases were moderately differentiated PDAC, with perineural invasion (PNI) in 19/23 cases (83%). According to western-blot analysis, PrPc was markedly expressed in PDAC tissues (273.5 ± 44.63 OD) respect to controls (100 ± 28.35 OD, p = 0.0018). Immunohistochemistry confirmed these findings, with higher linear staining of PrPc in PDAC ducts (127.145 ± 7.56 μm vs 75.21 ± 5.01 μm, p < 0.0001). PrPc and CD155 exactly overlapped in ductal tumoral cells, highlighting the possible relationship of PrPc with cancer stemness. Finally, PrPc expression related with cancer stage and there was a potential correspondence with PNI.

Conclusions: Our work provides evidence for increased levels of PrPc in PDAC. This might contribute to cancer aggressiveness and provides a potentially new biomarker. Work is in progress to decipher clinical implications.

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Introduction

Although pancreatic cancer is relatively rare, worldwide it is now the fourth most frequent cause of cancer death. Patients with pancreatic ductal adenocarcinoma (PDAC) have dismal prognosis as the overall median survival is 6 months and the 5-year survival rate is less than 10% [1–3]. At the time of diagnosis, only less than 20% of patients are eligible for surgical resection, while 80% of cases are considered advanced for the presence of regional infiltration or distant metastasis [4]. The poor prognosis of pancreatic cancer, even in cases eligible for surgical resection, is due to the extensive local infiltration and to the early lymphatic and hematogenous spread. In fact, even after surgical resection, pancreatic cancer is characterized by an early local or distant recurrence. One of the
main causes of such a disease course is the tumor spreading along perineural pathways, known as perineural invasion (PNI). The PNI is present in up to 100% of pancreatic cancers, being detected already at early disease stages, even in the absence of lymphatic or hematogenous metastasis, or in tumors smaller than 2 cm [5]. The high prevalence of local tumor recurrence even after curative resection is attributed to the residual tumor cells, undetected during the operation, in the remnant pancreas nerves and extra pancreatic nerve plexus [5]. Since PNI is an independent detrimental prognostic index in PDAC patients [6], an in depth knowledge of the biology of neuro-diffusion of the disease is expected to elucidate early key molecular events promoting the diffusion of pancreatic cancer.

Furthermore, the comprehension of those mechanisms involved in tumorigenesis should provide novel therapeutic strategies. It is now generally accepted that KRAS mutation is one of the earliest and most important genetic alterations underlying PDAC development [7–9]. However, all of the clinical trials targeting KRAS have failed. Thus, identifying novel molecules and pathways, which may serve as potential therapeutic targets to curb pancreatic cancer cell growth and/or metastasis is urgently needed. This is expected to improve our understanding on how cellular factors contribute to the tumorigenesis of PDAC to develop novel treatments.

Looking towards these new molecular frontiers in the comprehension of the biology of cancer, recent evidences obtained in a variety of malignancy indicate that the expression of Prion Protein (PrPc) in cancer cells could be associated with tumor aggressiveness and invasiveness. In particular, focusing on pancreatic cancer, several in vitro researches demonstrated that PrPc is expressed in a panel of human PDAC cell lines and that it interacts with different pathways, enhancing cellular growth, tumoral proliferation and invasion [10,11]. Moreover, an analogous significance for PrPc in glioblastoma has revealed how tumor diffusion is reminiscent of the spreading mechanisms in neurodegeneration, thus implicating a relationship between PrPc and neurotropism, which is also a biological peculiarity of PDAC [12].

No study so far investigated in vivo the occurrence of PrPc within PDAC. Therefore, the aim of this study was to prospectively evaluate and characterize the occurrence of PrPc in PDAC tissues from surgically resected patients.

Materials and methods

Patients and specimens

Between January 2018 and May 2020 samples from tumors of 28 patients surgically treated with pancreatic resections at our Institution were collected. Written informed consent was obtained from patients to use their surgical specimens and clinical pathological data for research purposes.

All patients had a preoperative suspicion of PDAC. Preoperative evaluation included medical history, physical, laboratory and radiological examinations, computed tomography (CT) and magnetic resonance imaging (MRI), often with magnetic resonance cholangiopancreatography (MRCP). In addition, abdominal ultrasound with and without contrast, endoscopic ultrasonography (EUS), and fine-needle aspiration (FNA) during EUS were also performed in selected patients. Preoperative data included age and gender.

All the specimens were frozen intraoperatively and further sliced and scored for histology. Pancreatic nodules not resulted adenocarcinomas were ruled out from the study.

Similarly, we could not proceed with this study when tumor specimens were too small. When PDAC diagnosis was confirmed, the pathologist took two specimens from the pancreatic tumor and two fragments from normal pancreatic tissue. One specimen per group (control and tumor) was fixed and kept in formalin for immunohistochemistry while the other one was rapidly frozen and kept at −80 °C for storage until for western blotting analyses (SDS-PAGE immunoblotting) to be carried out.

Histological data included: histological type of the tumor, the grade of differentiation, the tumor size, the number of harvested lymph nodes, the number of metastatic lymph nodes, the presence of angioinvasion and perineural infiltration, the presence of vascular infiltration in case of vascular resection, the presence of intraductal papillary mucinous neoplasm (IPMN) and the associated dysplasia.

Patients were staged after surgery according to the T and N definitions proposed for the AJCC 8th edition (pTNM) [13], basing on pathology results. Proposed T-stage definitions are the following: T1 ≤2 cm maximal diameter, T2 ≥2 ≤4 cm maximal diameter, T3 >4 cm maximal diameter, T4 = locally unresectable. Extra-pancreatic extension was not included in T-stage definitions. The N-staging included the following: N0 = node negative, N1 = 1–3 nodes positive for metastatic disease, N2 ≥4 nodes positive for metastatic disease.

Immunohistochemistry

Morphological study was carried out in control or tumor pancreatic tissues fixed in formalin 4%. For controls, we used non-affected neighboring tissue of the same patients. After fixation, samples were embedded in paraffin and 7–10 μm thick tissue sections were cut and mounted on slides for Haematoxylin & Eosin staining or immune-histochemical analysis. For Haematoxylin & Eosin the sections were plunged in the Haematoxylin solution (Sigma) for 20 min, washed in running water, and then immersed in the Eosin solution (Sigma) for a few minutes. Finally, they were dehydrated in increasing alcohol solutions, clarified in xylene and covered with DPX mounting medium (Sigma).

The immune-histochemical study was carried out using peroxidase or fluorescence-based immune-histochemistry. Pancreatic samples were analyzed to detect several antigens, in particular PrPc and CD155, a marker for stem cells.

For the immunohistochemical experiments the sections were first permeabilized by Triton X 0.1% for 15 min in TBS and then incubated in a blocking solution containing 10% normal goat serum (NGS) in TBS for 1 h at room temperature (RT). The sections were successively incubated with the Ab-I solution overnight at 4 °C. The different Ab-I solutions were prepared in PBS containing 2% NGS. After washing with BSA, the reaction with the Ab-I were revealed by using the secondary biotinylated or fluorescent antibody. For immune-peroxidase, samples were incubated with avidin-biotin complex (ABCkit vector) for 1 h at room temperature and stained with diaminobenzidine. For immunofluorescence, after the primary antibody, we used the anti-rabbit fluorescent secondary antibody Alexa Fluor anti488 (1:200, Life Technologies, Carlsbad, CA, USA) and the anti-mouse fluorescent secondary antibody Alexa Fluor 546 (1:200, Life Technologies) for 1 h and 30 min. The slides were mounted with the mounting medium DPX or Fluoroshield (Sigma) and were observed using the Nikon Eclipse 80i light microscope equipped with a fluorescent lamp and a digital camera connected to the NIS Elements software for image analysis (Nikon, Tokyo, Japan). The linear staining in the pancreatic ducts of control and PDAC was measured using the free software IMAGEJ. Randomly light microscope pictures at 10X magnification from each sample group (control or PDAC) were analyzed: in particular, we selected the region of stained duct using the straight-line selection tool of IMAGEJ. Data were expressed as the mean length ± SEM.
SDS-PAGE immunoblotting

Pancreatic tissue to be immunoblotted were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 5 mM EDTA, 1% SDS, 0.1% IGEPAL (NP40) and Complete Protease Inhibitor Cocktail Tablet (leupeptin, pepstatin, aprotanin) (Santa Cruz Biotechnology, Dallas, TX, USA). Then tissues were sonicated, and homogenates were centrifuged at 5000×g for 5 min. An aliquot of supernatant was used to determine the protein concentration by a protein assay kit (Sigma).

Samples (25 µg) were separated on 4—20% sodium dodecyl sulfate-polyacrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Biorad; Milano, Italy). The Membrane was then immersed in blocking solution containing PBS with 0.05% Tween-20 (PBS-T) and 5% not fat dried milk (Sigma). Then the membrane was incubated overnight at 4°C with primary antibody anti-protein PrPc (1:2000, Abcam) diluted in PBS-T containing 1% not fat dried milk (Sigma). The blots were washed three times with PBS-T and incubated for 1 h with goat anti-rabbit horseradish peroxidase-labeled secondary antibody (1:2000; KPL, Maryland, USA) diluted in PBS-T containing 2% not fat dried milk (Sigma).

The bands were visualized with enhanced chemiluminescence reagents (ImmuNo-Star HRP Substrate; Bio-RadLaboratories) and image analysis was carried out by ChemiDoc System (Bio-RadLaboratories).

β-Actin was used as an internal standard for semi-quantitative protein measurement, so-called “house-keeping protein”. Densitometric analysis was performed with ImageJ software and the unit of measure was the Optical Density (OD). Immunohistochemistry and western blotting of PDAC tissues were compared with control tissues. Furthermore, the degree of PrPc expression in PDAC was reported and compared also on the basis of cancer stage according to AJCC 8th edition, of the presence of PNI and of the detection of IPMN in the surrounding peritumoral pancreas.

Statistical analysis

Continuous variables with normal distribution are expressed as mean ± standard deviation (SD) and compared using Student’s t-test or ANOVA where appropriate. A p-value of less than 0.05 was considered statistically significant. The statistical analysis was performed using SPSS (Statistical Production and Service Solution for Windows, SPSS Inc, Chicago, IL, USA), version 23.

Results

Perioperative data were summarized in Table 1. We collected surgical specimens of 28 patients of which 5 were excluded because the tumor was too small for an adequate sampling or because the frozen section excluded the presence of PDAC. Of the remaining 23 patients included, 13 (56.5%) were male and 10 (43.5%) were female. The mean age was 72.5 ± 7.9 years (range 52–87). The definitive histological examination confirmed the presence of PDAC in all cases. The grading of the pancreatic tumor was “moderately differentiated” (G2) in all cases. The mean tumor dimension was 3.2 ± 1.1 cm (range 1.5–5.0). The mean harvest lymph nodes were 40.4 ± 14.5 (range 24–79) with the presence of metastatic lymph nodes in 20/23 cases (87%) and a mean number of metastatic lymph nodes of 5.3 ± 5.5 (range 1–23). The presence of angioinvasion was reported in 3/23 cases (13%), while the presence of perineural infiltration was reported in 19/23 cases (83%). In 6 patients (26%) IPMN areas with high grade of dysplasia were identified in the surrounding peritumoral tissue.

Three cancer stage groups were identified according to pTNM, stage I (n = 3, 13%), stage II (n = 9, 39%) and stage III (n = 11, 48%).

Histological changes in PDAC

Representative pictures from normal and PDAC tissue are shown in Fig. 1. The histological organization of pancreatic healthy tissue possesses a normal acinus architecture with well-preserved ductal system, while the stroma of PDAC tumors is composed of abundant extracellular matrix with increasingly evident loss of cellular architecture. The ducts are very enlarged and irregularly shaped (Fig. 1).

Table 1

<table>
<thead>
<tr>
<th>Pancreatic ductal adenocarcinoma, n (%)</th>
<th>23 (100%)</th>
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<tr>
<td>Grade of differentiation, n (%)</td>
<td>23 (100%)</td>
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<tr>
<td>G2/3</td>
<td>23 (100%)</td>
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<tr>
<td>Mean tumor dimension, cm</td>
<td>3.2 ± 1.1 (1.5–5.0)</td>
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<td>Mean harvest lymph nodes, n</td>
<td>40.4 ± 14.5 (24–79)</td>
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<tr>
<td>Mean metastatic lymph nodes, n</td>
<td>5.3 ± 5.5 (1–23)</td>
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<td>T status, n (%)</td>
<td>2 (8.7%)</td>
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<td>T1</td>
<td>2 (8.7%)</td>
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<td>T2</td>
<td>14 (60.9%)</td>
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<td>T3</td>
<td>7 (30.4%)</td>
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<td>N status, n (%)</td>
<td>3 (13.1%)</td>
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<td>N0</td>
<td>3 (13.1%)</td>
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<tr>
<td>N1</td>
<td>9 (39.1%)</td>
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<td>N2</td>
<td>11 (48.7%)</td>
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<tr>
<td>Stage, n (%)</td>
<td>3 (13%)</td>
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<td>I</td>
<td>3 (13%)</td>
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<tr>
<td>II</td>
<td>9 (39%)</td>
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<td>III</td>
<td>11 (48%)</td>
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<tr>
<td>Angioinvasion, n (%)</td>
<td>3 (13%)</td>
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<tr>
<td>Perineural infiltration, n (%)</td>
<td>19 (83%)</td>
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<tr>
<td>Vascular infiltration, n (%)</td>
<td>1/23 (4%)</td>
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Expression of PrPc in pancreatic tissue

The PrPc-specific labeling was present in PDAC ductal cells of all enrolled patients (n = 23). The labeling in PDAC tissues was selective for the ductal system, while the extracellular matrix was not labeled. In normal human pancreas, only a few ductal epithelial cells were moderately PrPc-stained (Fig. 2). Accordingly, with the semi-quantitative immune-blot analysis, PrPc was markedly expressed in tumor pancreatic tissues (273.5 ± 44.63 OD) and there was a limited expression in non-cancer tissues (100 ± 28.35 OD) (p = 0.0018) (Fig. 3).

Immunohistochemistry of PDAC revealed an increased PrPc, which was expressed in a linear dimension. In fact, measuring the linear staining of PrPc in ductal cells, we found a marked difference between PDAC compared to controls. In detail, in PDAC, the linear staining was 127.145 ± 7.56 µm, while in controls it was 75.21 ± 5.01 µm (p < 0.0001) (Fig. 4).

The degree of PrPc expression in patients with IPMN lesions in peritumoral tissue at pathology was not significantly different with respect to those without dysplastic areas (173.80 ± 26.16 OD vs 308.66 ± 57.53 OD, p = 0.191).

Expression of PrPc and predicted patients’ prognosis

Comparing the degree of PrPc expression within the cancer stage groups, we found a significantly higher expression of PrPc for advanced stages. In particular, PrPc expression at Western Blotting was 125.23 ± 50.08 OD in stage I, 157.79 ± 20.13 OD in stage II and 408.56 ± 72.39 OD in stage III (p = 0.0079).

When comparing the expression of PrPc between patients with
and without PNI, we found a higher expression in the group with PNI according to Western blot analyses, although not statistically significant (290.12 ± 52.73 OD vs 194.43 ± 47.15 OD respectively, p = 0.429).

Expression of CD155 in pancreatic tissue

In the ducts of tumor specimens, we found CD155 labeling, which was instead absent in control ducts. Therefore, we evaluated the presence of a merging between PrPc and CD155 expression and we found that PrPc and CD155 were co-expressed selectively within ductal cells of PDAC. The representative double staining indicated a co-localization of these markers in PDAC group (Fig. 5).

Discussion

Cellular prion protein is a cell surface glycoprotein which was initially discovered as the normal counterpart of the pathological scrapie prion protein (PrPSC), the main component of the infectious agent of Transmissible Spongiform Encephalopathies. A prion like structure characterizes diffusible proteins in a variety of neurodegenerative disorders. In line with neurodegeneration, within the central nervous system, glioblastoma multiform spreads in PrPc-dependent patterns [12]. Studies on the physiological function of PrPc have long been overlooked. PrPc was proposed to protect neurons against cell death and oxidative stress [14], to control copper metabolism [15], to regulate cell cycle [16], synaptic transmission [17], and cell adhesion [18], and to activate the immune system [19]. Interestingly, PrPc also plays a role in pluripotency and differentiation of embryonic stem cells [20], cell proliferation and differentiation [21–26] and muscle cell regeneration [27]. Moreover, recent evidence indicates that PrPc regulates not only self-renewal of stem/progenitor cells and stem cell fate, but also proliferation and resistance to apoptosis in cancer stem cells [20]. Such changes, in the case of stem cells, deregulate the interactions with their environment. This scenario is considered as a potential cause of cancer stem cells emergence [28]. Prion-mediated changes may represent initiating events that promote the emergence of the hallmarks of cancer, including self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion and metastasis,
**Fig. 3.** Representative immune-blots for PrPc and the house keeping protein b-actin in control tissues and PDAC tissues. Values are given as the mean ± S.E.M. Comparisons between two groups were made by using Student t-test. **P = 0.0018.**

**Fig. 4.** Immunohistochemical evaluation of PrPc expression. The histogram compares the length of the PrPc staining in the control ducts (no PDAC) and in tumor ducts (PDAC) expressed as mean ± SEM. In particular, we selected the region of PrPc stained duct using the straight-line selection tool of IMAGEJ in order to obtain the linear measure. Statistical analysis to compare groups was performed by t-test. The threshold of statistical significance was set at P < 0.05. **P < 0.0001. (10x magnification, scale bar 25 μm).**
limitless replicative potential and inhibition of apoptosis [29]. Generally, over-expression of PrPc induces resistance to cancer therapeutics, while repression or knockdown of PrPc induces sensitization to cancer therapeutics [30]. Starting from these observations PrPc has been involved in the development of human tumors even beyond glioblastoma [31,32] including gastric [33], breast [34], prostate [35], and colorectal [36] carcinomas.

All these reports indicate that the expression of PrPc is related with tumor stemness, invasiveness and resistance to chemotherapy. Furthermore, PrPc is markedly expressed within the tumor-initiating cells, which are responsible for development, dissemination and relapse of the disease.

Recently, several in vitro researches demonstrated that PrPc is expressed also in a panel of human PDAC cell lines and that it interacts with different pathways enhancing cellular growth, tumoral proliferation and invasion [10,11]. Conversely, silencing PrPc in PDAC cell lines would switch to a more favorable biologic behavior [10]. However, no study so far investigated its expression in vivo. The presence of PrPc within PDAC tissue might be a marker that could contribute to explain the biology of the disease in terms of aggressiveness, explaining the uniquely preferred perineural invasion of this neoplasm, based on the relationship of prions with neurotropism and neurodegenerative diseases [37].

Our results confirmed in tissue samples of surgically resected patients for PDAC the over-expression of PrPc uniquely within ductal epithelial cells in contrast with extracellular matrix, witnessing for a specific marker of ductal compartment. We also measured quantitatively a higher staining of PrPc in PDAC samples, compared with control ones. Moreover, the expression of PrPc was independent to the presence of dysplastic areas in surrounding “healthy” pancreas: this could indicate the correlation of PrPc with invasive cancer and not with preneoplastic lesions, confirming its role as marker of aggressiveness. Our data are in line with current literature, since PrPc seems not to be expressed in Pan-IN lesions, but only in PDAC cells [38]. Finally, in our sample the patients with high grade dysplastic IPMN in surrounding pancreas were all at stage I or II according to pTNM, while in the other group they were mainly at stage III, and this could explain why in the latter the mean PrPc expression was higher, despite not significantly.

Interestingly, our results also reveal an association of PrPc expression with other cellular proteins, known as staminal markers, such as CD155. The expression of CD155 is up-regulated in progenitor cells of various tissues and CD155 up-regulation ceases when the cells reach their differentiated states. By using immunofluorescence, we found CD155 labeling only in the ducts of tumor specimens, while it was absent in control ducts. Even more surprisingly, we noticed that the expression of PrPc and CD155 fully merged within ductal cancer cells (Fig. 5). This highlights a potential relationship of PrPc with cancer PDAC stemness and dedifferentiation and confirms recent studies in other tumors and neurodegenerative disorders, which suggest that PrPc plays a role in pluripotency and differentiation of cancer stem cells, and cell-to-cell disease spreading as well.

Since the quite recent start of our project, the mean patients’ follow-up is of 13 months, and it is too short to correlate the degree of PrPc expression to clinical outcomes. However, cancer stage according to pathology examination indicates the patient’s predicted prognosis: hence to obtain a preliminary prognostic correlation, we analyzed the possible relationship between PrPc and the cancer stage after resection based on pTNM [13], finding a significant difference between groups. Even if these parameters cannot completely define the prognostic value of PrPc detection, this can be encouraging in order to validate the role of prion protein in PDAC with further analyses.

Moreover, we investigated the correlation with PNI, which is considered to be another independent prognostic factor for PDAC. Although not statistically significant, there was a trend toward a higher degree of PrPc expression in the 19 patients with PNI.
respect to the 4 patients without PNI. Obviously, the very small number of the latter group does not allow to draw conclusions; however, we think that this interesting data is in line with the correlation of high levels of PrPc expression to more advanced cancer stage previously discussed, and should be considered coherent to our aims, thus representing a starting point to keep on investigating.

This project, as far as we know, is the first one to prospectively investigate the expression of PrPc in vivo in patients with PDAC treated with surgery and our results seem to represent a novelty applying to the biology of PDAC aggressiveness. In fact, even if our group of patients is limited, the results are significant and could lead to a novel insight in the biology of this tumor. Our findings could open new research perspectives: in fact when PrPc is markedly expressed in PDAC cells, it could be used also as novel diagnostic marker of PDAC, by looking at its presence as a circulating protein, since high levels of soluble PrPc have already been detected in the culture supernatants of cell lines [39]. Soluble PrPc may be present in the circulation or body fluid of patients with PDAC and its detection may provide an early and noninvasive method for detecting this tumor.

This study is a preliminary work on surgically resected specimens of PDAC and it represents the first step in a wider project in which we are enrolling more patients to study also the prognostic value of PrPc expression in PDAC tissues. The pool of our surgically treated patients will be followed-up in order to evaluate whether a significant correlation exists between PrPc expression and disease prognosis. The working hypothesis is that those with higher expression of PrPc, may undergo a poorer prognosis, with specific ways of recurrence and tumor spreading (i.e. a higher neurotropism, a higher presence of carcinosis, a shorter disease-free survival, a higher mortality, etc.) and/or a different response to normal therapeutic agents. Our preliminary results are encouraging because they seem to go in that direction: a higher expression of PrPc related to advanced cancer stage, discloses a potential higher biological aggressiveness. Moreover, the trend toward a higher expression in patients with PNI, although not significant so far, would show a potential relationship between PrPc and PDAC neurotropism.

The demonstration that PrPc levels are significantly higher in PDAC patients is expected to open new perspectives. When PrPc plays a role in the biology of PDAC, a higher expression of PrPc should occur in the most aggressive PDACs. An attempt to stratify the patients with different prognosis only according to PrPc expression in the resected specimen would be ideal. This could predict a higher relapse trend, more invasiveness and resistance to normal adjuvant therapies.

The use of specific therapeutic agents targeted at altering to PrPc metabolism should be able to decrease the expression of staminal markers, to reduce a pathological cell growth and reverting chemo-resistance.

Conclusions

Our results show that PrPc is markedly over-expressed within duct cells of PDAC tissues compared with control, and that PrPc over-expression selectively occurs within duct cells. Over-expression of PrPc overlaps with that of the staminal marker CD155 and this could be related to the role of PrPc in enhancing the emergence of the hallmarks of cancer in PDAC cells, promoting their aggressiveness and neurotropism. These data provide a step forward in the comprehension of PDAC biology. In fact, PrPc is likely to represent PDAC a marker of disease severity. Further studies with a higher number of patients, are needed to validate these results and to investigate potential clinical applications.

Statement of ethics

The study was approved by Ethics committee of “Area Vasta Nord Ovest (CEAVNO)”. All patients signed an informed consent to authorize the scientific use of the collected data.

Declaration of competing interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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