Name of Journal: *World Journal of Gastroenterology*
Manuscript Type: BASIC STUDY

Relationship between Pancreatic Cancer Autophagy and Perineural Invasion, Clinicopathological Features and Prognosis

Yan-Hui Yang, Jiang-Bo Liu, Yang Gui, Liang-Liang Lei, Shui-Jun Zhang

Yan-Hui Yang, Shui-Jun Zhang, Department of Hepatobiliary and Pancreatic Surgery, the First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China.

Jiang-Bo Liu, **Liang-Liang Lei**, Department of General Surgery, First Affiliated Hospital, College of Clinical Medicine, Henan University of Science and Technology, Luoyang 471000, Henan Province, China.

Yan-Hui Yang, Yang Gui, Department of Hepatobiliary Surgery, First Affiliated Hospital, College of Clinical Medicine, Henan University of Science and Technology, Luoyang 471000, Henan Province, China.

Author contributions: Yang YH and Liu JB contributed equally to this work; Yang YH and Liu JB performed the majority of the experiments; Gui Y and Lei LL assisted with various experiments and helped to analyze the data; Zhang SJ and Liu JB drafted and edited the manuscript; Yang YH, Liu JB performed critical revision of the manuscript.

Supported by the National Natural Science Foundation of China, No. U1504815.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of First Affiliated Hospital of Zhengzhou University.

Conflict-of-interest statement: The authors declare no competing interests.

Data sharing statement: No additional unpublished data are available.

Address correspondence to: Shui-Jun Zhang, MD, PhD, Department of Hepatobiliary and Pancreatic Surgery, the First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, HenanProvince, China. zhangshuijun@zzu.edu.cn.

Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China.

Henan Key Laboratory of Digestive Organ Transplantation.

Open and Key Laboratory of Hepatobiliary & Pancreatic Surgery and Digestive Organ Transplantation at Henan Universities.

Zhengzhou Key Laboratory of Hepatobiliary & Pancreatic Diseases and Organ Transplantation.

Telephone: +86-0371-66964992

Fax: +86-0371-66964992

Abstract

AIM: To investigate the relationship between autophagy and perineural invasion（PNI）, clinical features and prognosis in patients with pancreatic cancer.

METHODS: Clinical and pathological data were retrospectively collected from 109 patients with pancreatic ductal adenocarcinoma who underwent resection at the First Affiliated Hospital of Zhengzhou University from January 2011 to August 2016. 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. The correlations among LC3 expression, perineural invasion, and clinical pathological features in pancreatic cancer were analyzed. The patients were followed up for further survival analysis.

RESULTS: In 109 cases of pancreatic cancer, 68.8% (75/109) had evidence of perineural invasion and 61.5% (67/109) had high LC3 expression. Perineural invasion was associated with lymph node metastasis, pancreatitis and CA19-9 levels (P<0.05). LC3 expression was related to lymph node metastasis (P<0.05) and was positively correlated with neural invasion (P<0.05, r=0.227). Multivariate logistic regression analysis indicated that LC3, lymph node metastasis, pancreatitis, and CA19-9 level were factors that influenced neural invasion, whereas only neural invasion itself was an independent factor of high LC3 expression. Univariate analysis showed that LC3 expression, neural invasion and CA19-9 level were related to the overall survival of pancreatic cancer patients (P<0.05). Multivariate COX regression analysis indicated that perineural invasion and LC3 expression were independent risk factors for poor prognosis in pancreatic cancer (P<0.05).

CONCLUSION: Perineural invasion in patients with pancreatic cancer is positively related to autophagy. Neural invasion and LC3 expression are independent risk factors for pancreatic cancer with poor prognosis.

Key words: Pancreatic cancer; Perineural invasion; Autophagy; Clinical pathological features; Prognosis

Core tip: The relationship between autophagy and perineural invasion (PNI) was explored for the first time in pancreatic cancer. Pancreatic cancer PNI is related to LC3 expression-determined autophagy. PNI and LC3 expression were independent prognostic factors in pancreatic cancer. There might be a special association between autophagy and PNI, contributing to pancreatic cancer progression. This study might provide a new insight for the mechanism of PNI in pancreatic cancer.

Yang YH, Liu JB, Gui Y, Lei LL and Zhang SJ. Relationship between Pancreatic Cancer Autophagy and Perineural Invasion, Clinicopathological Features and Prognosis

Introduction

Pancreatic cancer, also known as "the king of cancer", is a malignant tumor with a poor prognosis that has almost equal mortality and morbidity in patients. The incidence of pancreatic cancer is increasing yearly[1]. Surgical resection is the only possible cure of pancreatic cancer, although less than 20% of patients are eligible for radical surgery [2]. At the time of diagnosis, most pancreatic cancer patients have distant metastases due to early occult symptoms, a lack of effective screening, and perineural growth characteristics. The incidence of perineural invasion (PNI) in pancreatic cancer is up to 80-100% and is an important factor leading to postoperative pancreatic cancer recurrence. Previous studies have shown a higher recurrence rate after surgery and shorter disease-free and overall survival rates in cases of pancreatic cancer with PNI compared with those of cases without PNI. PNI evaluation of pancreatic cancer can predict disease recurrence and prognosis after surgery [3, 4]. However, the pathogenesis of PNI has not yet been defined.

Autophagy has a dual role in promoting and inhibiting tumor growth [5, 6]. Autophagy, as a mechanism of anoikis avoidance in pancreatic cancer, is closely related to the survival of pancreatic cancer cells. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a typical marker of autophagy. LC3 labeling has been used to evaluate autophagy, and high levels of LC3 expression have been found in pancreatic cancer cells [7]. In addition, previous study also showed that pancreatic cancer cells with PNI have higher levels of autophagy [7].

No study has examined the relationship between autophagy and PNI in pancreatic cancer cells. However, it can be inferred that only those pancreatic cancer cells that can survive within nerve tissues can eventually develop into a clinically visible form of pancreatic cancer PNI. Autophagy is likely one of the mechanisms involved in cancer cell survival. Therefore, 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.

Materials and Methods

General data

Retrospective data were collected from 109 cases of pathology-confirmed pancreatic ductal adenocarcinoma patients who underwent radical surgery for pancreatic cancer from January 2011 to August 2016 at the First Affiliated Hospital of Zhengzhou University. 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. Tissue specimens were fixed in formalin and paraffin embedded for histological study. Clinical and pathological data were collected, and all cases were followed up. The date of surgical resection was considered as the starting time, and August 2016 was the deadline. The primary end-point was death due to pancreatic cancer. Eighty cases were followed up for more than 12 months. 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).

Out of the 109 patients studied, 61 cases were male, and 48 cases were female. Their ages ranged from 19 to 81 years, and the median age was 59 years. Tumor diameters ranged from 0.7–12 cm with a median value of 4.0 cm. 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. By the end of the follow-up period, 38 patients had died. The clinical stages were

classified according to the AJCC 2011 standard.

Immunohistochemistry

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. The pancreatic cancer tissues were routinely embedded in paraffin and sliced into 4-μm-thick continuous sections. The sections were then warmed in an autoclave to remove residual wax and were hydrated before antigen retrieval. Endogenous peroxidase activity was eliminated by incubating the tissue sections at room temperature with 3% H_2O_2 for 10 min. The tissue sections were washed 3 times with PBS continuously and incubated with a small amount of goat serum at room temperature in a closed chamber for 15 min. The goat serum was then poured off the slides (not washed), and anti-LC3 (Proteintech Group, diluted 1:120) and-UCH antibodies (Proteintech, diluted 1:100) were added, and the slides were incubated overnight at 4°C. The next day, the slides were thoroughly washed with PBS, a biotinylated secondary antibody in a working liquid was added, and the slides were incubated at room temperature for 15 min. After the slides were washed carefully with PBS again, horseradish peroxidase was added, and the slides were incubated for 5 min at room temperature. The slides were then rinsed with PBS three times, and a drop of DAB buffer was placed on the tissue slice and rinsed after 1 min to stop the color reaction. Then, the nucleus was then stained with hematoxylin, and the samples were placed in gradient alcohol, dehydrated with formaldehyde, covered with transparent balata, and observed by microscopy. PBS was used in place of the first antibody to serve as a negative reference sample, and a known positive slice served as the positive control. The immunohistochemical results were evaluated in a double-blind manner by two pathologists. If the results were

inconsistent, a third pathologist reviewed the data, and a consensus was reached.

LC3 immunohistochemical score

LC3-positive cells by immunohistochemistry required the following characteristics: (1) clear cell structure; (2) accurate localization of the positive particle; and (3) obviously higher pigmentation than that of the background and clear contrast. Positive LC3 expression was mainly localized in pancreatic cancer cell cytoplasm. Five random power fields (400X magnification) were observed for each case using an optical microscope. One hundred homogeneous cells were counted, and the staining intensities and the proportion of positive cells were observed. A semi-quantitative analysis was conducted using the product method. (1) Dye intensity was scored as follows: no yellow, 0 points; light yellow, 1 point; yellow or deep yellow, 2 points; and brown or tan, 3 points. (2) The expression range was scored as follows: <10%, 0 points; 10 to 25%, 1 point; 26 to 50%, 2 points; 51 to 75%, 3 points; and >75%, 4 points. The value from step (1) was then multiplied by the value from step (2): 0 points, negative (-); 1 to 3 points, weakly positive (+); 4 to 6 points, moderately positive (+ +); and 7 to 12 points, strongly positive (+ + +). More than 3 points indicated high expression, and 3 points or fewer indicated low expression.

PNI judging

UCH was expressed in the cytoplasm of all nerve fibers. The location of nerve fibers can be clearly defined by UCH, and then determined the invasion of cancer cells to the nerve tissue. According to previous reports, a positive PNI status was determined as cancer cells being found in the perineural spaces, perineurium or nerve tract [8].

Survival analysis

The effects of clinicopathological factors such as LC3 expression and perineural invasion on the overall survival rate in pancreatic cancer were analyzed in 80 patients with more than 12 months of follow-up data. The following factors were included in the survival analysis: LC3 expression, age, gender, tumor location, tumor size, histological grade, clinical stage, vascular invasion, lymph node metastasis, pancreatitis, diabetes status and preoperative CA19-9 level.

Data analysis

Statistical analyses were performed using SPSS19.0 and GraphPad Prism 5.0 statistical software. Enumeration data were checked by the Chi-square test or the four-grid table Fisher exact probability method. Correlations between clinicopathological factors such as LC3 expression and PNI were analyzed using the Spearman correlation method. LC3 expression and the factors that independently influenced neural infiltration were analyzed using two categories and unconditional logistic regression. Univariate and multivariate analyses were performed on factors that might affect the prognosis according to a COX risk regression model. The survival curve was plotted according to the Kaplan-Meier method. Results were considered significant when $P<0.05$.

Results

Evaluation of LC3 expression and perineural invasion in pancreatic cancer

LC3 expression was mainly localized to the cytoplasm. In contrast to normal paraneoplastic pancreatic tissues (Fig 1A), the expression of LC3 in pancreatic cancer tissues ranged from low to high in four grades: negative, weak positive, moderately positive and severe positive(Fig 1B-E). The immunohistochemical results indicated that LC3 protein expression was observed in pancreatic ducts, acinar epithelial cells, islet cells and pancreatic cancer tissues. There was significantly increased LC3 protein expression in pancreatic cancer

tissues and peritumoral tissues. 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). PNI by pancreatic cancer cells occurred mainly in the pancreatic cancer stroma. According to previous studies[9], 4 types of relationships exist between PNI and cancer nests: no perineural invasion, perineurium invasion, perineural space invasion, and invasion to the nerve fiber tracts. In 109 cases of pancreatic cancer, 75 cases were positive(Fig 1F -I), and 34 cases were negative for nerve invasion. The positive rate of nerve invasion was 68.8%. High LC3 expression was also found in the nests surrounding the PNI (Fig 1I).

Relationship between LC3 expression and perineural invasion in pancreatic cancer

The analysis of LC3 expression and PNI of pancreatic cancer demonstrated a significant positive correlation between these two parameters (P=0.018, correlation coefficient r=0.227). LC3 expression in pancreatic cancer tissues with PNI was significantly higher than that in pancreatic cancer tissues without PNI (Table 1).

Relationship between pancreatic cancer cell autophagy and perineural invasion and clinicopathological features

LC3 expression was associated with lymph node metastasis (P<0.05). LC3 expression was not related to sex, gender, tumor location, tumor size, histological grade, clinical stage, vascular invasion, or diabetes mellitus status. PNI was related to lymph node metastasis, pancreatitis and CA19-9 levels (P<0.05) and was not related to sex, age, tumor location, tumor size, histological grade, clinical stage, vascular invasion, or diabetes mellitus status (Table 2).

Logistic regression multivariate analysis of LC3 expression and perineural invasion

The clinical pathological factors possibly associated with LC3 expression and PNI were evaluated by multivariate analysis using a logistic regression model. The clinical pathological factors included LC3 expression and nerve infiltration, age, tumor site, tumor size, histological grade, clinical stage, vascular invasion, lymph node metastasis, diabetes, pancreatitis, and preoperative CA19-9 level. The results showed that LC3 expression, lymph node metastasis, pancreatitis and CA19-9 level were the factors that influenced the occurrence of PNI, which was an independent factor affecting LC3 expression (Tables 3 and 4).

Survival analysis

According to LC3 expression, patients were divided into high-expression or low-expression groups. The overall survival rate of the low-expression group was better than that of the high-expression group, and the risk of death was 2.78-times higher in the LC3 high-expression group than that in the low-expression group. This difference between the two groups was significant (Fig. 2A). The patients were also divided into a nerve-invasion group and a no-nerve-invasion group according to whether there was nerve infiltration. The overall survival rate of the patients without nerve invasion was better than that of the nerve-invasion group. The risk of death was 2.93-times greater in the PNI-positive group than that in the PNI-negative group, and the difference between the two groups was significant (Fig. 2B). Univariate analysis showed that the CA19-9 level, PNI and LC3 expression influenced the prognosis (Table 5). A factor of $P<0.2$ was added into the COX risk regression model. Multivariate analysis was performed using the stepwise conditional method. The results showed that PNI and LC3 expression were independent prognostic factors that influenced pancreatic cancer (Table 6).

Discussions

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.

This study found that high expression of LC3 was present in the cancer nests around the nerve infiltration, consistent with the discovery of Yang[7]. 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.

There is dissidence in the correlation between PNI and lymph node metastasis[26-28]. This study showed that PNI was associated with lymph node metastasis, pancreatitis, and CA19-9 levels (P<0.05), while it had no association with sex, age, tumor location, tumor size, histological grade, clinical stage, vascular invasion or diabetes mellitus. LC3 was related to lymph node metastasis but no other clinicopathological features. Multivariate analysis of logistic regression also showed that LC3, lymph node metastasis, pancreatitis and CA19-9 levels were the factors that influenced PNI, while PNI was an independent factor affecting LC3 expression. Tanaka, A[26], Duraker N[28] , etc., believe that lymph node metastasis can promote PNI. The cancer cells in lymph node metastases can form a lymphatic satellite around the nerve and then break through the nerve membrane; then, nerve invasion occurs. This study is consistent with results suggesting the need for regional lymph node dissection during surgical treatment[27]**.** Through this study, we can conclude that high LC3 expression, lymph node metastasis, pancreatitis and CA19-9 levels usually indicate the possibility of nerve invasion. Gender, age, tumor site, tumor size, histological grade, clinical stage, vascular invasion, and diabetes are not effective indicators of neural invasion and autophagy and cannot be used as a determinant of resection of the peripancreatic nerve during surgery.

The overall survival rates of the LC3 high-expression group and the nerve-invasion group were significantly lower than those of the low-expression group and the no-nerve-invasion group. Univariate analysis showed that the level of CA19-9, PNI and autophagy were associated with prognostic factors. Multivariate analysis showed that PNI and high LC3

expression were independent prognostic factors in pancreatic cancer patients. 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.

In summary, autophagy and PNI of pancreatic cancer cells are independent risk factors for adverse prognosis. There is a significant correlation between them, and there may be a pathway between them through which they interact with each other to promote the malignant progression of pancreatic cancer. How to control the role of autophagy in PNI of pancreatic cancer and then improve cancer prognosis requires further studies into the molecular mechanisms involved.

FULL TEXT GUID

Research background

Pancreatic cancer is a malignant tumor with a poor prognosis that has almost equal mortality and morbidity in patients. At the time of diagnosis, most pancreatic cancer patients have distant metastases due to early occult symptoms, a lack of effective screening, and perineural growth characteristics. The incidence of perineural invasion (PNI) in pancreatic cancer is 80-100% and is an important factor leading to postoperative pancreatic cancer recurrence. PNI evaluation of pancreatic cancer can predict disease recurrence and prognosis after surgery. However, the pathogenesis of PNI has not yet been defined .Autophagy, as a mechanism of anoikis avoidance in pancreatic cancer, is closely related to the survival of pancreatic cancer cells. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a typical marker of autophagy. LC3 labeling has been used to evaluate autophagy, and high levels of LC3 expression have been found in pancreatic cancer cells. In addition, another previous study showed that pancreatic cancer cells with PNI have higher levels of autophagy. No study has examined the relationship between autophagy and PNI in pancreatic cancer cells.

Research motivation

The relationship between autophagy and perineural invasion (PNI) was explored for the first time in pancreatic cancer. Pancreatic cancer PNI is related to LC3 expression-determined autophagy. PNI and LC3 expression were independent prognostic factors in pancreatic cancer. There might be a special association between autophagy and PNI, contributing to pancreatic cancer progression. This study might provide a new insight for the mechanism of PNI in pancreatic cancer.

Research objectives

This study focused on the relationship between pancreatic cancer cell autophagy and PNI, clinicopathological features and prognosis . We found that autophagy and PNI of pancreatic cancer cells are independent risk factors for adverse prognosis. There is a significant correlation between them, and there may be a pathway between them through which they interact with each other to promote the malignant progression of pancreatic cancer. Controlling the role of autophagy in PNI of pancreatic cancer may improve cancer prognosis ,which requires further studies into the molecular mechanisms involved.

Research methods

Clinical and pathological data were retrospectively collected from 109 patients with pancreatic ductal adenocarcinoma who underwent resection at the First Affiliated Hospital of Zhengzhou University from January 2011 to August 2016. 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. The correlations among LC3 expression, perineural invasion, and clinical pathological features in pancreatic cancer were analyzed. The patients were followed up for further survival analysis.

Research results

In this study, we found that LC3, lymph node metastasis, pancreatitis, and CA199 level were factors that influenced neural invasion, whereas only neural invasion itself was an independent factor of high LC3 expression. Perineural invasion and LC3 expression were independent risk factors for poor prognosis in pancreatic cancer. There is a significant correlation between them.

Research conclusions

This study focused on the relationship between pancreatic cancer cell autophagy and PNI, clinicopathological features and prognosis . We found that autophagy and PNI of pancreatic cancer cells are independent risk factors for adverse prognosis. There is a significant correlation between them, and there nust be a pathway between them through which they interact with each other to promote the malignant progression of pancreatic cancer. Controlling the role of autophagy in PNI of pancreatic cancer may improve cancer prognosis ,which requires further studies into the molecular mechanisms involved.

References

1 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* 2016;**66**:7-30 [PMID: 26742998 DOI: 10.3322/caac.21332]

2 **Zhang Y**, Dang C, Ma Q, Chen W, Nagata K. Predictors of systemic chemotherapy contraindication in pancreatic cancer patients with distant metastasis. *Journal of Modern Oncology* 2007;**54**:254-259 [PMID: 17419272]

3 **Hidalgo M.** Pancreatic cancer. *N Engl J Med* 2010;**362**:1605-1617 [PMID: 20427809 DOI: 10.1056/NEJMra0901557]

4 **Gillen S**, Schuster T, Meyer ZBC, Friess H, Kleeff J. Preoperative/Neoadjuvant Therapy in Pancreatic Cancer: A Systematic Review and Meta-analysis of Response and Resection Percentages. *PLoS Med* 2010;**7**:e1000267 [PMID:20422030DOI: 10.1371/journal.pmed.1000267]

5 **Zhou Y,** Zhou Q, Chen R. Pancreatic stellate cells promotes the perineural invasion in pancreatic cancer. *Med Hypotheses* 2012;**78**:811-813 [PMID: 22513235 DOI: 10.1016/j.mehy.2012.03.017]

6 **Brech A,** Ahlquist T, Lothe RA, Stenmark H. Autophagy in tumour suppression and promotion. *Mol Oncol* 2009;**3**:366-375 [PMID: 19559660 DOI: 10.1016/j.molonc.2009.05.007]

7 **Yang S,** Wang X, Contino G, Liesa M, Sahin E, Ying H, Bause A, Li Y, Stommel JM, Dell'Antonio G. Pancreatic cancers require autophagy for tumor growth. *Genes Dev* 2011;**25**:717-729 [PMID: 21406549 DOI: 10.1101/gad.2016111]

8 **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]

9 **Zhu Z,** Friess H, Dimola FF, Zimmermann A, Graber HU, Korc M, Büchler MW. Nerve growth factor expression correlates with perineural invasion and pain in human pancreatic cancer. *J Clin Oncol* 1999;**17**:2419-2419 [PMID: 10561305 DOI: 10.1200/JCO.1999.17.8.2419]

10 **Moschidis A,** Papageorgiou A, Atmatzidis K, Tsalis K, Moschidis E, Livanis J, Chrysogelou E, Mourelatos D, Tsavdaridis D, Harlaftis N. Synergistic Antitumor Activity of Oxaliplatin in Combination with Gemcitabine in Pancreatic Tumor-Bearing Mice. *Chemotherapy (Los Angel)* 2007;**53**:153-159 [PMID: 17347561 DOI: 10.1159/000100513]

11 **Takahashi H,** Akita H, Gotoh K, Kobayashi S, Marubashi S, Miyoshi N, Sugimura K, Motoori M, Kishi K, Noura S. Preoperative gemcitabine-based chemoradiation therapy for pancreatic ductal adenocarcinoma of the body and tail: impact of splenic vessels involvement on operative outcome and pattern of recurrence. *Surgery* 2015;**157**:484-495 [PMID: 25444512 DOI: 10.1016/j.surg.2014.09.022]

12 **Ryan DP,** Hong TS, Bardeesy N. Pancreatic adenocarcinoma. *N Engl J Med* 2014;**371**:1039-1039 [PMID: 25207767 DOI: 10.1056/NEJMra1404198]

13 **Lee MG,** Lee SH, Lee SJ, Lee YS, Hwang JH, Ryu JK, Kim YT, Kim DU, Woo SM. 5-Fluorouracil/Leucovorin Combined with Irinotecan and Oxaliplatin (FOLFIRINOX) as Second-Line Chemotherapy in Patients with Advanced Pancreatic Cancer Who Have Progressed on Gemcitabine-Based Therapy. *Chemotherapy (Los Angel)* 2013;**59**:273-279 [PMID: 24457620 DOI: 10.1159/000356158]

14 **Agarwal JP,** Jain S, Gupta T, Tiwari M, Laskar SG, Dinshaw KA, Chaturvedi P, D'Cruz AK, Shrivastava SK. Intraoral adenoid cystic carcinoma: prognostic factors and outcome. *Oral Oncol* 2008;**44**:986-993 [PMID: 18329324 DOI: 10.1016/j.oraloncology.2008.01.004]

15 **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: 21941281 DOI: 10.1038/nrc3131]

16 **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]

17 **Liu B,** Lu KY. Neural invasion in pancreatic carcinoma. *Hepatobiliary Pancreat Dis Int* 2002;**1**:469-469[PMID: 14607730]

18 **Cavel O,** Shomron O, Shabtay A, Vital J, Trejoleider L, Weizman N, Krelin Y, Fong Y, Wong RJ, Amit M. Endoneurial macrophages induce perineural invasion of pancreatic cancer cells by secretion of GDNF and activation of RET tyrosine kinase receptor. *Cancer Res* 2012;**72**:5733-5743-[PMID: 22971345 DOI: 10.1158/0008-5472.CAN-12-0764]

19 **Li X,** Ma G, Ma Q, Li W, Liu J, Han L, Duan W, Xu Q, Liu H, Wang Z. Neurotransmitter substance P mediates pancreatic cancer perineural invasion via NK-1R in cancer cells. *Mol Cancer Res* 2013;**11**:294-294 [PMID: 23345604 DOI: 10.1158/1541-7786.MCR-12-0609]

20 **Abiatari I,** Deoliveira T, Kerkadze V, Schwager C, Esposito I, Giese NA, Huber P, Bergman F, Abdollahi A, Friess H. Consensus transcriptome signature of perineural invasion in pancreatic carcinoma. *Mol Cancer Ther* 2009;**8**:1494-1504 [PMID: 19509238 DOI: 10.1158/1535-7163.MCT-08-0755]

21 **Terman A,** Dalen H, Eaton JW, Neuzil J, Brunk UT. Mitochondrial recycling and aging of cardiac myocytes: the role of autophagocytosis. *Exp Gerontol* 2003;**38**:863-876 [PMID: 12915208 DOI: 10.1016/S0531-5565(03)00114-1]

22 **Levine B,** Yuan J. Levine B, Yuan J. Autophagy in cell death: an innocent convict? *J Clin Invest* 2005;**115**:2679-2688 [PMID: 16200202 DOI: 10.1172/JCI26390]

23 **Michaud M,** Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, Shen S, Kepp O, Scoazec M, Mignot G. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science* 2011;**334**:1573-1577 [PMID: 22174255 DOI: 10.1126/science.1208347]

24 **Noman MZ,** Janji B, Kaminska B, Van MK, Pierson S, Przanowski P, Buart S, Berchem G, Romero P, Mamichouaib F. Blocking hypoxia-induced autophagy in tumors restores cytotoxic T-cell activity and promotes regression. *Cancer Res* 2011;**71**:5976-5986 [PMID: 21810913 DOI:10.1158/0008-5472.CAN-11-1094]

25 **Notte A,** Leclere L, Michiels C. Autophagy as a mediator of chemotherapy-induced cell death in cancer. *Biochem Pharmacol* 2011;**82**:427-434 [PMID: 21704023 DOI: 10.1016/j.bcp.2011.06.015]

26 **Tanaka A,** Matsumura E, Yosikawa H, Uchida T, Machidera N, Kubo R, Okuno K, Koh K, Watatani M, Yasutomi M. An evaluation of neural invasion in esophageal cancer. *Surg Today* 1998;**28**:873-878 [PMID: 9744393 DOI: 10.1007/s005950050245]

27 **Ayala GE,** Dai H, Ittmann M, Li R, Powell M, Frolov A, Wheeler TM, Thompson TC, Rowley D. Growth and survival mechanisms associated with perineural invasion in prostate cancer. *Cancer Res* 2004;**64**:6082-6090 [PMID: 15342391 DOI: 10.1158/0008-5472.CAN-04-0838]

28 **Ozaki H,** Hiraoka T, Mizumoto R, Matsuno S, Matsumoto Y, Nakayama T, Tsunoda T, Suzuki T, Monden M, Saitoh Y. The prognostic significance of lymph node metastasis and intrapancreatic perineural invasion in pancreatic

cancer after curative resection. *Surg Today* 1999;**29**:16-22 [PMID: 9934826 DOI: 10.1007/BF02482964]

29 **Schmukler E,** Grinboim E, Schokoroy S, Amir A, Wolfson E, Kloog Y, Pinkaskramarski R. Ras inhibition enhances autophagy, which partially protects cells from death. *Oncotarget* 2013;**4**:142-152 [PMID:23370967 DOI: 10.18632/oncotarget.703]

30 **Mathew R,** Karantza-Wadsworth V, White E. Role of Autophagy in Cancer. *Nat Rev Cancer* 2007;**7**:961–967 [PMID: 17972889 doi: 10.1038/nrc2254]

31 **Fujii S,** Mitsunaga S, Yamazaki M, Hasebe T, Ishii G, Kojima M, Kinoshita T, Ueno T, Esumi H, Ochiai A. Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. *Cancer Sci* 2008;**99**:1813-1813 [PMID: 18616529 DOI: 10.1111/j.1349-7006.2008.00893.x]

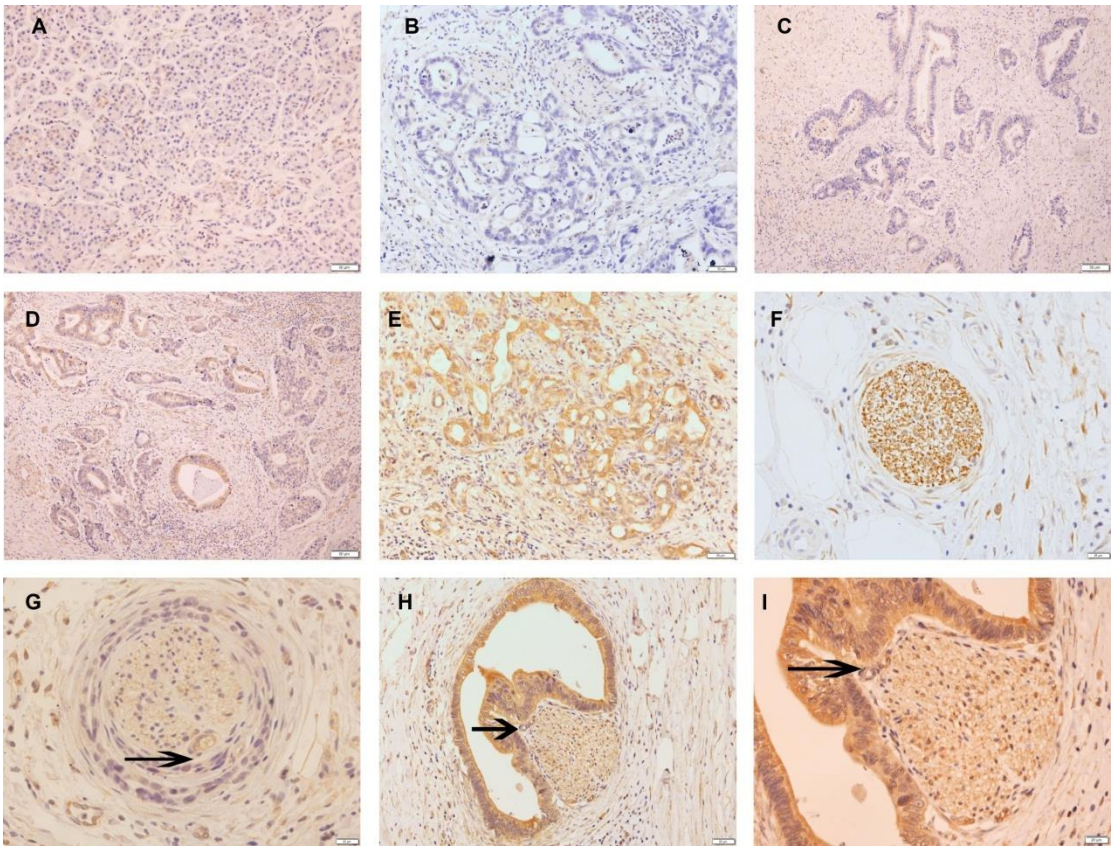

Fig. 1 Representative immunohistochemical results of LC3 and PNI. (A) Negative expression of LC3 in normal paraneoplastic pancreatic tissues (× 200); (B) Negative expression of LC3 in pancreatic cancer tissues(× 200); (C) Weakly positive expression of LC3 in pancreatic cancer tissues(× 200); (D) Moderately 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 represents 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).

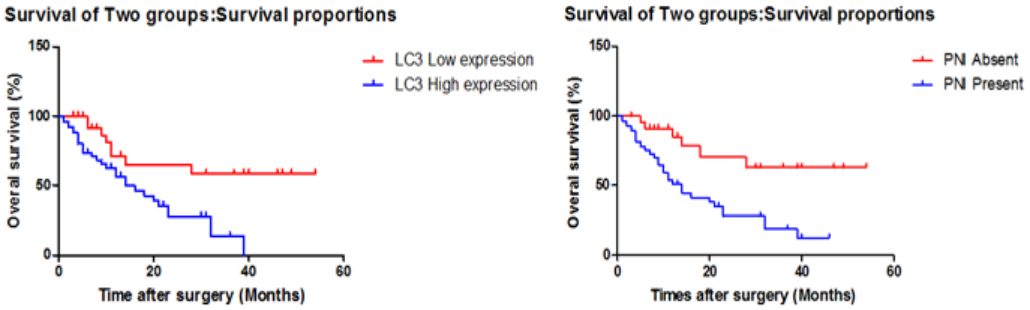

Fig 2 Kaplan–Meier estimates of overall survival in patients underwent radical surgery . (A) The overall survival rate of the LC3 low-expression group was better than that of the high-expression group (P<0.05). (B) The overall survival rate of the patients without nerve invasion group was better than that of those with nerve infiltration (P<0.05).

Table 1 Relationship between LC3 and perineural invasion expression in pancreatic cancer.

LC3	PNI		*P*	r
	Absent	Present		
low expression	18	22	0.018[a]	0.227
high expression	16	53		

[a] P <0.05, PNI Absent *vs* Present.

Table 2. Relationships of PNI and LC3 expression with clinicopathological features.

Parameters	*n*	PNI		*P*	LC3		*P*
		Absent	Present		Low expression	High expression	
Age (years)							
≤58	54	19	35	0.373	23	31	0.206
＞58	55	15	40		17	38	
Gender							
Male	61	15	46	0.093	26	35	0.148
Female	48	19	29		14	34	
Tumor location							
Head	66	17	49	0.129	28	38	0.124
Body/tail	43	17	26		12	31	
Tumor size							
≤2cm	18	6	12	0.83	8	10	0.455
>2cm	91	28	63		32	59	
Histologic grade							
Well or moderate	74	24	50	0.685	23	51	0.077
Poorly	35	10	25		17	18	
Vascular invasion							
Negative	83	26	57	0.957	31	52	0.801
Positive	26	8	18		9	17	
Lymph node metastasis							
Negative	36	17	19	0.011[a]	18	18	0.043[a]
Positive	73	17	56		22	51	
AJCC stage							
I+II	69	22	47	0.838	25	44	0.895
III+IV	40	12	28		15	25	

Pancreatitis							
Negative	30	15	15	0.009[a]	12	18	0.659
Positive	79	19	60		28	51	
Diabetes							
Negative	89	27	62	0.684	33	56	0.862
Positive	20	7	13		7	13	
CA19-9 level							
≤37U/ml	37	19	19	0.001[a]	13	24	0.808
>37 U/ml	72	15	57		27	45	

1 [a]*P* <0.05, LC3 Low expression *vs* High expression ; PNI Absent *vs* Present.

2

Table 3 Logistic regression multivariate analysis of perineural invasion with clinicopathological features in pancreatic cancer.

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; LC3: Microtubule-associated protein 1A/1B-light chain 3 ; PNI **:** Perineural invasion .

Table 4 Logistic regression multivariate analysis of LC3 expression with clinicopathological features in pancreatic cancer.

Parameters	Estimate, B	Standard error	Wald statistic	*P*-value	Odds ratio	95% CI
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 .

Table 5. Univariate analysis of survival in patients who underwent radical surgery.

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 .

Table 6 Multivariable analysis of survival in patients who underwent radical surgery.

Parameters	Hazard ratio	95% CI	*P*
PNI (Present *vs* Absent)	2.962	1.212-7.238	0.017[a]
LC3 (high *vs* Low)	2.491	1.107-5.608	0.027[a]

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