DR. Li-Juan Wei
Scientific Editor
World Journal of Gastroenterology
Email: l.j.wei@wignet.com
September 12, 2017

Dear DR. Li-Juan,

Thank you very much for your and the reviewers' thoughtful evaluations and positive review about our manuscript titled "Relationship between pancreatic cancer autophagy and perineural invasion, clinicopathological features and prognosis" (Ms.: WJG-D-17- 35255).

In the revision of our manuscript, comments and issues raised by the reviewers have been carefully considered and appropriate changes (highlighted in yellow) have been made. Please find a point-by point response to the reviewers' comments (below). To clarify, we present those requests in *italics* followed by our responses.

We are pleased that the reviewers agree that the manuscript will be a valuable contribution to the literature in this area. We hope that the revised manuscript will now be found acceptable for publication in your journal.

Your prompt consideration of our revision will be greatly appreciated.

Sincerely yours,

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Point-by-point response

Response to Reviewer 03271124

Comments To Authors:

-Dear Authors, This well design study demonstrated the relationship between pancreatic cancer autophagy and PNI which is one of the poor risk factor for pancreatic cancer. There are no previous reports about this topic.

Response:

We are pleased that the Reviewer feels that the manuscript is interesting and readable. We are very grateful to the Reviewer for his/her constructive comments/suggestions for improving our manuscript.

This is my comments.

-1. There are many various poor prognostic factors for pancreatic cancer such as vascular invasion, lymph node metastasis. ***Why PNI are the only important factor for autophagy?***

Response:

Thanks for your careful thinking and positive review. In this study, we analyzed the factors that influenced the occurrence of PNI, and the results showed that LC3 expression, lymph node metastasis, pancreatitis and CA199 level were the independent factors influenced PNI occurrence; we also attempted analyzed the factors that influenced the expression of LC3, conversely, the results yield that PNI was the only independent factor affecting LC3 expression in this study setting. For this result, we may guess PNI could be indeed correlated with LC3 expression and even affected the autophagy development; however, the reason for this may need other experiments to investigate the underlying mechanisms.

-2. From the introduction part, you mentioned that "The incidence of perineural invasion (PNI) in pancreatic cancer is 80-100% and is an important factor leading to postoperative pancreatic cancer recurrence." And "Autophagy, as a mechanism of anoikis avoidance in pancreatic cancer, is closely related to the survival of pancreatic cancer cells". That's mean almost all of pancreatic cancer patients have this poor prognostic factors (PNI) and the patients who have autophagy show poor prognosis. ***What is the reason to find the relationship of the autophagy in pancreatic cancer to PNI?***

Response:

Thank you very much for your suggestions. PNI is one of the main clinical features of pancreatic cancer, and mechanisms of its development is unclarified. According to previous studies and literature review, we speculate that pancreatic cancer cells invade into nervous tissue (PNI) because of a variety of physical and molecular mechanisms in multiple molecular factors (growth factors, chemotatic factors, neurotrophic factors, etc.) and tumour microenvironment (hypoxia, reactive oxygen species, reactive nitrogen species, etc.). Yang et al. 2011 [1] suggested that pancreatic cancers require autophagy for tumour growth. Therefore, we speculate the pancreatic cancer cells invaded and resident in nervous tissue will be confront with survival problem in new environment, and need continuous energy supply for the persistent proliferation and invasion, while survival signalling pathways activation and cell autophagy change of the cancer cells may participate in the maintenance of these resident cancer cells having continuous energy supply, and persistent proliferation and invasion from pancreas to other organs and tissues along the nerve. Therefore, cell autophagy may be one of molecular mechanisms of pancreatic cancer cells resident in nervous tissue obtaining energy supply and maintaining survival to develop PNI, while PNI is an important factor leading to postoperative pancreatic cancer recurrence and poor survival.

-3. In the discussion part, you explained "This study found that high expression of LC3 was present in the cancer nests around the nerve infiltration (Fig.1 E),

*consistent with the discovery of Yang[8]. In histology terms, it has been suggested that high LC3 expression is related to PNI. Further analysis of immunohistochemistry showed that there was a significant positive correlation between LC3 expression and PNI in pancreatic cancer tissues. Therefore, we speculated that, in the PNI process, a high autophagy level may assist cancer cells in escaping apoptosis, avoiding the damage of adverse stress and providing energy for the invasion and metastasis of pancreatic cancer"***. Is there any others evidence to support this mechanism?*** *Because you mentioned that in the aim of the study "this study focused on the relationship between pancreatic cancer cell autophagy and PNI, clinicopathological features and prognosis and provides a clinical basis for further study of the autophagy mechanisms affecting the pathogenesis of pancreatic cancer PNI."*

Response:

Thanks for your careful comments. PNI is an important factor leading to postoperative pancreatic cancer recurrence and poor survival, and mechanisms of its development is unclarified. We speculate that pancreatic cancer cells invade into nervous tissue (PNI) because of a variety of physical and molecular mechanisms in multiple molecular factors and tumour microenvironment. Yang et al. 2011[1] found that there existed high autophagy level in pancreatic cancer, and suggested that pancreatic cancers require autophagy for tumour growth. We must recognize there were some limitations in the study. In this study, we found that pancreatic cancer PNI is positively related to autophagy. Even though our granted project will explore the basic mechanisms of autophagy affecting PNI development in pancreatic cancer, we cannot find any other evidence to support this mechanism because of limited clinical pathological and prognostic analysis on LC3 expression determined autophagy associated with PNI and treatment outcome in the patients with pancreatic cancer. So, we are performing other experiments for

the basic mechanisms of autophagy related to PNI, and will also design a prospective prognostic study to further investigate the impact of autophagy on prognosis in pancreatic cancer patients (including the patients stratified by PNI).

-4. From previous study show lymph node metastasis and large tumor size is the poor prognostic factor (Tarantino et al, Br J Surg 2017, Marchegiani et al. Ann Surg 2017). ***Do you have any comment why this factor is not the independent factor for survival in this study?***

Response:

Thank you very much for your suggestions. As we know, the most well-known prognostic factors are related to the characteristics of patient and tumour, such as lymph node metastasis and large tumour size. The above-mentioned two studies (Tarantino et al, Br J Surg 2017 [2], Marchegiani et al. Ann Surg 2017 [3]) explored accurately and objectively prognostic role of lymph node metastasis and tumour size in pancreatic cancer patients. However, conventional prognostic factors, such as lymph node and tumour staging, do not always efficiently estimate clinical outcomes in individual pancreatic cancer patients because of the complex characteristics of the disease. Therefore, the discovery of molecular markers (determining characteristics of the disease) to aid in tumour-type stratification and pancreatic cancer surveillance is critical. In this study, we found CA199 level, PNI and LC3 expression influenced the prognosis of pancreatic cancer patients in a univariate analysis, while PNI and LC3 expression were independent prognostic factors in multivariate statistics. In view of this study was designed and completed in a retrospective manner to evaluate the relationship between pancreatic cancer autophagy and PNI and patient survival, and we will design a prospective prognostic study to further investigate the impact of LC3 expression determined autophagy on prognosis

in pancreatic cancer patients including the groups stratified by detailed lymph node staging, tumour size staging, and PNI recruited more samples and followed-up in the future near years (3-5 years).

-5. Is there any limitations in this study?

Response:

Thanks for your careful thinking about this. We agree with you that there were some limitations in the study. Firstly, this study was designed and completed in a retrospective manner to evaluate the relationship between pancreatic cancer autophagy and PNI and patient survival. The included pancreatic cancer patients were all received proper and right post-operative adjuvant therapy but in a non-random way, such as gemcitabine- or non-gemcitabine-based chemotherapy, and/or radiotherapy. Furthermore, semi-quantitative IHC detection may affect the precision of LC3 expression results, rendering the related analyses biased. And, in this study, we found that pancreatic cancer PNI is positively related to autophagy, but didn't detect the underlying mechanism. Finally, we only analysed the treatment outcome of the patients with followed up more than 12 months by August 2016. Thus, we suggest that the impact of LC3 expression on neural invasion and poor prognosis in pancreatic cancer patients should be interpreted together with other prognostic factors carefully. Aim at the above-mentioned problems, we are performing other experiments for the basic mechanisms of autophagy related to PNI, and will design a prospective prognostic study to further investigate the impact of autophagy on prognosis in pancreatic cancer patients (including the patients stratified by PNI) recruited and followed-up in the future near years (3-5 years).

-6. In the PNI judging part, you mentioned that "According to previous reports, a positive PNI status was determined as cancer cells being found in the perineural spaces, perineurium or nerve tract". ***But there is no reference.***

Response:

Thanks. We have carefully checked the above-mentioned content and added an appropriate reference (highlighted in yellow), and are listing in here.

Li J, Ma Q, Liu H, Guo K, Li F, Li W, Han L, Wang F, Wu E. Relationship between neural alteration and perineural invasion in pancreatic cancer patients with hyperglycemia. PLoS One 2011; 6: e17385 [PMID:21386984 DOI:10.1371/journal.pone.0017385]

-7. In discussion part, "At present, previous pancreatic cancer studies have suggested that PNI is the main cause of abdominal pain in patients and is also one of the important causes of difficult radical operation and local recurrence of pancreatic cancer [5]". ***Is this reference correct?***

Response:

Thanks. We have carefully checked the reference 5 and found that we mistake the reference about above-mentioned content. Now, we have remove the wrong reference and added a correct reference (highlighted in yellow), and are listing in here.

Wrong reference:

Régis DBSI, Kowalski LP, Cavalcante DAV, Flávia LA, Magrin J. Multivariate analysis of risk factors for neck metastases in surgically treated parotid carcinomas. *Arch Otolaryngol Head Neck Surg* 2001;**127**:56-56[PMID: 11177015 DOI: 10.1001/archotol.127.1.56]

Correct reference:

Akerberg D, Ansari D, Andersson R. Re-evaluation of classical prognostic factors in resectable ductal adenocarcinoma of the pancreas. *World J*

Gastroenterol 2016;**22:**6424-6433[PMID: 27605878 DOI: 10.3748/wjg.v22.i28.6424]

Bapat AA, Hostetter G, Von Hoff DD, Han H. Perineural invasion and associated pain in pancreatic cancer. Nat Rev Cancer 2011;11:695-707[PMID: 21941281DOI: 10.1038/nrc3131]

Response to Reviewer 02462687

Comments To Authors:

-This is a retrospective study to evaluate the relationship between pancreatic cancer autophagy and perineural invasions. The authors concluded that autophagy and perineural invasions were significantly associated to pancreatic cancer progression. This was well written, but there were several points to be clarified.

Response:

Thank you very much for your kind words and constructive suggestions.

Major comments

-1. In figure 1C, D and E, please indicate ***which part is the perineural invasion by using arrow****.*

Response:

Good points. We agree with you that the part of the perineural invasion should be indicated by using arrow. We add the information in the revised manuscript (**Fig. 1G-I**) and are listing here as well.

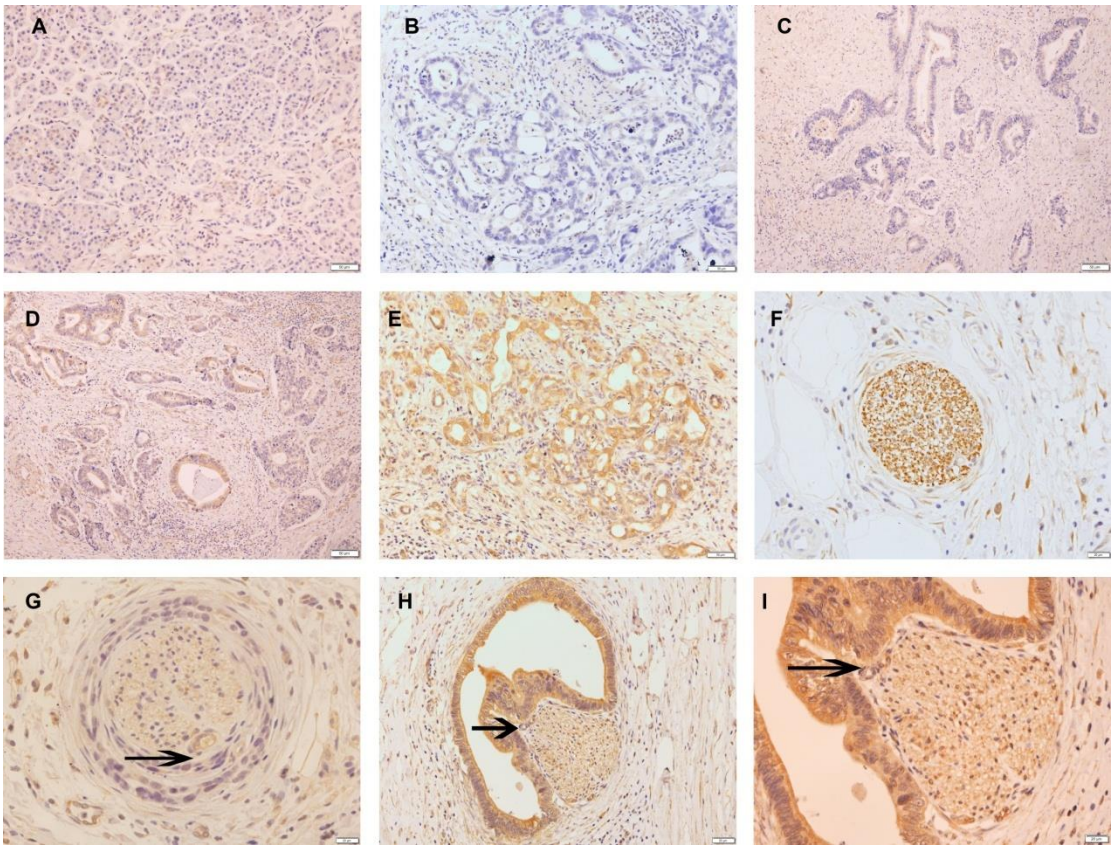

Fig. 1 Representative immunohistochemical results of LC3 and PNI. (A) Negative expression of LC3 in normal paraneoplastic pancreatic tissues (× 200); (B) Negative expression of LC3 in pancreatic cancer tissues(× 200); (C) Weakly positive expression of LC3 in pancreatic cancer tissues(× 200); (D) Moderately positive expression of LC3 in pancreatic cancer tissues(× 200); (E) Strongly positive expression of LC3 in pancreatic cancer tissues(× 200); (F-G) Perineural invasion in pancreatic cancer tissues(× 400, arrow represent cancer cells infiltrating into nerve tissue); (H) Pancreatic cancer cells with high LC3 expression enclosing and invading into nerve tissue (× 200, arrow represents cancer cells infiltrating into nerve tissue); (I) Pancreatic cancer cells with high LC3 expression enclosing and invading into nerve tissue (×400, arrow represents cancer cells infiltrating into nerve tissue).

-2. As shown in Figure 2, overall survival in LC3 low expression and PNI absent patients was surprisingly high over 50%, even though both groups have stage III and IV patients. ***I would concern about too much censor in both groups. The***

authors should explain and discuss about this point.

Response:

Thanks for your careful thinking. We have carefully checked the Figure 2 and raw survival data and calculated overall survival in all related groups including LC3 low expression, LC3 high expression, PNI absent and PNI present patients, and, finally, the overall survivals were correct. In this study, eighty cases were followed up for more than 12 months, and by the end of the follow-up period, 38 patients had died. The survival time of 80 patients who were followed up for more than 12 months was 1 to 54 months, and the median survival time was 20 months (we wrote 9.5 months instead of 20 months mistakenly during first submission). In this study, we made effort to obtain the follow-up information of the all included patients, and meanwhile removed the patients with follow-up time less than 12 months, for providing the accurate prognostic results. As to overall survivals in LC3 low expression and PNI absent patients were high over 50% even though both groups have stage III and IV patients, we performed further analysis of LC3 expression and PNI status stratified by tumour staging, and attached the detailed median survival time for your review (**Table A and B**). According to the survival data, we found that the lowest median survival time was 14 months in PNI present (56/80), LC3 high expression (51/80), vascular invasion positive (22/80), and CA19-9 level >37 (51/80) groups, while overall survival was high over 50% in PNI absent (24/80), LC3 low expression (29/80), and CA19-9 level ≤37 (29/80) groups; and, the prognostic significance of PNI in all stage patients (III+IV or I+II) was vital importance, where regardless of III+IV stage or I+II stage, PNI always increased the risk of death in the pancreatic cancer patients; however, LC3 expression may only increase the risk of death in patients with I+II stage disease, while no influence the survival in the patients with III+IV stage. For avoiding the too much censor and much accurate prognostic

significance, we will continue the follow-up of the included patients, and will design a prospective prognostic study to further investigate the impact of autophagy on prognosis in pancreatic cancer patients (with more samples, including the patients stratified by PNI) recruited and followed-up in the future near years (3-5 years).

Table A Univariate analysis of survival in patients who underwent radical surgery.

Parameters		N	Median survival time (months)	Hazard ratio	95% CI	P
PNI	Present	56	14	3.701	1.539-8.903	0.003[a]
	Absent	24	-			
LC3	High	51	14	3.196	1.433-7.126	0.005[a]
	Low	29	-			
Sex	Male	50	20	1.154	0.590-2.260	0.676
	Female	30	18			
Age	>58	40	20	1.176	0.621-2.225	0.619
	≤58	40	18			
Tumor location	Body/tail	28	21	1.102	0.570-2.131	0.773
	Head	52	20			
Histologic grade	Poorly	26	32	1.287	0.636-2.604	0.484
	Well or moderate	54	20			
Tumor size	>2cm	61	18	0.94	0.444-1.991	0.871
	≤2cm	17	20			
Vascular invasion	Positive	22	14	1.821	0.883-3.755	0.105
	Negative	58	21			
Lymph node metastasis	Positive	56	20	0.871	0.449-1.688	0.682
	Negative	24	18			
AJCC stage	III+IV	28	14	1.473	0.752-2.889	0.259
	I+II	52	23			
Diabetes	Present	17	21	1.105	0.522-2.337	0.795
	Absent	63	20			
Pancreatitis	Present	24	20	1.075	0.520-2.222	0.845
	Absent	56	18			
CA19-9 level	>37	51	14	2.648	1.286-5.454	0.008[a]
	≤37	29	-			

Table B Univariate analysis of survival in patients who underwent radical surgery stratified by tumour staging with LC3 expression and PNI status.

Analysis		Hazard ratio	95% CI	P
III+IV	PNI (present vs. absent)	4.597	1.013-20.871	0.048
	LC3 (high vs. low)	3.159	0.870-11.474	0.081
I+II	PNI (present vs. absent)	3.474	1.172-10.299	0.025
	LC3 (high vs. low)	3.271	1.188-9.005	0.022
	PNI absent (III+IV vs. I+II)	1.355	0.229-8.030	0.738
	PNI present (III+IV vs. I+II)	1.609	0.778-3.328	0.200
	LC3 high (III+IV vs. I+II)	1.959	0.875-4.387	0.102
	LC3 low (III+IV vs. I+II)	1.186	0.280-5.034	0.817

Minor comments Genera data

-1. Please provide about ***the post-operative adjuvant therapy.***

Response:

Thanks for your careful and constructive suggestions. We agree with you that we should describe the post-operative adjuvant therapy. According to the suggestions recommended by you, we have added the information of post-operative adjuvant therapy and amended the related contents in the Materials and methods section of this revision.

We are listing here as well.

The included pancreatic cancer patients were not treated with radiation or chemotherapy prior to surgery, but received post-operatively adjuvant gemcitabine- or non-gemcitabine-based chemotherapy, and/or radiotherapy. (Page 6, line9-12)

-2. What kind of ***informed consent did patients provide****? About surgery or participating this study?*

Response:

Thanks for your positive review and careful thinking. We agree with you that the informed consent provided by patients should be introduced and defined early in the text. In this study, the obtained informed consent from all the patients were for the collection of biological samples such as tissue samples and blood samples. Now, we have added these contents (highlighted in yellow) in the Materials and Methods section of this revision.

We are listing the added section here as well.

All patients provided informed consent for the collection of biological samples, and this study was approved by the Institutional Review Board of First Affiliated Hospital of Zhengzhou University (Scientific Research No. 5, 2015). (Page 6, line 17-20)

-3. Please provide the number of IRB approval.

Response:

Thanks for your careful suggestions. We have added the approval number assigned by the Institutional Review Board of First Affiliated Hospital of Zhengzhou University in the Materials and Methods section of this revision and highlighted in yellow.

We are listing here as well.

All patients provided informed consent for the collection of biological samples, and this study was approved by the Institutional Review Board of First Affiliated Hospital of Zhengzhou University (Scientific Research No. 5, 2015). (Page 6, line 17-20)

Results

*-1. The word, "**high autophagy rate**", is not defined in the materials and methods. Please provide its definitions.*

Response:

Thanks. We must recognize we made a mistake in expressing this "high autophagy rate". Here, the "high autophagy expression" (from the sentence "High autophagy expression was observed in 61.5% of cases") should be "high autophagy level". In this study, we called pancreatic cancer with high LC3 expression (including moderate and strong positive expression) as high autophagy level pancreatic cancer in the narrow sense, and we found that the high autophagy level is closely related to pancreatic cancer PNI, and speculated that high autophagy level may assist cancer cells in escaping apoptosis, avoiding the damage of adverse stress and providing energy for the invasion and metastasis of pancreatic cancer. In the revised manuscript, for avoiding confusion, we modified the sentence and are listing here as well (highlighted in yellow in text).

In 109 pancreatic cancer tissues, forty-two cases had low LC3 expression (termed "low autophagy level"), including 6 (5.5%) negative cases and 36 (33%) weakly positive cases (Fig 1B and 1C); while 67 cases had high LC3 expression (termed "high autophagy level"), including 50 (45.9%) that were moderately positive and 17(15.6%) that were strongly positive (Fig 1D and 1E). (Page 10, line 1-6).

-2. In survival analysis, the risk of death was 2.78-times higher both in the LC3 high-expression group and in the PNI-positive group. Please provide how to calculate 2.78. Please re-confirm that the number is same in both situations.

Response:

Thanks for your careful suggestions. We must recognize we made a mistake in transcribing the univariate analytical result of risk of death between PNI present group and PNI absent group. Here, the "the risk of death was 2.78-times higher in the PNI-positive group" (from the sentence "The risk of

death was 2.78-times greater in the PNI-positive group than that in the PNI-negative group") should be "2.93-times higher". In this study, we performed survival analysis and univariate or multivariate analysis of risk of death in GraphPad Prism 5.0 statistical software, obtaining the univariate analytical result of risk of death between PNI present group and PNI absent group (HR=2.93) and LC3 high expression and LC3 low expression (HR=2.78). We have modified these results in the revised manuscript and are listing here.

The risk of death was 2.93-times greater in the PNI-present group than that in the PNI-absent group, and the difference between the two groups was significant (Fig. 2B). (Page 11, line23-24).

Discussion

-1. The relationship between PNI and LC3 is the most important issue in this manuscript. Please discuss about "Chicken or Eggs" problem.

Is autophagy the result of PNI? Or does PNI induce autophagy?

Table 1. In table 3 and 4, please spell out B, S.E., Wals., Sig., and Exp(B) in the legend.

Response:

Thank you very much for your suggestions. We agree there may be a "Chicken or Eggs" problem in exploring the relation between PNI and autophagy. PNI is one of the main clinical features of pancreatic cancer, and mechanisms of its development is unclarified. According to previous studies, we speculate that pancreatic cancer cells invade into nervous tissue (PNI) because of a variety of physical and molecular mechanisms in multiple molecular factors (growth factors, chemotatic factors, neurotrophic factors, etc.) and tumour microenvironment (hypoxia, reactive oxygen species, reactive nitrogen species, etc.). The pancreatic cancer cells invaded and resident in nervous tissue will be confront with survival problem in new

environment, and need continuous energy supply for the persistent proliferation and invasion, while survival signalling pathways upregulation and cell autophagy change of the cancer cells may participate in the maintenance of these resident cancer cells having the ability (continuous energy supply, and persistent proliferation and invasion) to PNI and spread from pancreas to other organs and tissues. Therefore, cell autophagy may be one of molecular mechanisms of pancreatic cancer cells resident in nervous tissue obtaining energy supply and maintaining survival.

We have made a modification in table 3 and 4 and spell out full name of B, S.E., Wals., Sig., and Exp(B) in the tables.

We are listing here as well.

Table 3 Logistic regression multivariate analysis of perineural invasion with clinicopathological features in pancreatic cancer. (Page 28, line 1-4).

Parameters	Estimate, B	Standard error	Wald statistic	P-value	Odds ratio	95%CI
Lymph node metastasis (positive vs. negative)	1.068	0.499	4.581	0.032[a]	2.911	1.094-7.743
CA199 (>37 vs.≤37)	1.508	0.493	9.368	0.002[a]	4.520	1.720-11.874
Pancreatitis (present *vs.* absent)	1.301	0.514	6.419	0.011[a]	3.673	1.343-10.049
LC3 (high vs. low)	1.032	0.491	4.406	0.036[a]	2.806	1.071-7.351
Constant	-7.209	1.799	16.058	0	0.001	

[a]*P* <0.05, CI: Confidence interval.

Table 4 Logistic regression multivariate analysis of LC3 expression with clinicopathological features in pancreatic cancer. (Page 29, line 1-3).

Parameters	Estimate, B	Standar	Wald	P-value	Odds	95% CI

		d error	statistic		ratio	
PNI (present vs. absent)	0.997	0.427	5.451	0.02[a]	2.71	1.174-6.259
Constant	-1.115	0.732	2.316	0.128	0.328	

[a]*P*<0.05, PNI **:** Perineural invasion .

-2. In table 5, please provide the meaning of hazard ratio in each category. For example, in sex category, male vs female or female vs male?

Response:

Thank you very much for your suggestions. We agree with that we should provide the meaning of hazard ratio in each category in table 5. Now, we have made a modification in table 5 and are listing here as well.

Table 5. Univariate analysis of survival in patients who underwent radical surgery. (Page 29, line 1-4).

Parameters	Hazard ratio	95% CI	*P*
PNI (present vs. absent)	3.701	1.539-8.903	0.003[a]
LC3 (high vs. low)	3.196	1.433-7.126	0.005[a]
Sex (male vs. female)	1.154	0.590-2.260	0.676
Age (>58 vs.≤ 58)	1.176	0.621-2.225	0.619
Tumor location (body/tail vs head)	1.102	0.570-2.131	0.773
Histologic grade (poorly vs. well or moderate)	1.287	0.636-2.604	0.484
Tumor size (>2cm vs. ≤2cm)	0.94	0.444-1.991	0.871
Vascular invasion (positive vs. negative)	1.821	0.883-3.755	0.105
Lymph node metastasis (positive vs. negative)	0.871	0.449-1.688	0.682
AJCC stage (III+IV vs. I+II)	1.473	0.752-2.889	0.259
Diabetes (present vs. absent)	1.105	0.522-2.337	0.795
Pancreatitis (present vs. absent)	1.075	0.520-2.222	0.845
CA19-9 level (>37 vs. ≤37)	2.648	1.286-5.454	0.008[a]

[a]*P*<0.05, LC3: Microtubule-associated protein 1A/1B-light chain 3 ; PNI **:**

Perineural invasion .

Reviewer 03538158

-1. In abstract section, Is UCH a ubiquitin carboxy terminal hydrolase?

Response:

Thank you very much for your suggestions. Yes, the full name for UCH is ubiquitin carboxy-terminal hydrolase. In abstract section, we neglected to describe the full name of the UCH. In the revised manuscript, we added respectively the full name of the UCH in the Abstract and Materials and Methods section.

We are listing here as well.

Abstract:

Expression levels of the autophagy-related protein microtubule-associated protein 1A/1B-light chain 3 (LC3) and perineural invasion marker ubiquitin carboxy-terminal hydrolase (UCH) in pancreatic cancer tissues were detected by immunohistochemistry. (Page 3, line 8-11)

Materials and Methods:

Expression levels of LC3 and the nerve fiber marker ubiquitin carboxy-terminal hydrolase (UCH) were detected by immunohistochemistry using a standardized streptavidin–peroxidase (SP) method and the SP immunohistochemical Kit (ZSGB-Bio, Beijing, China) according to the manufacturer's instructions. (Page 7, line 4-8)

-2. Authors should show the control staining and the normal paraneoplastic pancreatic tissues stained by LC3?

Response:

Thank you for your suggestion. We have added the photo of normal paraneoplastic tissue stained by LC3 in the revised manuscript.

We are listing here as well.

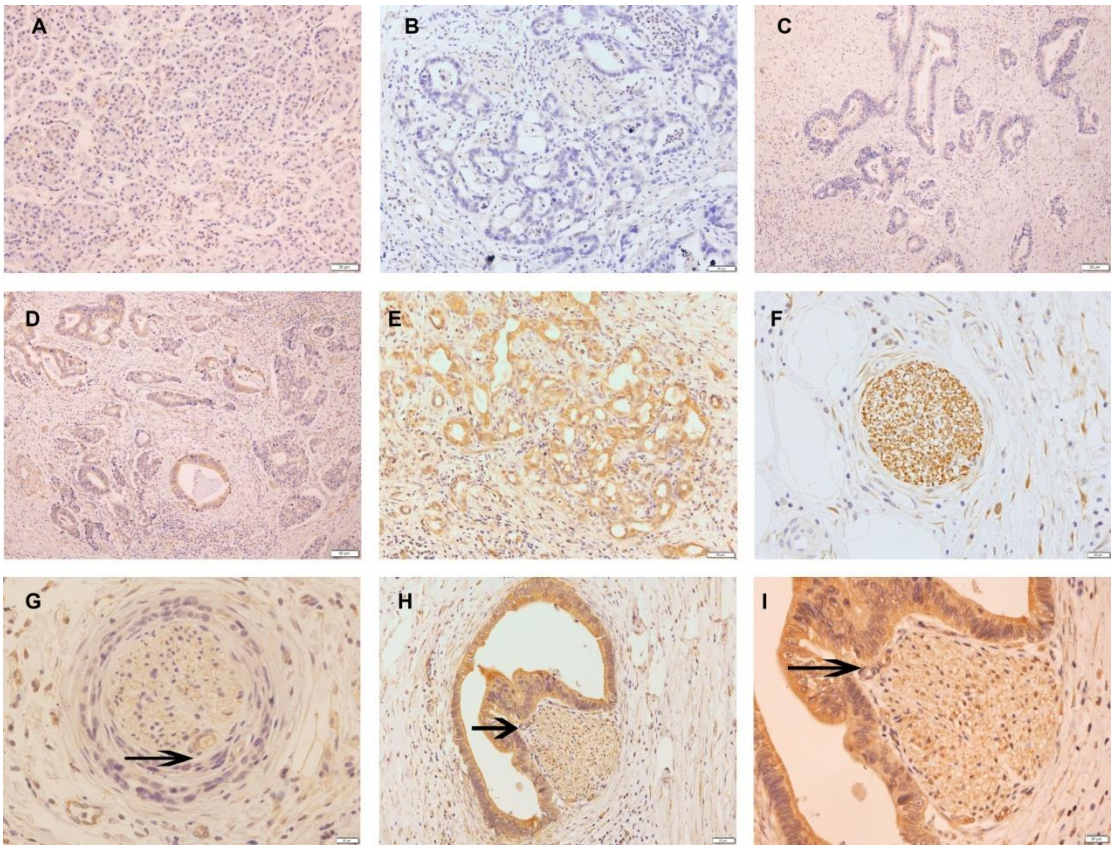

Fig. 1 Representative immunohistochemical results of LC3 and PNI. (A) Negative expression of LC3 in normal paraneoplastic pancreatic tissues (× 200); (B) Negative expression of LC3 in pancreatic cancer tissues(× 200); (C) Weakly positive expression of LC3 in pancreatic cancer tissues(× 200); (D) Moderately positive expression of LC3 in pancreatic cancer tissues(× 200); (E) Strongly positive expression of LC3 in pancreatic cancer tissues(× 200); (F-G) Perineural invasion in pancreatic cancer tissues(× 400, arrow represent cancer cells infiltrating into nerve tissue); (H) Pancreatic cancer cells with high LC3 expression enclosing and invading into nerve tissue (× 200, arrow represents cancer cells infiltrating into nerve tissue); (I) Pancreatic cancer cells with high LC3 expression enclosing and invading into nerve tissue (×400, arrow represents cancer cells infiltrating into nerve tissue).

-3. Authors should show LC3-I and LC3-II by western blotting.

Response:

Thank you for your suggestion. In this study, we detected the expression and location of LC3 in pancreatic cancer tissue using IHC assay, and investigated the significance of LC3 expression in pancreatic cancer. In this retrospective study, we did not detect LC3 expression in pancreatic cancer tissue from the included patients by western blotting, because there were no more reserved frozen tissue samples and limited formalin-fixed paraffin-embedded samples. However, we validated the expression of the levels of LC3 protein in pancreatic cancer PANC-1 cells by western blotting using the same antibody. Moreover, we will design a prospective prognostic study to further investigate the impact of autophagy on prognosis in pancreatic cancer patients recruited and followed-up in the future near years (3-5 years), where LC3 expression analysis in pancreatic cancer tissue by western blotting will also be included.

We are listing here (the levels of LC3 protein in pancreatic cancer PANC-1 cells by western blotting; unpublished data).

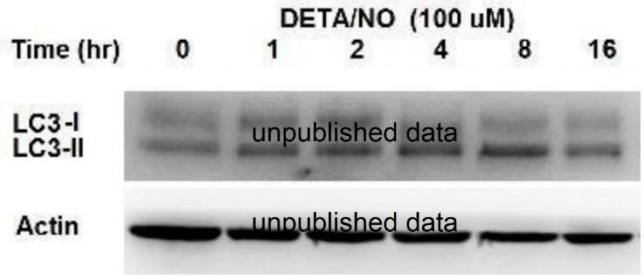

Figure. the impact of a nitric oxide donor on the level of LC3 protein in pancreatic cancer PANC-1 cells (**unpublished data**).

Response to Reviewer 00068723

Comments To Authors:

-The authors investigated perineural invasion and autophagy of pancreatic cancer. They concluded that autophagy was related with perineural invasion, and poor prognosis. The results were rationale. But photos were not clear enough to confirm the results.

Response:

Thanks for your positive review and careful thinking. We have added much clear photos for confirming related results.

-LC3 immunohistochemical score should be presented in figures.

Response:

Thank you for your thoughtful suggestion. We have added different photos (Fig. 1B-E) of increasing LC3 immunohistochemical expression level in related figures.

We are listing here as well.

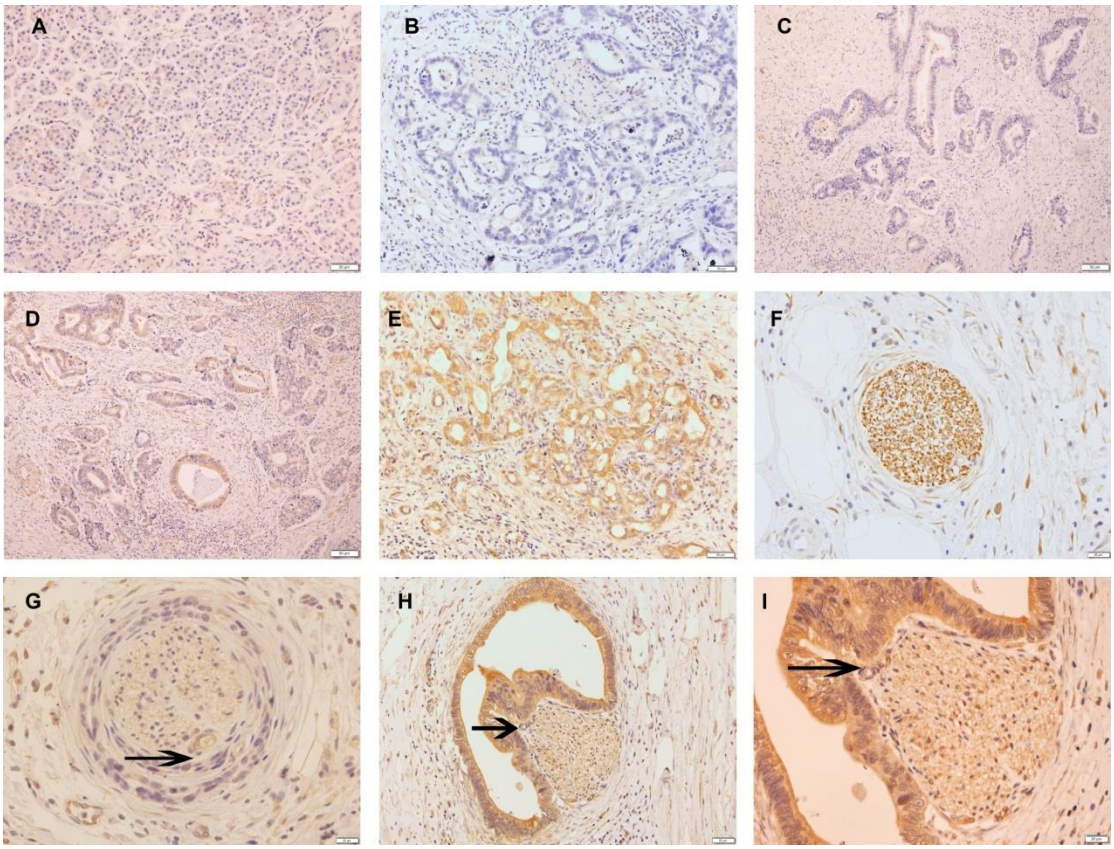

Fig. 1 Representative immunohistochemical results of LC3 and PNI. (A) Negative expression of LC3 in normal paraneoplastic pancreatic tissues (× 200); (B) Negative expression of LC3 in pancreatic cancer tissues(× 200); (C) Weakly positive expression of LC3 in pancreatic cancer tissues(× 200); (D) Moderately positive expression of LC3 in pancreatic cancer tissues(× 200); (E) Strongly positive expression of LC3 in pancreatic cancer tissues(× 200); (F-G) Perineural invasion in pancreatic cancer tissues(× 400, arrow represent cancer cells infiltrating into nerve tissue); (H) Pancreatic cancer cells with high LC3 expression enclosing and invading into nerve tissue (× 200, arrow represents cancer cells infiltrating into nerve tissue); (I) Pancreatic cancer cells with high LC3 expression enclosing and invading into nerve tissue (×400, arrow represents cancer cells infiltrating into nerve tissue).

-Darkness and color of A and B were different. It was not appropriate to evaluate with photos with different conditions. Neural tissues of C and D were not clear. Please change the photos to those that would show perineural invasion more clearly. In D,

what was the tissues that were positive?

Response:

Thank you for your careful thinking. We have carefully checked and modified the related photos for evaluation in identical conditions and better readability and are listing here.

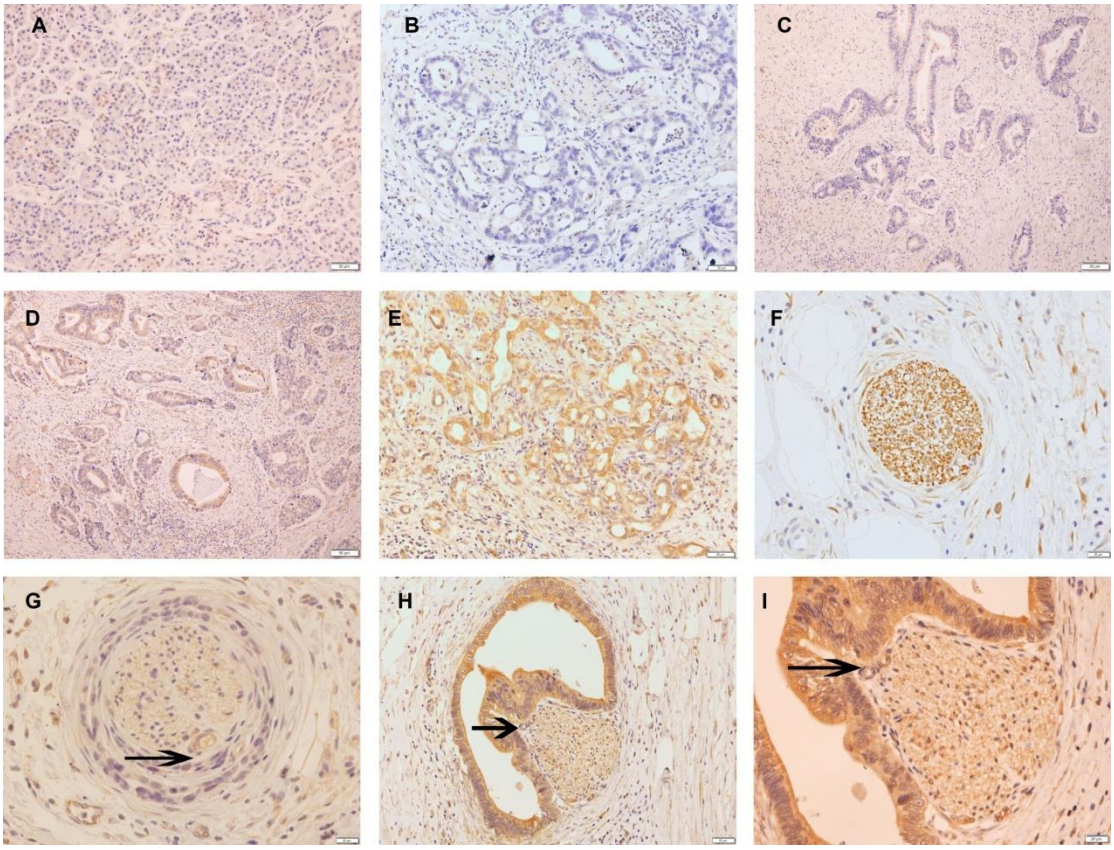

Fig. 1 Representative immunohistochemical results of LC3 and PNI. (A) Negative expression of LC3 in normal paraneoplastic pancreatic tissues (× 200); (B) Negative expression of LC3 in pancreatic cancer tissues(× 200); (C) Weakly positive expression of LC3 in pancreatic cancer tissues(× 200); (D) Moderately positive expression of LC3 in pancreatic cancer tissues(× 200); (E) Strongly positive expression of LC3 in pancreatic cancer tissues(× 200); (F-G) Perineural invasion in pancreatic cancer tissues(× 400, arrow represent cancer cells infiltrating into nerve tissue); (H) Pancreatic cancer cells with high LC3

expression enclosing and invading into nerve tissue (× 200, arrow represents cancer cells infiltrating into nerve tissue); (I) Pancreatic cancer cells with high LC3 expression enclosing and invading into nerve tissue (×400, arrow represents cancer cells infiltrating into nerve tissue).

-Discussion. First and second paragraphs of discussion were repeats of Introduction.

Response:

Thank you for your careful thinking. We made some modifications in the first and second paragraphs of Discussion section in the revised manuscript and are listing here.

Pancreatic cancer has a poor treatment outcome because of a low resection rate, early invasion and metastasis, and insensitivity to radiotherapy and chemotherapy[10-13]. PNI is common in pancreatic cancer, and also found in breast, prostate and rectal cancers[14]. A lot of studies have suggested that PNI is the main cause of abdominal pain in patients and is also one of the important causes of local recurrence of pancreatic cancer[15-19]. PNI is a continuous process involved with multiple molecular factors and tumor microenvironment, but it is unclarified how cancer cells maintain their survival and proliferation from pancreatic cancer tissues to the external pancreatic plexus [20]. Autophagy is the process of degrading cytoplasmic proteins or organelles through lysosomes. Under physiological conditions, autophagy plays a major role in maintaining the intracellular environment stability[21, 22]. Autophagy is an important mechanism of escaping apoptosis for tumor cells. Moreover, autophagy may mediate resistance to chemotherapy in pancreatic cancer[23-25]. Therefore, this study was designed and completed in a retrospective manner to evaluate the relationship between pancreatic cancer autophagy and PNI and patient survival. (Page12, line2-18)

-Discussion. Please speculate the rationality of relationship among perineural

invasion, autophagy, and poor survival in more detail.

Response:

Thank you for your suggestion. Autophagy is very complex and plays an important role in tumour progression. In this study, the multivariate analytical results showed that PNI and high LC3 expression were independent prognostic factors in pancreatic cancer patients. Interestingly, this study also found that high autophagy level is closely related to PNI. Therefore, we speculated that the autophagy associated with poor survival in pancreatic cancer could be explain by the properties of autophagy assisting cancer cells to evade stress-induced apoptosis in PNI environment, while the results undoubtedly promote tumour cell survival and persistent proliferation, and invasion from pancreas to other organs and tissues along the nerve. Therefore, the high autophagy of cancer cells may promote the malignant progression of pancreatic cancer, resulting in PNI and the poor treatment outcome in patients with the disease. Also, we made some modifications in the Discussion section of the revised manuscript and are listing here.

Autophagy is very complex and often plays an important role in tumor progression[6, 29]. Interestingly, this study found that high autophagy level is closely related to PNI, while both of which are independent risk factors for pancreatic cancer with a poor prognosis. The autophagy associated with poor survival in pancreatic cancer could be explain by the properties of autophagy assisting cancer cells to evade stress-induced apoptosis in PNI environment undoubtedly promote tumor cell survival[30, 31]. Therefore, the high autophagy of cancer cells may promote the malignant progression of pancreatic cancer, resulting in PNI and the poor treatment outcome in patients with the disease. (Page 14, line2-11)

References

[1] **Yang S,** Wang X, Contino G, et al. Pancreatic cancers require autophagy for tumor growth. *Genes Dev*. 2011. **25**(7): 717-29-.

[2] **Tarantino I,** Warschkow R, Hackert T, et al. Staging of pancreatic cancer based on the number of positive lymph nodes. *Br J Surg*. 2017. **104**(5): 608-618.

[3] **Marchegiani G,** Andrianello S, Malleo G, et al. Does Size Matter in Pancreatic Cancer?: Reappraisal of Tumour Dimension as a Predictor of Outcome Beyond the TNM. *Ann Surg*. 2017. **266**(1): 142-148.