Appendix Detailed Response to Reviewer's Comments

Reviewer #1: It is an interesting article entitled "Detailing the ultrastructure's increase of prion protein in pancreatic adenocarcinoma." The study results showed compartmentalization of PrPc in pancreatic cancer cells and an association between PrPc expression in PDAC and recurrence at 12 months after surgery. I have several questions and comments. 1) Please indicate which lanes are the PDAC in Fig.1. 2) The typical ultrastructure of PDAC is shown in Fig.4; compared to the structure in Fig.6, the morphology of the nucleus is a little broken, vacuolation in the cytoplasm is conspicuous, and the endoplasmic reticulum structure around the nucleus is disrupted. Is it possible to distinguish between viable cells and necrotic cells of PDAC? 3) A magnitude in TEM is ranged from x6000 to x8000. Do the authors use constant magnification to quantify the number of immuno-gold particles? Although arrows refer to some particles, some particles seem to be not referred. How many immune-gold particles are there in Fig.6? 4) Please confirm the description that distribution is express by SD, not S.E.M in statistical analysis. 5) Please describe the number of patients in Fig.10 and 11. 6) Was the number of immune-gold particles associated with recurrence, as was the PrPc expression? Was there a relationship between PrPc expression and prognostic variables such as Stage? 7) Is compartmentalization of PrPc likely to be associated with the recurrence of PDAC? Do the authors think that PrPc in the nucleus and PrPc in the cytoplasm have different roles?

RESPONSE: Thank you for your precious suggestions and questions.

<u>Point 1-</u> *Please indicate which lanes are the PDAC in Fig.1:* Done. The figure uploaded in the manuscript is now been updated with the labeled lanes (PDAC and Controls).

Point 2- *The typical ultrastructure of PDAC is shown in Fig.4; compared to the structure in Fig.6, the morphology of the nucleus is a little broken, vacuolation in the cytoplasm is conspicuous, and the endoplasmic reticulum structure around the nucleus is disrupted. Is it possible to distinguish between viable cells and necrotic cells of PDAC?*

This is surely an interesting point. In order to visualize the cellular ultrastructure of PDAC in our study, electron microscopy was performed. As described in materials and methods section, *"when preparing embedded pancreas tissue blocks for electron microscopy analysis, firstly we carried out semi-thin sections in order to better focus on those areas of the tissue where ductal and parenchymal area could be evidenced"*. The ductal tissue visualized at semi-thin sections, where the typical characteristics of PDAC were evident, without necrotic phenomena, was the topographical reference to proceed with electron microscopy analysis. Actually, the presence of vacuolation in the cytoplasm, alteration of the normal nuclear pattern and disruption of other cellular organelles are also typical patterns of PDAC cells, especially when we have more dedifferentiated cells. Moreover, since electron microscopy is performed on fixed tissues, we cannot analyze the real viability of our cells, but we can visualize some indirect signs of the presence of viable cells at the moment the fixation was done: the high expression of PrPc itself either in the nuclear compartment and in the cytosol is the expression of an active gene transcription and protein synthesis, thus excluding the presence of necrosis.

Point 3-a *A magnitude in TEM is ranged from x6000 to x8000. Do the authors use constant magnification to quantify the number of immuno-gold particles?*

As already reported in materials and methods section, TEM analysis was performed at a magnification of 6000-8000x because this was considered a good compromise in order to allow the concomitant visualization of immuno-gold particles and cellular ultrastructure (organelles). For this reason, the magnification ranged between these two values, being higher magnification when it was necessary to better detect the immune-gold particles (for which we used a constant magnification to answers directly to the question) and at lower magnification, when we wish to better visualize the cellular ultrastructure. In order to better explain this concept and following your precious advice, we updated the manuscript with this sentence in materials and methods section: *"TEM analysis was performed at a magnification of 6000-8000x which allowed the concomitant visualization of immuno-gold particles and all cell organelles, using higher magnification when it was necessary to better visualize the immune-gold particles and lower magnification when an ensemble view of the whole ultrastructure was requested for our analysis".*

Point 3-b *Although arrows refer to some particles, some particles seem to be not referred. How many immune-gold particles are there in Fig.6?*

There are 21 immune-gold particles in Fig. 6a and 34 in Fig 6b. We decided to refer with specific arrows only some of them in order to not overcrowd the picture with too many symbols. Following your suggestion, we updated the figure legend as follows "*A: The arrows highlight some of the PrPc immuno-gold particles in the nucleus (8000x magnification, scale bar 500nm).*

B: The arrows highlight some of the PrPc immuno-gold particles in the cytosol (7000x magnification, scale bar 600nm)".

Point 4 *Please confirm the description that distribution is express by SD, not S.E.M in statistical analysis.*

The distribution is expressed by SD and the adequate corrections were done in the manuscript.

Point 5 *Please describe the number of patients in Fig.10 and 11.*

As described in the results section, we analyzed clinical data of 24 patients with available follow-up. Among these patients, 11 did not experience any relapse of the disease during follow-up, while in 13 cases a recurrence occurred (as reported in the manuscript, "*The 12-months recurrence rate was 54.1% (n=13)*"). These data have been updated in Fig 10 legend, by adding: "*The histogram compares the expression of PrPc with western blot in specimen from patients surgically resected for PDAC with no evidence of disease at 12-months follow-up (n=11) with those from patients with disease recurrence (n=13)".*

Moreover, as already reported in results section, "*21 patients out of 24 received adjuvant chemotherapy (CT). Of these, 10/21 (47.6%) were without evidence of disease recurrence at 12 months, while 11/21 (52.4%) experienced a relapse of the disease*". These data have been updated also in Fig.11 legend as follows: "*The histogram compares the expression of PrPc with western blot in specimen from patients surgically resected for PDAC with no evidence of disease at 12 months and treated with adjuvant CT (n=10) with those from patients treated with adjuvant CT with disease recurrence (n=11)".*

Point 6-a *Was the number of immune-gold particles associated with recurrence, as was the PrPc expression?*

As described in materials and methods section, immune-gold particles were detected by using immune electron microscopy. With this technique obviously we gained a lot in details, which is fundamental in order to analyze the cellular ultrastructure. For this reason, when the goal was to disclose the subcellular compartmentalization of PrPc, we used immune electron microscopy and indeed we found a peculiar location of PrPc, with differences between tumoral and normal pancreatic cells, as described. In order to obtain not only qualitative data, but also a kind of quantitative analysis, we counted the single immune-gold particles found at TEM analysis in different patients. As we explained, in all cases the distribution was similar, with a higher number of particles in PDAC cells respect to normal ones and with a higher nuclear concentration in PDAC cells respect to cytosol. However, being data obtained from ultrastructural magnification, they are representative of specific groups of cells magnified and consequently analyzed, and they are not equivalent to the real number of immune-gold particles in the whole tissue fragment. In fact, with TEM inevitably we lose this global information and any correlation of the ultrastructure of a group of cells with clinical data could bring some biases and could not be really representative. Conversely, Western blot analysis was the technique of choice to quantify the global expression of PrPc in our tissue fragments, because it detected the amount of the single protein analyzed (PrPc) in the whole fragment, even if it inevitably loses the ultrastructural detail of TEM analysis. For this reason, for the correlation of PrPc expression with clinical data, Western blot analysis was preferred and performed, being it more representative of the real expression of PrPc in each single tissue fragment and in each specific patient.

Point 6-b *Was there a relationship between PrPc expression and prognostic variables such as Stage?*

In our previous work we already demonstrated the relationship of PrPc expression and patients' prognosis according to cancer stage based on pathology results. Indeed, the present manuscript is an advancement in our research project and we managed to link our findings also with the preliminary data from patients' follow-up. This is an important aspect, since clinical data from patients' follow-up correlate with the real patients' prognosis, not that predicted basing on pathology results. Anyway, following your suggestions, we related the degree of expression of PrPc at western blotting with the cancer stage and we found again a significant difference between groups (p=0.0042), thus confirming our previous data. As a result, we reported and discussed this finding by adding the following sentences in the materials and methods section: *"The degree of PrPc expression in PDAC was reported and compared also on the basis of cancer stage according to AJCC 8th edition"*, the following sentences in the results section: *"When correlating within PDAC group the amount of PrPc expression with specific cancer stages, a significantly higher expression of PrPc for advanced stages was detected. In particular, PrPc expression at Western Blotting was 161.69±63.92 OD in stage I, 173.25±76.5 OD in stage II and 346.86±.55.26 OD in stage III (p=0.0042). "*, and the following sentences in the Discussion section: *"As already demonstrated in our previous preliminary work, the expression of PrPc correlated with predicted patients' prognosis based on cancer stage according to pathology results. These data are encouraging, since they are confirmed also with a wider pool of patients".*

Point 7-a *Is compartmentalization of PrPc likely to be associated with the recurrence of PDAC?* As explained in point 6-our data regarding PrPc compartmentalization cannot be used to obtain clinical correlation as western blot data. Actually, the elucidation of the PrPc compartmentalization could be associated not so much with the recurrence of PDAC, but

rather with the discovery of new molecular pathway that hopefully could be targeted by new therapeutical agents, with important clinical implication under this point of view.

Point 7-b *Do the authors think that PrPc in the nucleus and PrPc in the cytoplasm have different roles?*

Our data are a kind of novelty in literature regarding the specific location of PrPc in PDAC cells. Few studies exist about the possible role of PrPc in PDAC and none regarding its ultrastructural compartmentalization. Our findings surely should be the propeller for further investigation about the possible different roles of this protein according to its subcellular location. A hypothesis is that nuclear PrPc could have a role in signaling complexes that contribute to a regulation of proliferation and cell-cell adhesion through a nucleo-junctional interplay, by interaction with several pathways such as Wnt and Hippo pathways, which are modulated by cell contacts and are deregulated at high frequency in many human cancers. Similarly, the cytosol and plasma membrane location of PrPc have a role in cell-cell junctions and proliferation, but through other pathways such as interaction with Src kinase and desmosomal proteins, thus activating different pathways respect to nuclear PrPc. In this sense the nuclear and the cytosolic location of PrPc could have a double effect on cell-to-cell adhesion and proliferation, by activating different pathways that deserve to be discovered and further investigated.

Following your advice, we added these sentences in the discussion section:" *the detection of a peculiar and prominent concentration in the nucleus suggests an involvement of PrPc in regulating directly gene expression, "acting as a nucleo-junctional interplay in order to modulate the transcriptional activity of different pathways involved in carcinogenesis. In fact, PrPc could have a role in signaling complexes that contribute to a regulation of proliferation and cell-to-cell adhesion. Recently an association has been found in enterocytes between nuclear PrPc and Wnt and Hippo*

pathways, which are modulated by cell contacts and are de-regulated at high frequency in many human cancers. In this way, PrPc should be considered as an actor in oncogenic processes through its role in the dynamics of cell-to-cell junctions because its nuclear localization could lead to modulate transcriptional activity of Wnt and Hippo effectors, some of the pathways clearly involved in carcinogenesis [59]"

Reviewer #2: Dear Authors, my comments are totally in favor of publication, your work was very well done and very interesting, I hope you will continue your researches in the field.

RESPONSE: Thank you for your positive feedback. We really appreciate your comment, since we are enthusiastic with our project, which is the result of a multidisciplinary team work and we do hope to continue our research.

EDITORIAL OFFICE'S COMMENTS

(1) Science editor: 1 Scientific quality: The manuscript describes a Basic Study of the ultrastructure increase of prion protein in pancreatic adenocarcinoma. The topic is within the scope of the WJG. (1) Classification: Grade B and Grade C; (2) Summary of the Peer-Review Report: The work was very well done and very interesting. Authors should indicate the lanes in figure 1 and 2. Some details need to be added. The questions raised by the reviewers should be answered; (3) Format: There is 1 table and 11 figures; (4) References: A total of 59 references are cited, including 14 references published in the last 3 years; (5) Self-cited references: There are 2 self-cited references. 2 Language evaluation: Classification: Grade A and Grade A. The manuscript is reviewed by a native English speaker. 3 Academic

norms and rules: The authors provided the Biostatistics Review Certificate, the Institutional Review Board Approval Form. No academic misconduct was found in the Bing search. 4 Supplementary comments: This is an invited manuscript. No financial support was obtained for the study. The topic has not previously been published in the WJG. 5 Issues raised: (1) The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor; (2) The "Article Highlights" section is missing. Please add the "Article Highlights" section at the end of the main text. 6 Recommendation: Conditional acceptance.

RESPONSE: Thank you for your precious comments.

Issue raised: *(1) The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor:* DONE.

2) The "Article Highlights" section is missing. Please add the "Article Highlights" section at the end of the main text.

Article Highlights section has now been uploaded together with the main manuscript.

(2) Company editor-in-chief: I have reviewed the Peer-Review Report, the full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements of the World Journal of Gastroenterology, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the Criteria for Manuscript Revision by Authors. Before final acceptance, uniform presentation should be used for figures showing

the same or similar contents; for example, “Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...”.

RESPONSE: Thank you for your revision. Changes in figure legends has been made as suggested.