

Association of γ -aminobutyric acid type A receptor-associated protein with prognosis in patients after radical pancreatic cancer treatment

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Abstract

Purpose: To discuss the value and possible causes of γ -aminobutyric acid type A receptor-associated protein (GABARAP) expression and multiple clinicopathological factors in the prognosis of patients after radical pancreatic cancer treatment.

Methods: The pancreatic tissues of 76 pancreatic cancer patients after R0 resection were screened according to the criteria, and the expression levels of GABARAP were determined by using immunohistochemistry (MaxVision) to label the pancreatic cancer tissues and normal pancreatic tissues at the peri-cancer level in these two types of specimens, and the relationship between GABARAP and other factors and the disease-free and overall survival of patients after radical pancreatic cancer treatment was evaluated by single factor survival analysis and Cox regression analysis.

Results: The expression ratio of GABARAP in pancreatic cancer tissue was 55.26% (42/76), which was drastically higher than that in normal pancreatic tissue adjacent to cancer (21.42% (12/76)). In 76 patients, the middle relapse-free survival time after radical operation for pancreatic cancer was 12.8 months, and the middle overall survival time was 17.8 months. Forty-one patients found relapse on CT, and the recurrence site: local recurrence accounted for 17.1% (7/41) Distant metastasis accounted for 78.0% (32/41), and local recurrence with distant metastasis accounted for 4.9% (2/41). Among the three recurrence methods, there was no drastical difference in survival time from relapse to death ($P > 0.05$). Cox regression model evaluation showed that GABARAP ($P = 0.044$) and postoperative adjuvant chemotherapy ($P = 0.038$) were the overall survival of patients after radical pancreatic cancer Period independent prognostic indicators and both are protective factors for the prognosis of pancreatic cancer patients. Further data analysis found that postoperative chemotherapy had no drastical effect on the relapse-free survival and overall survival of 42 GABARAP-positive patients with cancer tissue, while in 34 GABARAP-negative patients, postoperative chemotherapy drastically increased the total patient Survival period ($P = 0.041$) but had no drastical effect on the patient's relapse-free survival period

Conclusion: The expression of GABARAP in pancreatic cancer tissues was drastically up-regulated, and patients with high expression of GABARAP in pancreatic cancer tissues had better prognosis, but had no drastical effect on the relapse-free survival of patients after radical operation of pancreatic cancer. The expression of GABARAP in pancreatic cancer tissues and Postoperative adjuvant chemotherapy is an independent indicator of patients' prognosis after radical pancreatic cancer resection. Both are protective factors. The high expression of GABARAP in pancreatic cancer may indicate that the adjuvant chemotherapy is low benefit.

Background

Pancreatic malignancy is a common malignancy known for its difficulty in detection, low resectable rate and high mortality rate, and although pancreatic cancer is statistically ranked 8th in incidence, it is ranked 5th in mortality^[1, 2], Only 2%-9% have a 5-year survival rate^[3], This is firstly because there is a lack of

effective screening methods to detect the disease. Second, the almost complete absence of symptoms associated with early pancreatic cancer, coupled with the abundance of blood vessels and lymphatic vessels in pancreatic tissues, thin peritoneal membrane and easy metastasis, resulting in the majority of pancreatic cancer patients were already stage IV tumors at the time of diagnosis, and only 20% of them were clinically viable for surgery^[4]. However, the prognosis of patients with pancreatic cancer that can be treated radically remains poor, with 5-year survival rates ranging from 13% to 25% in patients who are completely resected^[5]. Many scholars look for factors that affect the prognosis of pancreatic cancer patients after radical surgery and find that most pancreatic cancer chemotherapy is associated with poor outcomes^[6]. In recent years, many scholars have found that autophagy plays a very important regulatory role in the overall process of pancreatic cancer, and autophagy is closely related to the treatment and prognosis of pancreatic cancer, which makes it the current research hotspot of pancreatic cancer.

Cellular carcinoma is a process of unrestricted cell proliferation due to overactivation of proto-oncogenes or inactivation of oncogenes. The overgrowth of cancer cells and their incompatibility with neighboring normal cells is a predetermined characteristic of cancer cells, which require large amounts of material and energy. However, it has been shown that even after neovascularization, oxygen and glucose supplies are insufficient to support malignant proliferating cancer cells in locally advanced cancers.^[7, 8] Tumor hypoxia is used as a marker of poor prognosis^[9]. But tumors like pancreatic cancer, which have no blood supply, survive even when the blood supply is extremely low, and even become more malignant.^[10, 11] An alternative metabolic pathway is needed to provide energy when cancer cells are depleted of oxygen and glucose, and anaerobic glycolysis does not provide enough for growth.

Autophagy is the cell in the lack of nutrition, hormonal stimulation, oxygen deficiency, microbial invasion and environmental temperature changes and other external stimuli, or internal disorders such as organelle damage, abnormal protein accumulation, used to remove the cell's own harmful substances, synthetic proteins and production of ATP, in order to maintain intracellular homeostasis and improve cell survival. At the same time, autophagy is also a cell death mechanism, over-activated autophagy can lead to programmed cell death (also called type II programmed death), which is another programmed cell death pathway in addition to apoptosis. This is another programmed cell death pathway in addition to apoptosis. Autophagy can be stimulated by the overproliferation of tumor cells, increased cellular demand and environmental changes. Autophagy is caused by ULK1/2-Atg13-FIP200-Atg101 and the transmembrane autophagy protein Atg9A initiated, They were all recruited to the autophagosome formation site. Subsequently, they are recruited Beclin-1-Atg14-hp150-PIK3C3 to produce 1,2-palmitoylphosphatidylinositol-3-phosphate (PI3P). PI3P absorbs downstream effectors WIPI1/2 and DFCP1, and binds to autophagy proteins, reorganizes and expands the isolation membrane to form autophagosomes.^[12, 13] Autophagosomes are the core of autophagy, a dual membrane structure, and the autophagosomal membrane is formed with the assistance of GABARAP, a member of the Atg8 family of autophagy-related proteins, which helps to maintain ULK1 activation and substrate phosphorylation during the final stage of autophagy formation until the ULK1 complex dissociates and the autophagosome closes^[14], Interacts with NSF and TRPML3^[15], and priority recruitment PLEKHM1

through the LIR module. Autophagy-lysosomal fusion driven by HOPS recruitment, HOPS is mediated by GABARAP interaction with PLEKHM1^[16] and eventually allow the encapsulated substrate to be degraded by lysosomes in the autophagic vesicles. Autophagy, as a means of maintaining cellular homeostasis, can remove harmful substances from cells and reduce damage to DNA, thereby reducing gene mutation and cancer. Yang et al.^[17] found that mouse glandular vesicle cells were more susceptible to pancreatic ductal chemogenesis and precancerous lesions after inhibiting autophagy. This demonstrates that autophagy has an anticancer effect in early tumorigenesis.

Autophagy also plays a role in the development of pancreatic cancer. Not only is there an increased level of autophagy in pancreatic cancer tissues, but also an increased autophagy flux is found in the invaded nerve fibers and lymph nodes.^[18] And high levels of autophagy in the surrounding tissues of pancreatic cancer strongly suggest a poor prognosis for the patient^[19]. This reflects the carcinogenic role of autophagy in the development of pancreatic cancer.

However, when autophagy is overactivated, the degradation of large amounts of macromolecules, organelles, etc. can also lead to programmed cell death, which is also a way to inhibit pancreatic cancer cells. It has been shown that therapeutic measures such as cisplatin and ionizing radiation can activate autophagy and induce autophagic cell death in pancreatic cancer cells.^[20] Triptolide has also been found to induce up-regulation of autophagy levels and autophagic death in pancreatic cancer cells.^[21]

The mammalian autophagy-associated protein Atg8 family includes two subfamilies with at least seven proteins: MAP1LC3A, MAP1LC3B, MAP1LC3C (LC3C), GABARAP, GABARAPlike1 (GABARAPL1/gec1) and GABARAPlike 2 (GABARAPL2/GATE16). They perform three functions during autophagy: first, the whole process of expansion and closure of the autophagic cell membrane; second, as substrate receptors recruiting cytoplasmic substrates through the LIR (LC3 interaction region) base sequence^[22]; Third, as an adapter for signaling and transporting proteins to autophagosomes and for autophagy mechanisms^[23, 24]. Among them, GABARAP interacts with ULK1, an important mediator of autophagy formation, and assembles the ULK complex to form autophagosomes, and specifically recruits PLEKHM1 in the final stage, and mediates the recruitment of HOPS and drives the autophagy-lysosome fusion.^[16] Selective knockdown of GABARAPs significantly reduces the isolation of cytoplasmic material during autophagy (by approximately 80%)^[16], and lead to a larger autophagy hoard^[25], This suggests that GABARAPs play an indispensable and important role in macroautophagy, particularly autophagosomal-lysosomal fusion^[16]. Therefore, the level of GABARAP expression also reflects the level of autophagy in the cells. In fact, many studies have shown that pancreatic cancer cells have higher levels of autophagy than normal pancreatic tissue., Yang et al.^[18] The expression of LC3-II, which represents autophagic flux, and Atg7, an autophagy-associated protein, were observed to be significantly higher in eight pancreatic cancer cell lines than in normal pancreatic ductal cells, suggesting that increased autophagy is an acquired change and that autophagy promotes pancreatic cancer growth by blocking the accumulation of toxic levels of reactive oxygen species (ROS) and providing substances to maintain oxidative phosphorylation. These require the involvement of GABARAP, which is absent from the formation of autophagic vesicles that are

unable to degrade and provide the cells with substances and energy to remove harmful substances. In addition, GABARAP was found to be present not only on the bilayer membrane of autophagosomes, but also in Golgi bodies, pericentromeric substances and centromeric satellites.^[13, 26] This is also one of the mechanisms by which cells can respond rapidly to external stimuli. The presence of GABARAP pools in the Golgi complex and centrosomes laterally responds to the dependence of cellular autophagy on GABARAP and the role of GABARAP in autophagy.

In addition, it has been shown that GABARAP is a tumor suppressor gene. The mRNA and protein levels of GABARAP are lower in primary breast cancer tissues compared to normal breast tissues, and ectopic expression of the GABARAP gene in low-expressing breast cancer cell lines reduces tumor growth rate^[27]. This tumor suppressive property of GABARAP can be mediated through direct effects on autophagy and indirectly through the control of the translocation of receptors such as epidermal growth factor (EGFR)^[28, 29].

However, there are contrary reports that GABARAP acts as a tumor enhancer in vivo and that intact GABARAP function in host animals facilitates the growth of inoculated homozygous melanoma cells, which appears to be associated with inhibition of apoptosis and anti-tumor immune response^[30]. Their experiments showed that tumor formation mediated by the carcinogen DMBA was inhibited in GABARAP knockout mice, and the immune response was enhanced by stimulation of macrophage and lymphocyte inflammatory factors such as IL-1 β , IL-6, IL-2 and IFN- γ , which were not degraded by GABARAP-deficient, mitochondrial and other damaged organelles. Gene expression profiling of mammary glands showed significantly elevated levels of apoptosis-inducing genes and oncogenes Xaf1 in GABARAP knockout mice. Up-regulation of apoptosis-inducing, cell death and cell cycle inhibitor genes in the mammary gland was triggered by the use of the oncogenic chemical DMBA.^[30] Finally, tumor growth of B16 melanoma cells was inhibited after subcutaneous inoculation in GABARAP-deficient mice^[30]. This suggests that GABARAP expression in the tumor environment favors inhibition of tumor cell survival.

It has been argued that^[31], Combined surgery, radiotherapy and chemotherapy cannot change the genetic evolution of cancer cells and their biological behavior, so the efficacy of pancreatic cancer treatment is not significant. Further study of the mechanism of pancreatic cancer in its occurrence, development and therapeutic resistance from the microscopic perspective of genes and proteins may better explain the poor prognosis of pancreatic cancer patients, and thus improve the current status of pancreatic cancer diagnosis and treatment and provide theoretical basis and new treatment ideas for pancreatic cancer treatment in the future. Therefore, this study aims to discuss the role of autophagy in pancreatic cancer by exploring the relationship between GABARAP expression levels in pancreatic cancer tissues and patients' prognosis after radical treatment of pancreatic cancer.

Method

I. Material

1. Source of cases

Select pancreatic cancer patients from Huadong Hospital Affiliated to Fudan University, and their post-operative specimens from radical resection were collected from January 1, 2016 to December 31, 2018 in the pathology department, and the pathology of the specimens was pancreatic ductal adenocarcinoma. The inclusion criteria were patients with radical pancreatic cancer resection, negative margins, no neoadjuvant chemotherapy before surgery, pathology of pancreatic ductal adenocarcinoma and non-peripheral death, and 76 patients were finally enrolled. There were 50 cases of male and 26 cases of female, aged between 29~84 years, with a median age of 65.7 ± 9.6 years and a median age of 66.5 years. The tumors were located at the head of the pancreas in 48 cases and at the tail of the pancreas in 28 cases, with radical pancreaticoduodenectomy and radical pancreatic tail plus splenectomy respectively. The pathologic classification was pancreatic ductal adenocarcinoma with 1 case of high differentiation, 63 cases of medium differentiation and 12 cases of low differentiation. According to the American Cancer Society (AJCC) criteria, the cases in this group were clinically staged: 6 cases of stage I, 57 cases of stage II, and 13 cases of stage III. There were 19 cases of vascular invasion. 56 cases of nerve invasion. 41 cases of lymph node metastasis. Preoperative CA19-9 was increased in 68 cases. There were 53 cases of open surgery and 23 cases of minimally invasive surgery. Postoperative adjuvant chemotherapy was given in 60 cases. Most of the patients were reviewed regularly every 3~6 months after surgery, and postoperative follow-up was conducted by telephone, WeChat or outpatient. The following variables were selected for analysis, including sex, age (<65 years or ≥ 65 years), tumor location, tumor stage, pathological classification, maximum tumor diameter, lymph node metastasis, vascular invasion, nerve invasion, preoperative CA19-9 level (≤ 37 U/ml or >37 U/ml), surgical method (open or minimally invasive), postoperative adjuvant chemotherapy and GABARAP expression..

2. Antibodies and reagents

- a. GABARAP mouse polyclonal antibody (NOVUS, USA) at a dilution of 1:200
- b. TS-0831 Dewaxing Solution (Fuzhou Maixin Biotechnology Development Co., Ltd.)
- c. TW-0821 buffer (Fuzhou Maixin Biotechnology Development Co., Ltd.)
- d. TT-0801 DAB dyeing solution (Fuzhou Maixin Biotechnology Development Co., Ltd.)
- e. DNS-0811 Immunohistochemical Antigen Repair Buffer (Fuzhou Maixin Biotechnology Development Co., Ltd.)

3. Major instruments

- a. 4°C refrigerator (China Haier refrigerator)
- b. Paraffin slicer (Leica, Germany)
- c. Pathological tissue bleaching and drying instrument (Changzhou Zhongwei Electronic Instrument Co., Ltd.)
- d. Electrothermal incubator 65 °C (Shanghai Jinghong Experimental Equipment Co., Ltd.)

- e. Automatic immunohistochemical stainer (Fuzhou Maixin Biotechnology Development Co., Ltd.)
- f. NIKON Y-THR (NIKON, Japan).

II. Method

1. Immunohistochemical staining (MaxVision).

1. According to the pathology file, select the wax blocks containing tumor tissue and the wax blocks containing normal pancreatic tissue, slice them separately, line them with HE stain, observe the presence of tumor tissue and normal pancreatic tissue under the microscope, and select the wax blocks with clear boundaries between tumor tissue and non-tumor tissue and rich tissue.
2. Slice the wax pieces and put the wax pieces gently into the hot water of a drift oven, make them float, scald them flat, then attach the wax pieces to the slides, wait for the water to dry, and put them in a 65°C electric oven for 1 hour.
3. Select the Lumatas automatic immunohistochemistry stainer with the staining program in the computer and print out the label with the label printer, which corresponds to the antibody template to be performed.
4. Place the printed label on one end of the finished slide and place the slide on the staining rack of the stainer.
5. Place the kit containing the dewaxing solution, primary antibody, secondary antibody and DAB on the rack.
6. Place the antigen repair solution, buffer, alcohol, and distilled water in the appropriate container of the instrument.
7. Enter the operating software of the instrument, scan the section label and reagent label, then the automatic immunohistochemistry stainer will complete the steps of dewaxing, hydration, repair, add primary antibody, add secondary antibody, DAB color development, etc., and remove the slide after operation.
8. Remove the slides and re-stain with hematoxylin: rinse with water, stain with hematoxylin for 10~30 seconds, and return to blue with PBS solution, ammonia or tap water.
9. Dehydration: put into 85% ethanol, 95% ethanol and anhydrous ethanol, soak for 3 minutes each.
10. Transparency: using xylene for transparency
11. Sealing: Sealing with a neutral resin
12. Mirror examination.

2. Determination of a positive result

The positive control was a positive slide for known pancreatic cancer and the negative control was a TW-0821 buffer instead of GABARAP mouse polyclonal antibody. The negative control was TW-0821 buffer instead of GABARAP mouse polyclonal antibody. The pancreatic cancer or paraneoplastic pancreatic cell cytoplasm was found to be positive for GABARAP. The section was observed under 400 times

microscope, combined with cell staining intensity analysis and calculated the ratio of the number of brown positive cells to the number of all cells in the visual field, at least 10 visual fields were calculated, and the comprehensive ratio > 25% was determined to be positive.

3. Statistical analysis

All the data in this study were analyzed using IBM SPSS Statistics 26 statistical software. The percentage of utilization and the mean \pm standard deviation ($\bar{x} \pm s$) were expressed as count and measure data, respectively. Kaplan-Meier one-factor analysis, Log-Rank test, Landmark analysis, Empower statistics, and multifactorial analysis were performed using time-based Cox regression with $P < 0.05$ as the standard for statistical significance.

Results

1. GABARAP expression and its association with various clinicopathological factors in patients

The positive rate of GABARAP in pancreatic cancer specimens was 55.26% (42/76) in 42 out of 76 patients, mainly expressed in the cytoplasm of pancreatic cancer cells, while the positive rate was 21.42% (12/76) in 12 cases in the control group of normal parapancreatic tissue (Figure 1 and Table 2). The positive expression rate of GABARAP was significantly higher in pancreatic cancer tissues than in normal parapancreatic tissues ($P < 0.001$). The cardinal correlation test (Table 1) found that GABARAP expression was not significantly correlated with sex, age (<65 years or ≥ 65 years), tumor location, TNM stage, pathological grade, maximum tumor diameter (<3cm or ≥ 3 cm), lymphatic invasion, vascular invasion, nerve invasion, preoperative CA19-9 level (>37U/ml), postoperative chemotherapy (P values >0.05).

2. Disease free survival and prognosis of patients after radical pancreatic cancer treatment

A total of 76 patients undergoing radical treatment for pancreatic cancer were included in this study, and 27 patients died as of December 31, 2019, with 1 case missing and 48 survivors. Recurrence was found in 41 patients according to the changes of type-b ultrasonic, tumor index and CT, and the site of recurrence was found by CT: local recurrence accounted for 17.1% (7/41), distant metastasis accounted for 78.0% (32/41), local recurrence with distant metastasis accounted for 4.9% (2/41), see Fig. 2. Patients had a median relapse-free survival of 12.8 months and the cumulative survival analysis is shown in Fig. 3. The relapse-free survival is shown in Fig. 4.

3. Relationship between GABARAP, clinicopathological factors and disease-free survival and overall survival in patients after radical pancreatic cancer treatment

The median disease-free survival was 12.9 months and the median overall survival was 22.0 months in 34 patients with GABARAP-positive pancreatic cancer, and the median disease-free survival was 11.8 months and the median overall survival was 17.4 months in 34 patients with negative GABARAP. One-factor K-M survival analysis showed that the GABARAP expression level was not significantly related to the disease-free survival of the patients. However, GABARAP expression level ($P = 0.015$, Figure 5) and

tumor site ($P=0.018$, Figure 6) were significantly associated with the overall survival of patients after pancreatic cancer radical treatment, and the overall survival of GABARAP-negative patients was significantly lower than GABARAP-positive patients. The results of one-factor K-M survival analysis were as follows (Table 3): age ($P=0.048$, Figure 7) and postoperative adjuvant chemotherapy ($P=0.018$, Figure 8) were also significantly associated with the overall survival of patients after radical pancreatic cancer treatment, while sex, TNM stage, maximum tumor diameter, vascular invasion, nerve invasion, lymph node invasion, degree of pathological differentiation, surgical modality and normal preoperative CA19-9 were not significantly associated with the overall survival of patients after radical pancreatic cancer treatment.

Among 42 patients with GABARAP-positive pancreatic cancer tissues, one-factor survival analysis showed no significant difference between clinicopathological factors and overall survival between chemotherapy and non-chemotherapy groups ($P > 0.05$), while among 34 patients with GABARAP-negative cancer tissues, one-factor analysis showed that tumor site ($P=0.044$, Figure 9) and adjuvant chemotherapy ($P=0.005$, Figure 10) were significantly associated with overall survival, but not with disease-free survival..

4. Results of Cox regression multifactor analysis

In the one-factor analysis of total survival, the survival curves of GABARAP were crossed recently between tumor site and cancer tissue. The time-dependent covariates were also calculated by Cox regression: the time-dependent covariates of GABARAP for tumor site and cancer tissue were Wald card-square = 0.314, $P=0.575$; Wald card-square = 0.297, $P=0.586$, both of them $P > 0.05$, indicating that the variables did not change with time, which satisfied the Cox regression model. Cox regression multivariate analysis results (Table 4) suggest that GABARAP ($P=0.044$, Figure 11) and postoperative adjuvant chemotherapy ($P=0.038$, Figure 12) are independent prognosis of overall survival for patients after radical pancreatic cancer. The indexes and the OR values of both are less than 1, suggesting that postoperative adjuvant chemotherapy and GABARAP expression are protective factors for the prognosis of pancreatic cancer patients. However, age and tumor location have no significant effect on patients after radical resection of pancreatic cancer.

Among 34 GABARAP-negative patients, Cox regression analysis showed (Table 5) that postoperative chemotherapy was associated with patients' overall survival ($P=0.041$, Figure 13) but not tumor site, suggesting that in GABARAP-negative patients, postoperative chemotherapy significantly improved overall survival.

Discussion

1. GABARAP

The human *GABARAP* gene is located on the posterior strand of chromosome 17, 17p13.1, with a gene size of 1.5kb. The gene encodes a GABA(A) receptor-associated protein and is expressed in the human

pancreas at a median gene level \approx RPKM=38.499 \pm 4.242 <https://www.ncbi.nlm.nih.gov/gene/11337>. GABARAP is less expressed in pancreatic tissues than in other human organs. Two major pathways involved in GABARAP have been identified: autophagy signaling pathway and neurotransmitter signaling pathway. Diseases associated with the *GABARAP* gene include Stiff-Person syndrome and amyotrophic lateral sclerosis 1 (ALS1), with associated pathways including autophagy and human cytomegalovirus infection. Genetic ontology (GO) annotations associated with this gene include microtubule protein binding and GABA(A) receptor binding. Important paralogous homologs of this gene is GABARAPL1.

GABA(A) receptor-associated protein \approx GABARAP \approx is a 14kDa-sized cytoplasmic protein that is highly positively charged at its N terminus and shares sequence similarity with light chain 3 of microtubule-associated proteins 1A and 1B. ^[32], GABA(A) receptors can be involved in the aggregation of GABA(A) receptors on the cytoskeleton through mediated interactions with the cytoskeleton, and are also involved in autophagy. During the initial phase of autophagy, GABARAP interacts with ULK1, the core mediator of autophagosome formation, and assembles the ULK complex to begin autophagosome formation. In the intermediate stage, GABARAP, which lipidizes to the outer surface and edges of the autophagosome, recruits core autophagy proteins through the LIR module to promote the extension of the autophagosome membrane. LIR-containing core autophagy components

ULK1, ULK2, ATG13, FIP200^[33], VPS34, ATG14, Beclin 1^[34], ATG2A and ATG2B^[35] also highlights the central role of the GABARAP protein. And ATG4B^[36] preferentially combine GABARAP. Recent studies using knock-in mutations of the LIR base sequence of ULK1 and recombinant ATG13 knockout cells with WT and LIR mutant constructs suggest that the binding of GABARAP to ULK1 and ATG13 is important for ULK1 activity and autophagosome formation. These interactions may act synergistically to achieve optimal activation of ULK1 ^[37]. Lipidized to closed autophagosomes LC3B or GABARAP, respectively, recruits essential proteins via LIR modules such as PLEKHM1 or FYVE \approx Fab-1 \approx YGL023 \approx Vps27 \approx EEA1 structured domain \approx and curly helical structural domain of protein 1 \approx FYCO1 \approx , JNK interacting protein 1 \approx JIP1 \approx Promote autophagy fusion or translocation ^[38]. The mechanism of this lipidation effect in autophagy closure is not fully understood, but appears to be GABARAP-specific, as depletion of all LC3 has no effect on starvation-induced autophagy flux, but depletion of all GABARAP inhibits this effect ^[12, 39]. In addition, GABARAP proteins can bridge some of the divisional pores left by the final phase of autophagosome closure to facilitate organelle closure, consistent with the idea that lipidized GABARAPL1 accumulates at sites of high curvature and membrane-to-membrane juxtaposition ^[40] \approx GABARAP specifically recruits PLEKHM1 in the final phase and mediates HOPS recruitment to drive autophagy-lysosomal fusion ^[16], In cells lacking all GABARAP proteins after knockdown, PLEKHM1 was not recruited at the membrane ^[41], Also leads to failure of mutual fusion between autophagosomes and lysosomes to degrade substrates that are encapsulated by autophagosomes ^[42]. The GABARAP subfamily is essential for the later stages of autophagy cell maturation ^[43]. Through its interaction with the reticulocyte receptor TEX264, it participates in the remodeling of the endoplasmic reticulum substructure domain into autophagosomes under nutritional stress and then fuses with lysosomes to achieve endoplasmic reticulum renewal ^[44]. In addition, GABARAP may produce phosphatidylinositol-4-phosphate (PtdIns4P)

by recruiting phosphatidylinositol 4-kinase 2- α (PI4K2A) into autophagosomes, and PtdIns4P may complete autophagosomal maturation by recruiting membrane docking and fusion elements, thereby promoting fusion of bilayer membrane vesicles with lysosomes.^[45] Using electron microscopy, PtdIns4P was found to be located on the cytoplasm, but not necessarily on the luminal lobules of the internal and external autophagosome membranes^[46]. In addition, PtdIns4P co-localized with the late endosome RAB7, the binding spouse of PLEKHM1, further indicating the important role of GABARAP in the coordination of autophagy-lysosomal fusion events.

GABARAP has a unique role and is indispensable in autophagy, and its interaction with the autophagy-related proteins ATG2A and ATG2B has recently been reported to be necessary for efficient autophagosome shutdown^[35]. Additional studies have shown that selective knockdown of GABARAPs leads to greater autophagosome hoarding^[25], and the initial slowdown in autophagy formation^[12], and significantly reduced the isolation of cytoplasmic material during autophagy (by approximately 80%)^[16]. This all suggests that GABARAPs play an indispensable and important role in macroautophagy, particularly autophagosomal-lysosomal fusion^[16]. This is in line with the experiments of Engedahl et al.^[47], Human ATG8 family siRNA-mediated knockdown experiments conducted by their group showed that cellular autophagosomes fuse with lysosomes in large numbers independently of LC3 but require GABARAP. Weidberg et al.^[25] It has also been suggested that the GABARAP subfamily is required for the late maturation of autophagy. In addition, GABARAP subfamily members also play an irreplaceable role in selective autophagy of the LC3 subfamily.^[48] Therefore, the high or low expression^[48] of GABARAP also reflects the high or low level of cellular autophagy.

Yang et al.^[18] The expression of autophagy-associated proteins LC3-II and Atg7 was significantly increased in pancreatic cancer cell lines, and the fusion of autophagosomes and lysosomes in pancreatic cancer cells was also observed by electron microscopy. This indicates that autophagy is highly activated in pancreatic cancer cells after tumor formation. In this study, the positive rate of GABARAP in 76 pancreatic cancer tissues was 55.26% and was mainly expressed in pancreatic tumor tissues, whereas the positive rate was only 21.42% in the control group of paraneoplastic normal pancreatic tissues, with a significant difference in GABARAP expression between the two groups ($P=0.001$). Among the 64 patients with GABARAP-negative paraneoplastic normal pancreatic tissue, 53.12% (34/64) and 46.88% (30/64) of patients had increased GABARAP expression after pancreatic cell carcinoma, respectively. This result also indicates that autophagy is highly activated after pancreatic cell carcinoma..

2. Prognosis and survival analysis of patients with pancreatic cancer after radical treatment

In this study, 27 patients died, 1 was missed, 48 were definite survivors, and the overall median survival was 17.8 months. There were 41 patients with recurrence detected by CT and the site of recurrence: local recurrence accounted for 17.1% (7/41), distant metastasis for 78.0% (32/41) and local recurrence with distant metastasis for 4.9% (2/41). The patient's median recurrence-free survival time was 12.8 months. Univariate analysis indicated that the tumor location and the expression of GABARAP in cancer tissues

had no significant difference in the overall survival of patients in the early stage (<8.8 months) (survival curve staggered), but showed significant differences after the overall survival >8.8 months, suggesting a significant long-term effect. However, because the overall survival time of 8.8 months is far less than the overall median survival time of 76 patients of 17.8 months, only 6 people (7.9%) in actual statistics, and the time-dependent Cox analysis shows that the two are in line with the risk ratio model, so it may be early. The presence of confounding factors is not statistically significant. Cox regression analysis showed that GABARAP ($P=0.044$) and postoperative adjuvant chemotherapy ($P=0.038$) were independent prognostic indicators of overall survival after radical treatment of pancreatic cancer and both were protective factors for the prognosis of patients with pancreatic cancer. However, in the cancer tissues of untreated pancreatic cancer patients, autophagy was observed by LC3 immunohistochemical labeling, and it was found that high expression of LC3 in pancreatic cancer peripheral tissues was positively associated with poor prognosis of patients.^[19] This is not quite the same as our study.

Radical pancreatic cancer remains by far the most promising cure for pancreatic cancer, but the prognosis of patients after radical therapy remains poor, and the insensitivity of pancreatic cancer to postoperative chemotherapy is particularly important among the many influencing factors.^[6] According to the Guidelines for the Comprehensive Treatment of Pancreatic Cancer (2018 edition), first-line chemotherapy agents for pancreatic cancer include gemcitabine (GEM) alone or in combination with fluorouracil analogs such as capecitabine, tegio, and 5-fluorouracil (5-FU)/formyltetrahydrofolate. It has been found that gemcitabine also activates autophagy and induces autophagic death in pancreatic cancer cells, and that larger doses of gemcitabine are required to achieve the same effect when added to autophagy inhibitors.^[20] Pardo et al.^[49] Gemcitabine was shown to induce vesicle membrane protein 1 (VMP1)-mediated autophagy and lead to apoptosis in pancreatic cancer cells in a pancreatic cancer cell line. Cannabinoids inhibit cell growth by inducing reactive oxygen species-mediated autophagic death, thereby sensitizing drug-resistant pancreatic cancer cells to gemcitabine^[50]. Gemcitabine or 5-FU may enhance autophagy, modulate chemotherapy tolerance, and induce pancreatic cancer cell death in combination with omeprazole^[51]. Yet there are scholars who have found that^[52], Autophagy is an important cause of insensitivity to 5-FU in pancreatic cancer cells, and inhibition of autophagy by chloroquine (CQ) or lysosomal photoinjury increases susceptibility to 5-FU, which may be related to the use of 5-FU alone.

Among the 42 patients with GABARAP-positive pancreatic cancer, single-factor survival analysis showed no significant difference between the clinicopathological factors and the overall survival of the chemotherapy group and the non-chemotherapy group ($P > 0.05$), while among the 34 patients with GABARAP-negative cancer, single-factor analysis showed that tumor site ($P=0.044$) and adjuvant chemotherapy ($P=0.005$) were significantly associated with overall survival. Since 76 patients underwent radical treatment for pancreatic cancer, and the prognosis of the GABARAP-positive group was significantly better than that of the negative group, we believe that chemotherapy drugs such as gemcitabine induced the autophagic death of pancreatic cancer cells that might be residual, which up-regulated the GABARAP level in the GABARAP-negative cancer cells and improved the prognosis. In

pancreatic cancer cells with high GABARAP expression (i.e. pancreatic cancer tissues with high autophagy level), the autophagy induced by chemotherapy was not significant (could not further increase the autophagy level to induce autophagic death) or resisted, which was not significantly different from that without chemotherapy, suggesting that adjuvant chemotherapy may not be effective in these patients. For the first result, considering that none of the patients had undergone preoperative neoadjuvant chemotherapy and the autophagy level of normal pancreatic tissue was not high, the autophagy of pancreatic cancer cells was mainly caused by the harsh environment around the pancreatic cancer cells, i.e., there was insufficient blood supply, the probability of distant metastasis of pancreatic cells through blood vessels was low, and there were few residual tumor cells after radical resection, so the overall survival of patients was long. We believe that the high expression of GABARAP in patients whose tumors can be radically resected in the early stage reflects the good prognosis of the patients.

In our study, other clinicopathological factors such as sex, TNM stage, tumor classification, tumor size, lymph node metastasis, vascular infiltration, preoperative CA19-9 level, surgical modality and degree of differentiation were not significantly associated with patient prognosis after radical pancreatic cancer treatment. However, this does not mean that these factors are not important to the prognosis of patients, it may be that the number of cases is too small or the follow-up time is insufficient, which needs further study..

In summary, GABARAP is mainly involved in autophagy in neuronal cells, but the positive rate of GABARAP in pancreatic cells is only 21.42% under normal conditions. Considering that GABARAP is closely related to cellular autophagy, the high expression of GABARAP reflects the activation of pancreatic cancer cell autophagy, which also indicates that pancreatic malignant tumor cells may have insufficient blood supply before surgery, and cancer cells have a low probability of metastasis through blood vessels, and the prognosis is better. In further analysis, we found that the effect of chemotherapy in patients with high GABARAP expression was less pronounced than that in patients with low GABARAP expression, which may be related to the mechanism of chemotherapy drug action and autophagy resistance.^[52] When the two are considered together, it may not be that autophagy itself is beneficial to the prognosis of patients (as a result of the poor chemotherapeutic effect of patients with elevated autophagy in this study and other previous studies, autophagy may be beneficial to the survival of cancer cells), but the poor survival environment of pancreatic cancer cells (starvation, hypoxia, activation of autophagy, etc.) as a result of elevated autophagy, low preoperative blood supply to cancer tissues, and low probability of distant metastasis of cancer cells through the circulatory system, resulting in a better prognosis of patients. In addition, the surgical approach has a significant impact on the disease-free survival of patients, and patients with minimally invasive surgical approach are more likely to relapse. At the same time, the Cox risk model suggests that adjuvant chemotherapy also plays an independent role in predicting the prognosis of patients after radical treatment of pancreatic cancer, and the median overall survival of patients was significantly improved with adjuvant chemotherapy.

Conclusion

- 1.GABARAP is highly expressed in pancreatic ductal adenocarcinoma tissue
- 2.In PDAC,GABARAP expression levels in pancreatic cancer tissues were significantly correlated with overall survival of patients, and the overall survival of patients with high expression was significantly higher than that of patients with low expression
- 3.In PDAC,GABARAP expression levels in pancreatic cancer tissues and postoperative adjuvant chemotherapy have independent value in assessing the prognosis of patients after radical pancreatic cancer treatment.
- 4.In PDAC,high GABARAP expression in pancreatic cancer tissues may be a predictor of poor adjuvant chemotherapy benefit in patients

Declarations

Competing interests

The authors declare that they have no competing interests

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

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Author's contribution

ZC, TWH, TYY, YZR, WW, WZKand WWYconducted pathological design and analysis, ZC conducted sample collection and wrote the manuscript. XL and GY were stained and read by immunohistochemistry. All the authors read and approved the manuscript.

Ethics approval and consent to participate

All tissue samples were obtained with patients writing consent and the study was approved by the ethical committee of Huadong Hosipital Affiliated to Fudan University and performed in accordance with the ethical guidelines of the Declaration of Helsinki.

Abbreviations

GABARAP	Gamma-Aminobutyric Acid Receptor-Associated Protein
ATP	Adenosinetriphosphate
Unc-51	unc-51 Serine/threonine-protein kinase unc-51 Uncoordinated protein 51
ULK1/2	Unc-51 Like Autophagy Activating Kinase 1/2
Atg	Autophagy-Related Protein
DMBA	Dimethylolbutanoic acid,[2,2-Bis(hydroxymethyl)butyric Acid]
FIP200	(Focal adhesion kinase,FAK),FAK-family interacting protein of 200 kDa
hp150	Chromatin assembly factor 1 subunit A CAF-I p150 hp150
PIK3C3	Phosphatidylinositol 3-kinase catalytic subunit type 3
PI3P	Phosphatidylinositol 3-phosphate,1,2-dipalmitoyl
WIPI1/2	WD Repeat Domain, Phosphoinositide Interacting 1/2
DFCP1	Double FYVE-containing protein 1
NSF	N-ethylmaleimide-sensitive fusion protein/Vesicle-fusing ATPase
TRPML3	Transient receptor potential channel mucolipin 3/ Mucolipin-3
LIR	LC3 interacting region

LC3	Microtubule-associated proteins 1A/1B light chain 3
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PLEKHM1	Pleckstrin Homology And RUN Domain Containing M member 1
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HOPS	homotypic fusion and protein sorting
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AJCC	American Joint Committee on Cancer
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DAB	Diaminobenzidine
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SPSS	Statistical Package for the Social Science
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TNM	Tumor Node Metastasis
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OR	Odds ratio
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RPKM	Reads Per Kilobase of exon model per Million mapped reads
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ALS1	Amyotrophic Lateral Sclerosis 1
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GO	Gene Ontology
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FYCO1	FYVE and coiled-coil domain-containing protein 1
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JIP1	C-Jun-amino-terminal kinase-interacting protein 1
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TEX264	Testis Expressed 264
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PI4K2A	Phosphatidylinositol 4-Kinase Type 2 Alpha
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PtdIns4P	Phosphatidylinositol-4-phosphate
RAB7	(Rat sarcoma,Ras),Ras-related protein Rab-7a
siRNA	SmallinterferingRNA
GEM	Gemcitabine
VMP1	Vacuole Membrane Protein 1
5-FU	5-fluorouracil
CQ	Chloroquine

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Tables

Table 1 Relationship between GABARPAP expression and clinicopathological parameters in pancreatic cancer tissue

Variable	No. of cases	GABARAP-positive cases(%)	Univariate <i>P</i>
Sex			
Females	26	13(50.0%)	0.506
Males	50	29(58.0%)	
Age(years)			
<65	34	19(55.9%)	0.922
≥65	42	23(54.8%)	
Tumor location			
Head	48	24(50.0%)	0.227
Body or Tail.	28	18(64.3%)	
TNM			
I	6	3(50.0%)	0.925*
II	57	31(54.4%)	
III	13	8(61.5%)	
Tumor size			
<3cm	18	10(55.6%)	0.977
≥3cm	58	32(55.2%)	
Vascular invasion			
No	57	33(57.9%)	0.424
Yes	19	9(47.4%)	
Neuroaggression			
No	20	9(45.0%)	0.282
Yes	56	33(58.9%)	
Lymph node metastasis			
No	35	18(51.4%)	0.534
Yes	41	24(58.5%)	
Degree of differentiation			
undifferentiated	12	9(75.0%)	0.162*
neutralization	63	33(52.4%)	

highly differentiated	1	0(0.00%)	
Level of the perioperative CA19-9			
Normal	8	5(62.6%)	0.663
Elevate	68	37(54.4%)	
Post-operative chemotherapy			
No	16	9(56.3%)	0.929
Yes	60	33(55.0%)	

*Fisher text

Table 2 Expression of GABARAP in pancreatic adenocarcinoma tissues and normal pancreatic tissues at the paracellular level

Group	No. of cases	No. of GABARAP positive(%)	No. of GABARAP negative.(%)
Pancreatic cancer tissue	76	42(55.26%)	34(44.74%)
Paraneoplastic normal pancreatic tissue	76	12(21.42%)	64(78.58%)

Cardinality test $\chi^2=25.850, P=0.001$

Table 3 One-factor survival analysis of survival after radical pancreatic cancer treatment

Variable	No. of cases	Median DFS (months)	Univariate <i>P</i>	Median OS(months)	Univariate <i>P</i>
Sex					
Females	26	10.8	0.913	18.9	0.683
Males	50	12.9		21.3	
Age(years)					
≤65	34	15.4	0.241	24.6	0.048
≥65	42	11.4		17.4	
Tumor location					
Head	48	12.6	0.251	18.6	0.018^a
Body or Tail	28	13.2		22.7	
TNM					
I	6	10.5	0.978	17.4	0.611
II	57	12.8		18.4	
III	13	16.3		22.8	
Tumor size					
≤3cm	18	12.1	0.543	18.1	0.509
≥3cm	58	12.8		19.8	
Vascular invasion					
No	57	12.9	0.356	22.6	0.505
Yes	19	11.4		18.4	
Neuroaggression					
No	20	12.4	0.140	23.7	0.597
Yes	56	12.9		19.1	
Lymph node invasion					
No	35	13.8	0.189	22.6	0.594
Yes	41	11.0		19.0	
Pathological differentiation					
low differentiation	12	13.9	0.369	25.1	0.478

mid differentiation	63	12.7		18.9	
high differentiation	1	33.3		33.3	
Preoperative level of CA19-9					
normal	8	16.6	0.750	19.2	0.912
elevate	68	12.6		19.7	
Postoperative adjuvant chemotherapy					
No	16	12.2	0.524	15.6	0.018
Yes	60	12.8		22.5	
GABARAP (cancerous tissue)					
negative	34	11.8	0.701	17.4	0.015^b
positive	42	12.9		22.0	

a Survival curves cross, overall survival period 8.8 month, Log-rank test P-value

b Survival curves cross, overall survival period 8.8 month, Log-rank test P-value

Table 4 Cox regression multifactorial analysis of risk factors for overall survival after radical pancreatic cancer treatment

Factors	OR-value	Exp(B)	P-value
Age ≥ 65y	1.697	0.529	0.216
Tumor site body and tail of pancreas	0.458	-0.781	0.125
Postoperative chemotherapy Yes	0.413	-0.885	0.038
GABARAP positive	0.440	-0.821	0.044

Table 5 Cox regression multifactorial analysis of risk factors for overall survival after radical pancreatic cancer treatment in 34 patients with GABARAP-negative cancer tissue

Factors	OR-value	Exp(B)	P-value
Tumor site☐body and tail of pancreas☐	0.317	-1.150	0.137
Postoperative chemotherapy☐Yes☐	0.340	-1.078	0.041

Figures

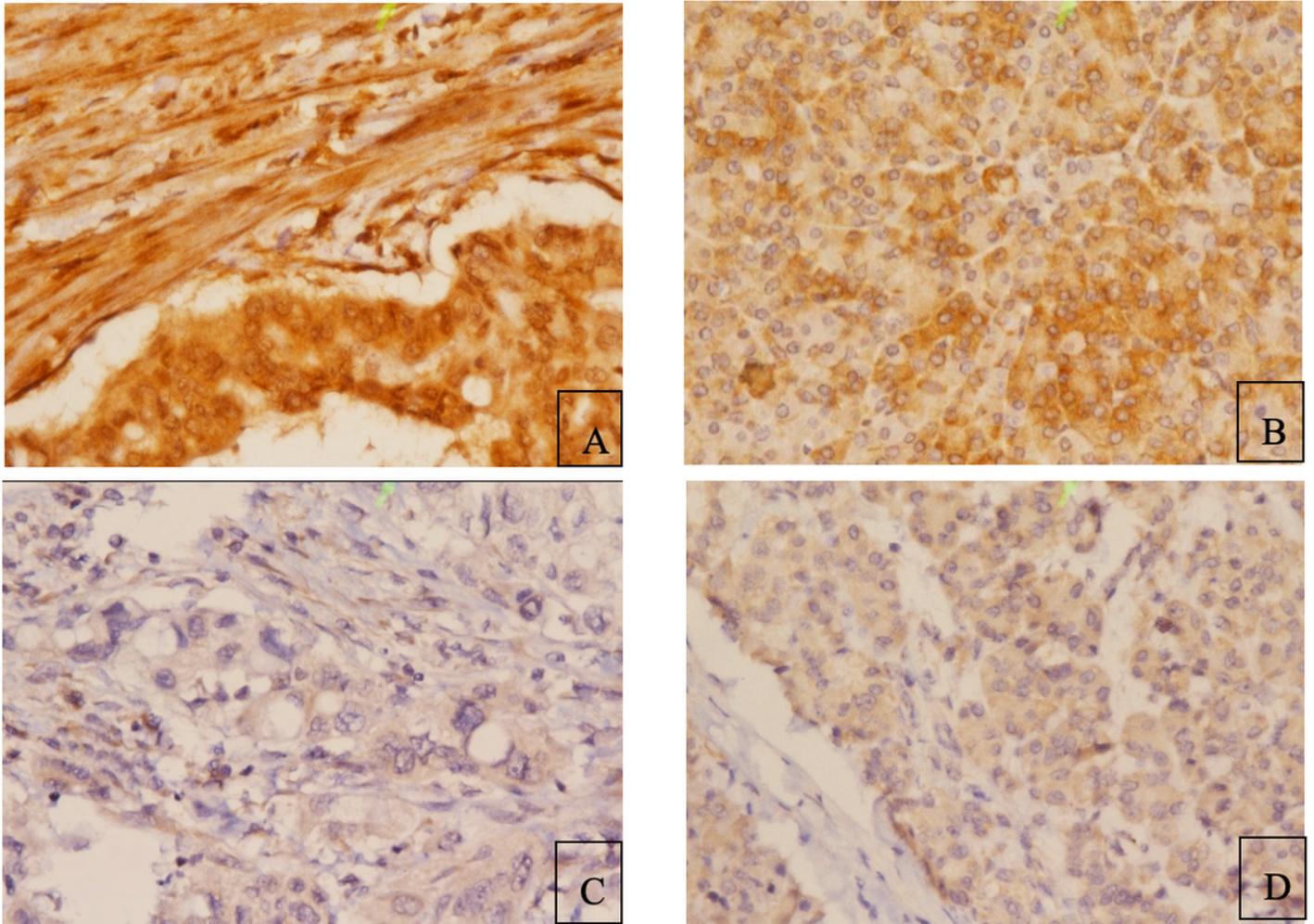


Figure 1

A: Immunohistochemically positive control staining for GABARAP in human pancreatic adenocarcinoma tissue B: Immunohistochemically positive results for GABARAP in paraneoplastic normal pancreatic tissue C: Immunohistochemically negative control staining for GABARAP in human pancreatic adenocarcinoma tissue D: Immunohistochemically negative results for GABARAP in paraneoplastic normal pancreatic tissue

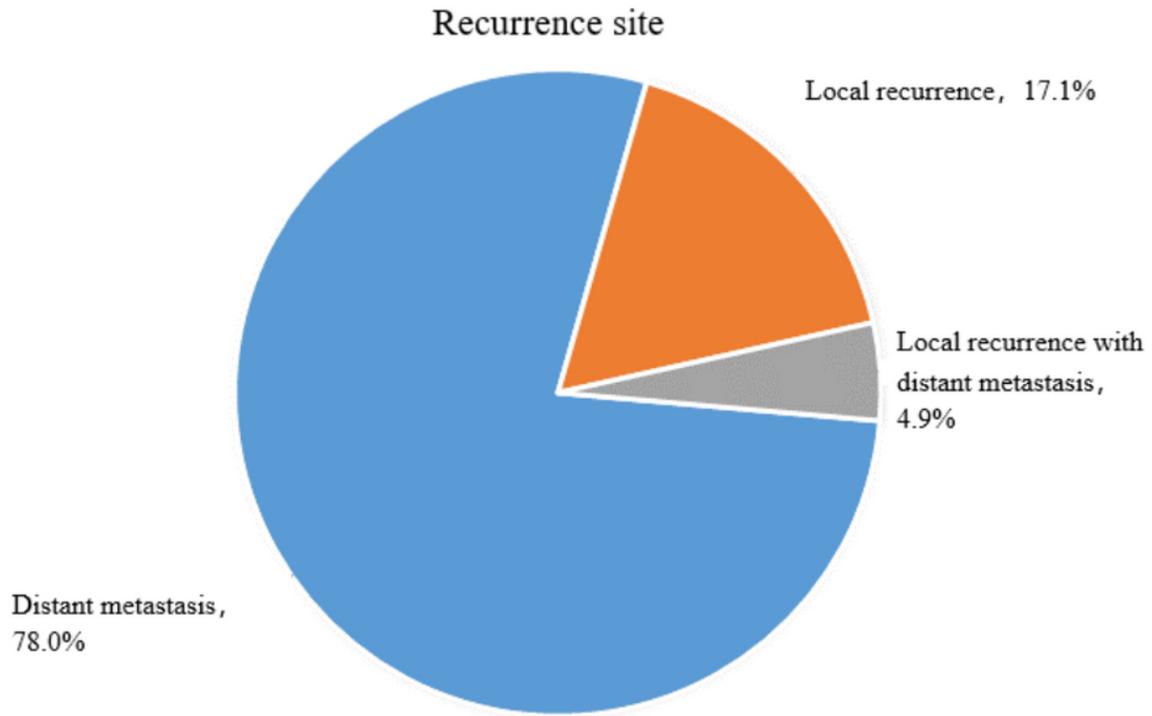


Figure 2

Recurrence site

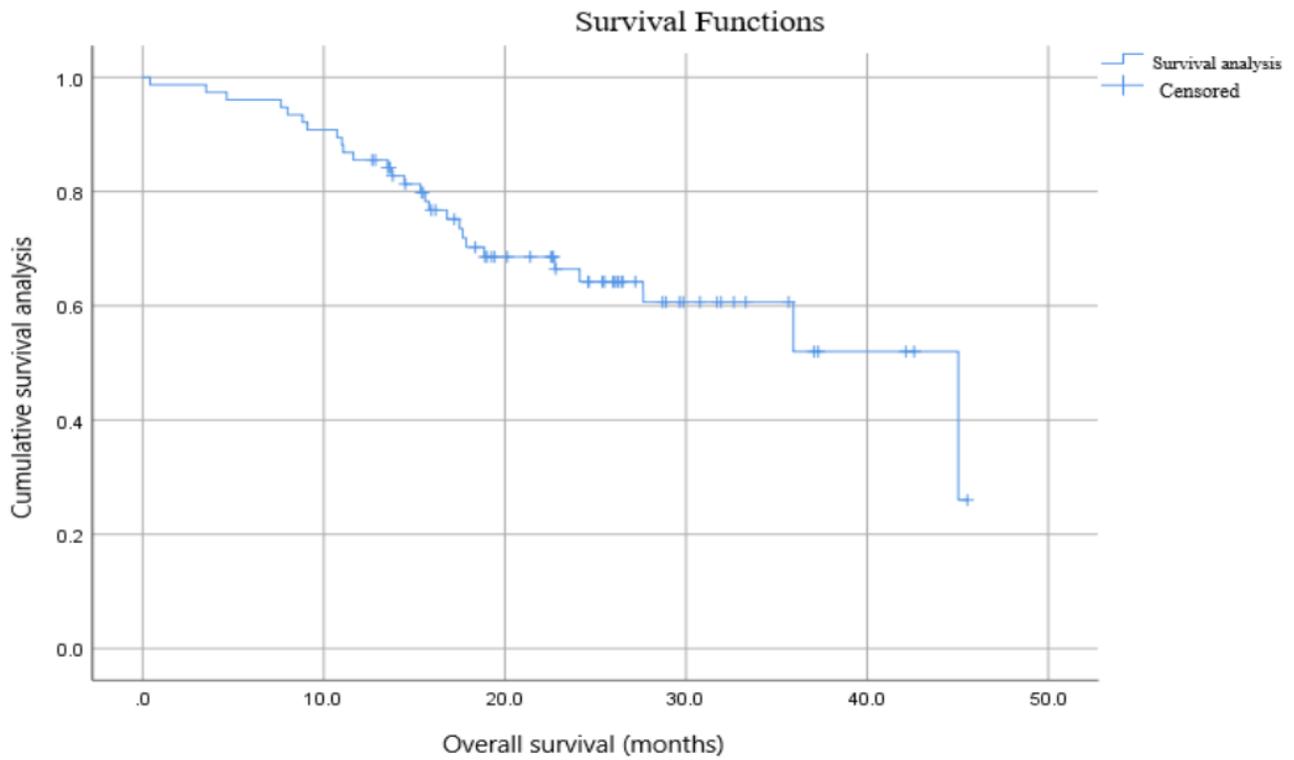


Figure 3

Overall survival of 76 patients

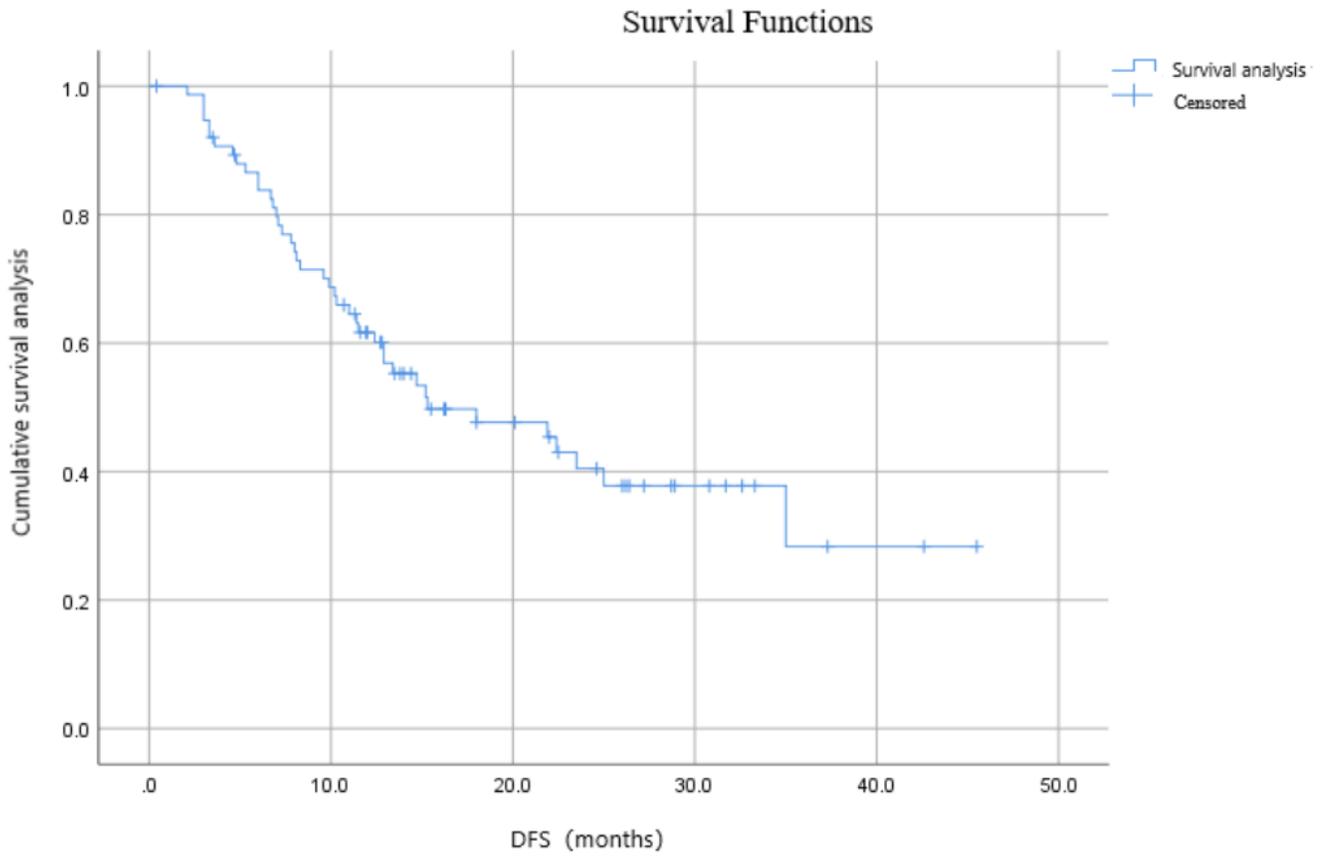


Figure 4

Disease-free survival of 76 patients

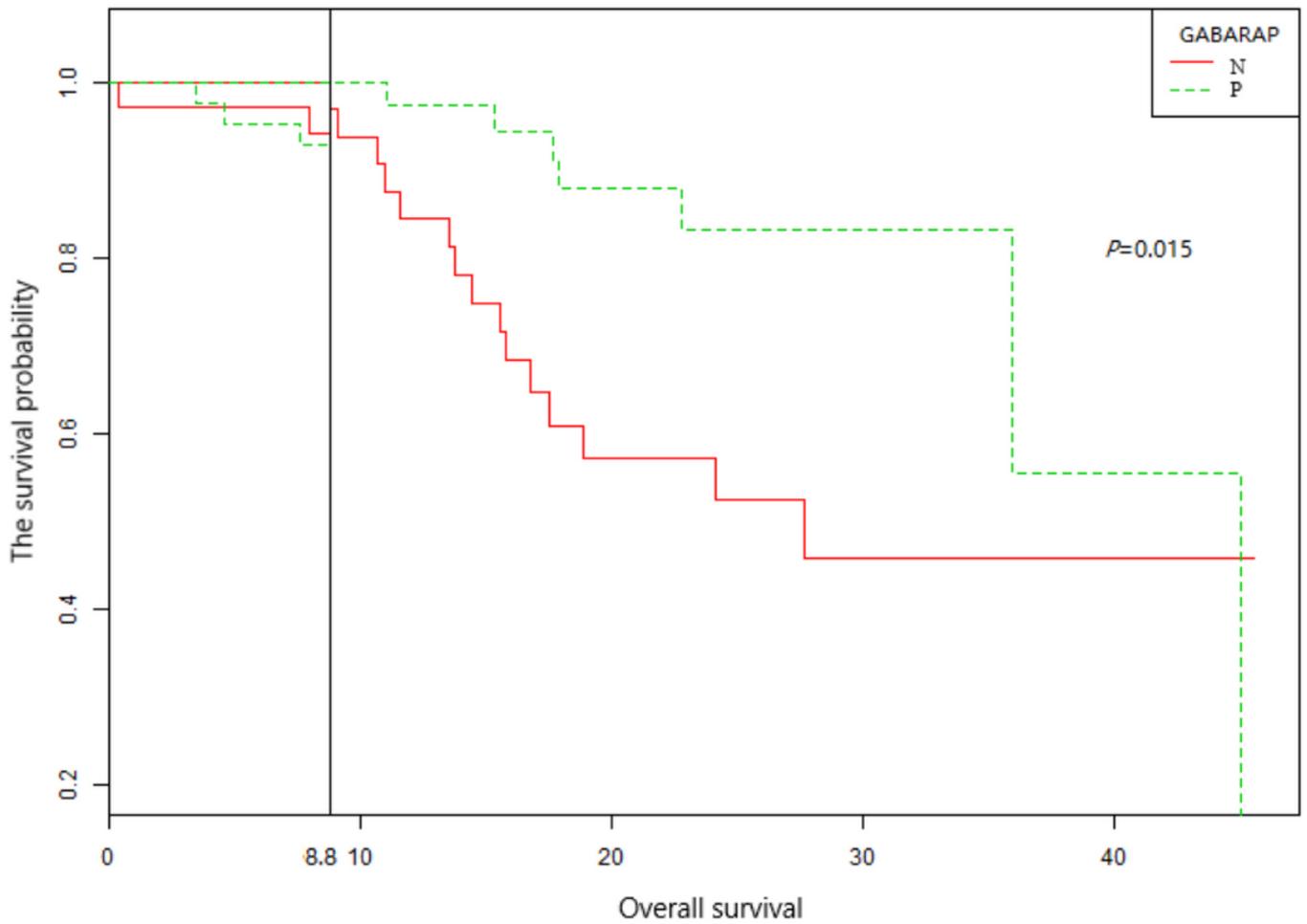


Figure 5

Relationship between GABARAP and overall survival in cancer tissue (N: negative P: positive)

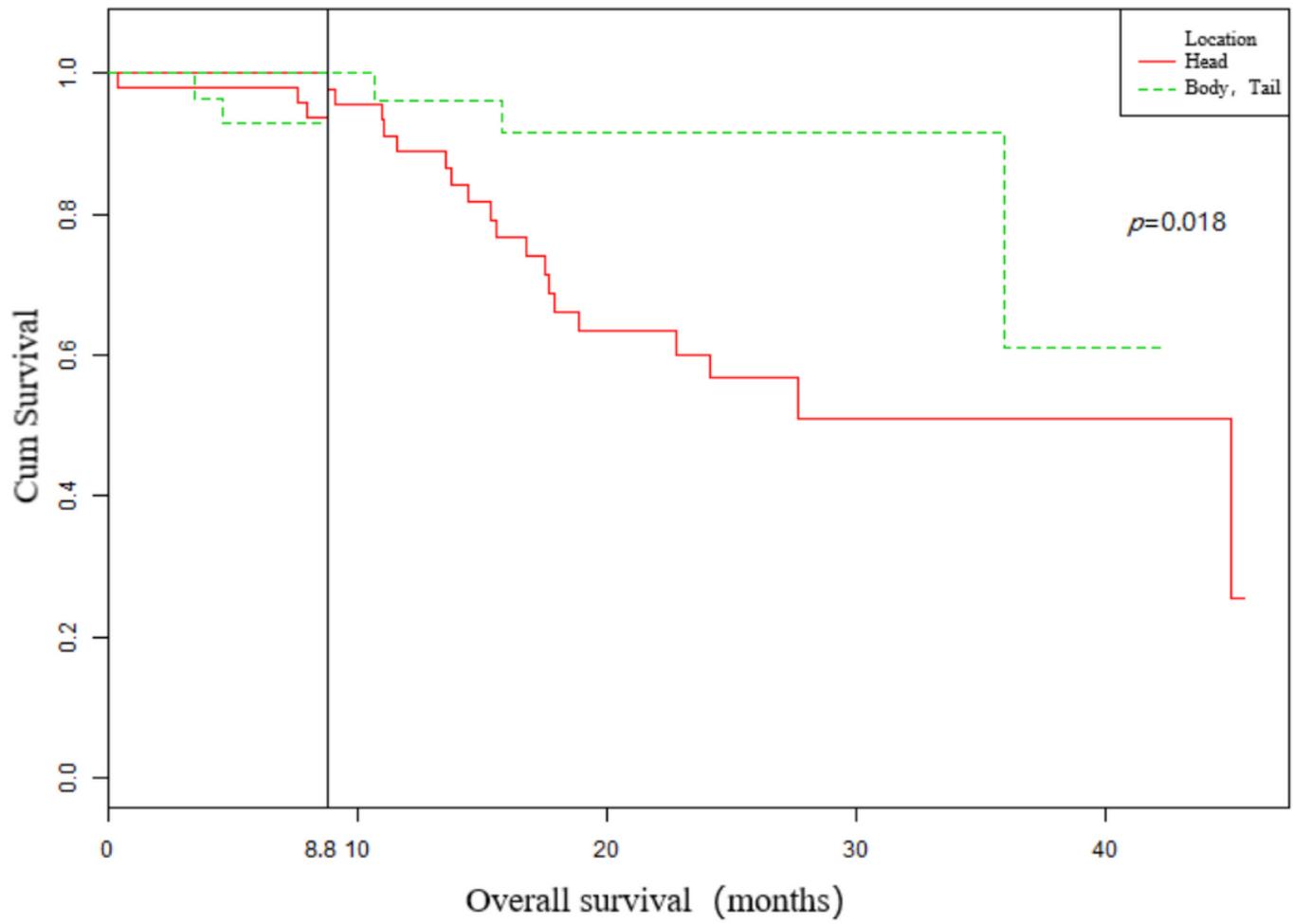


Figure 6

Tumor site versus overall patient survival

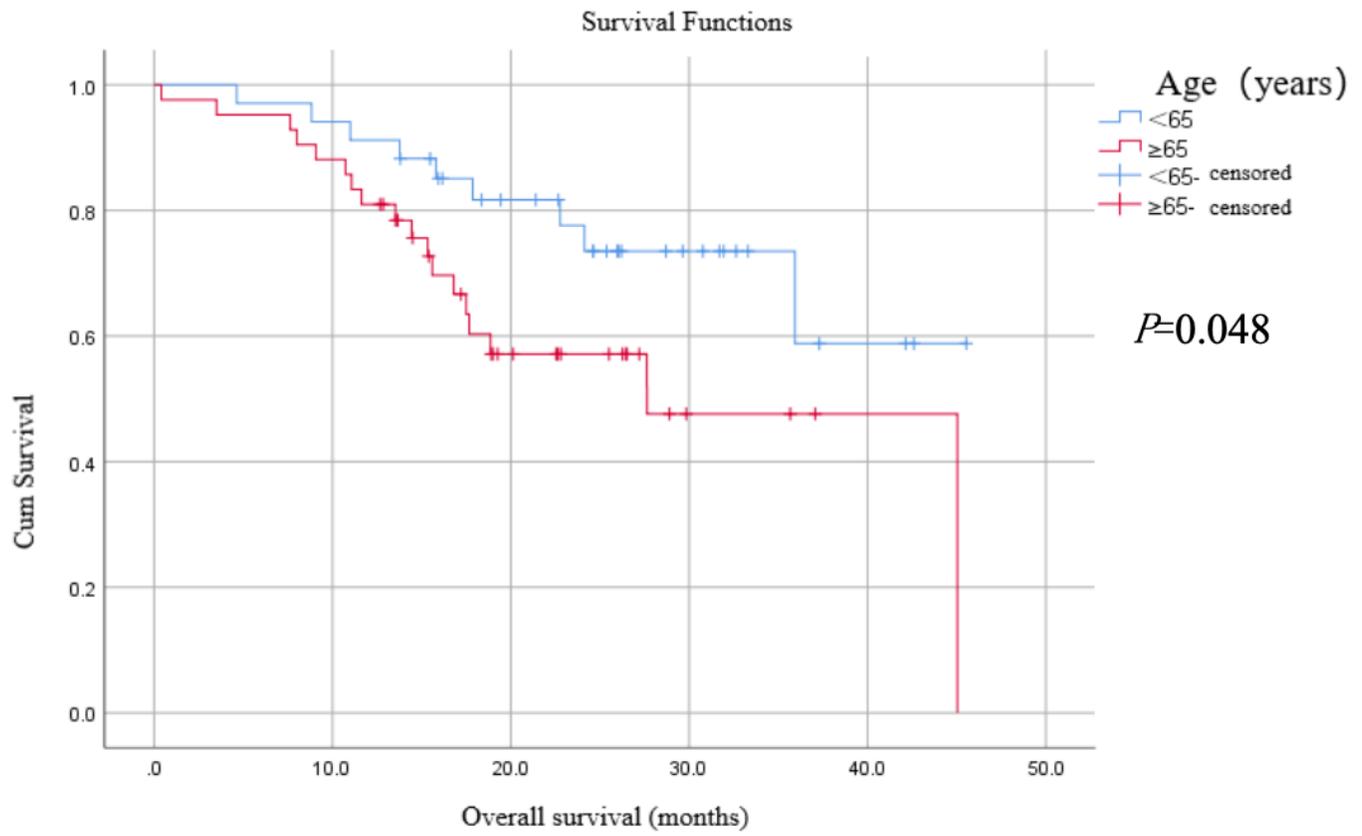


Figure 7

Relationship between age and overall patient survival

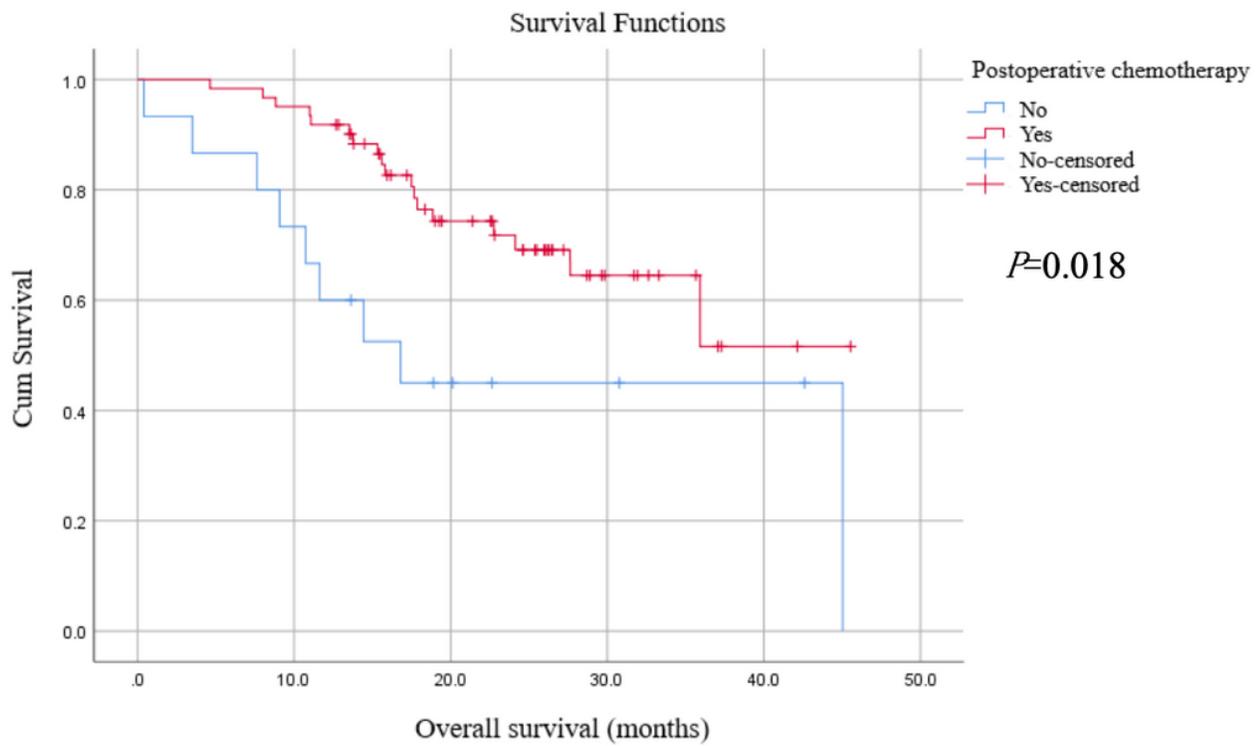


Figure 8

Relationship between postoperative adjuvant chemotherapy and overall patient survival

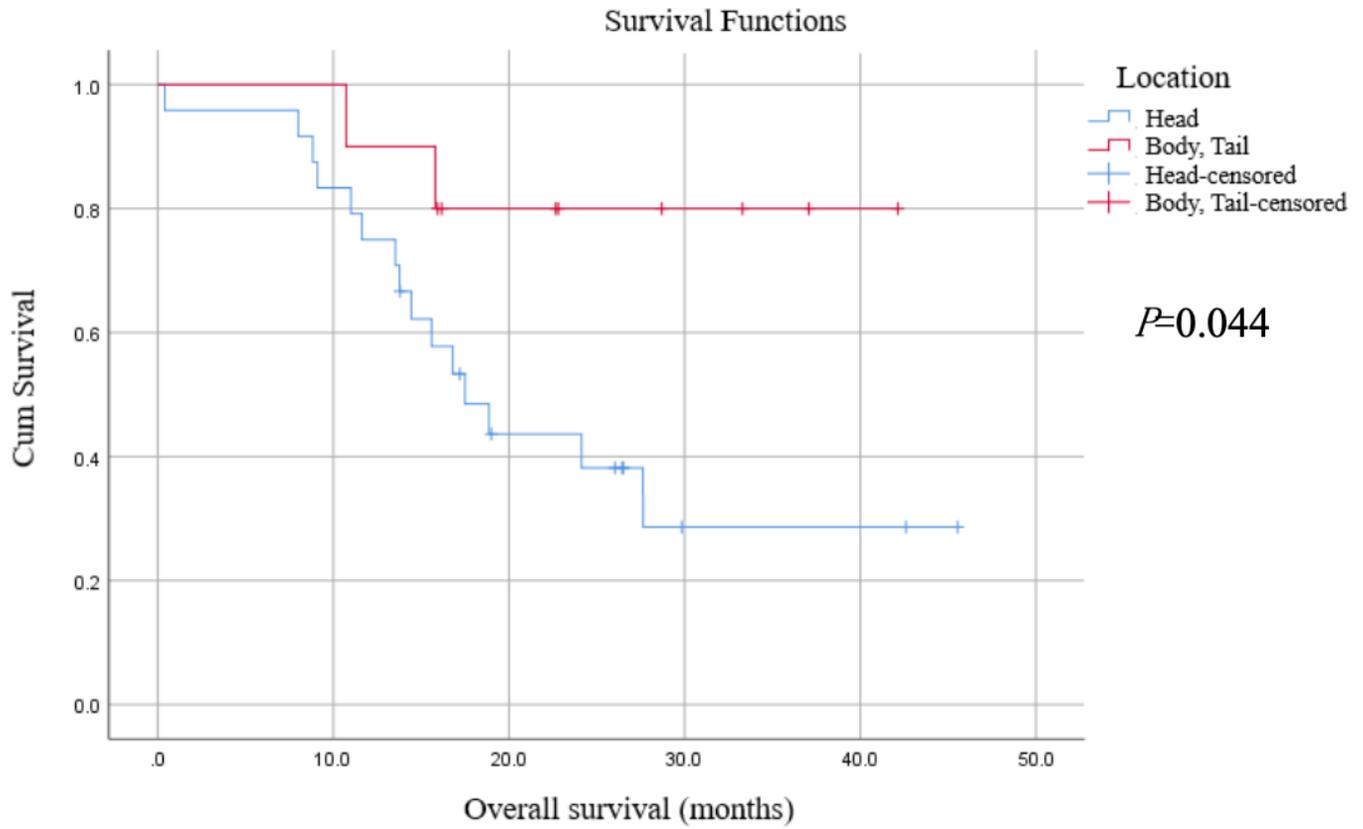


Figure 9

Comparison of pancreatic head and pancreatic tail survival analysis of 34 patients with GABARAP-negative cancer tissue

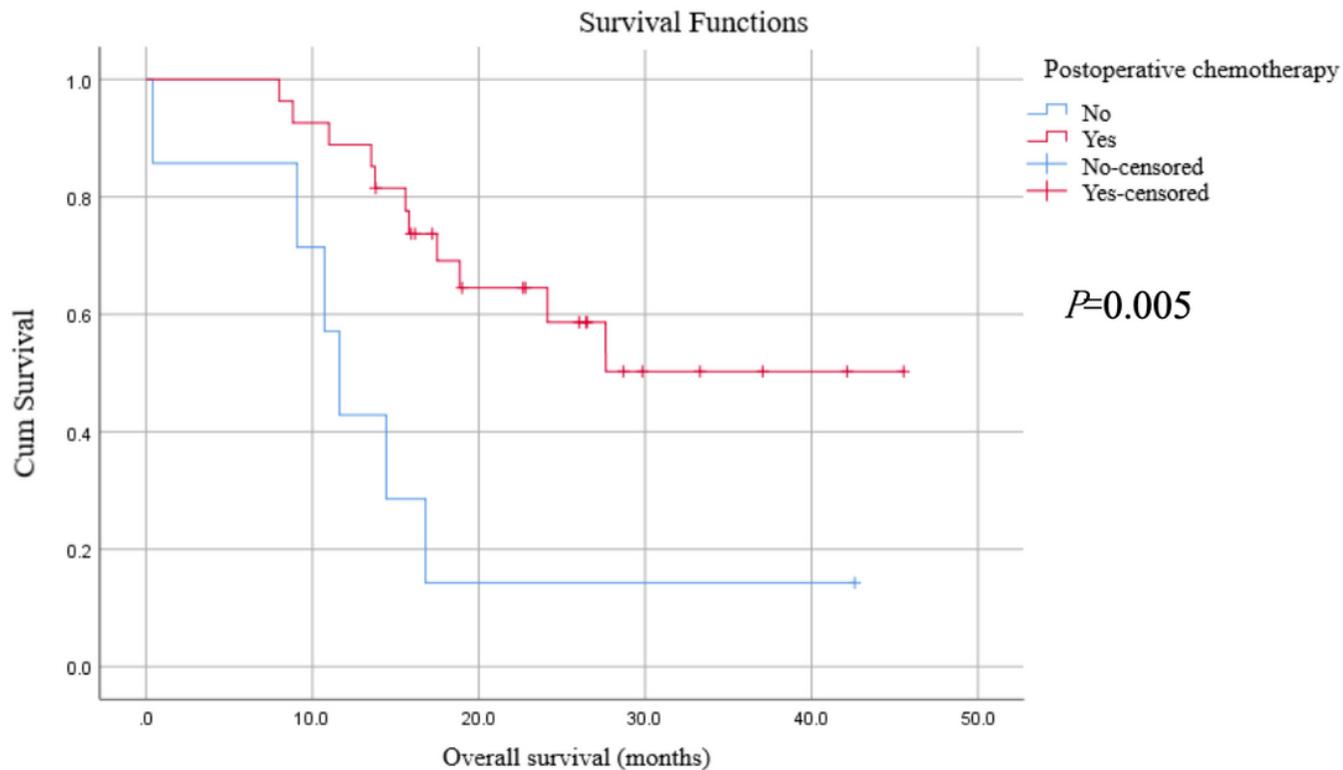


Figure 10

Comparison of chemotherapy and non-chemotherapy survival analysis of 34 cancer tissues with GABARAP-negative group

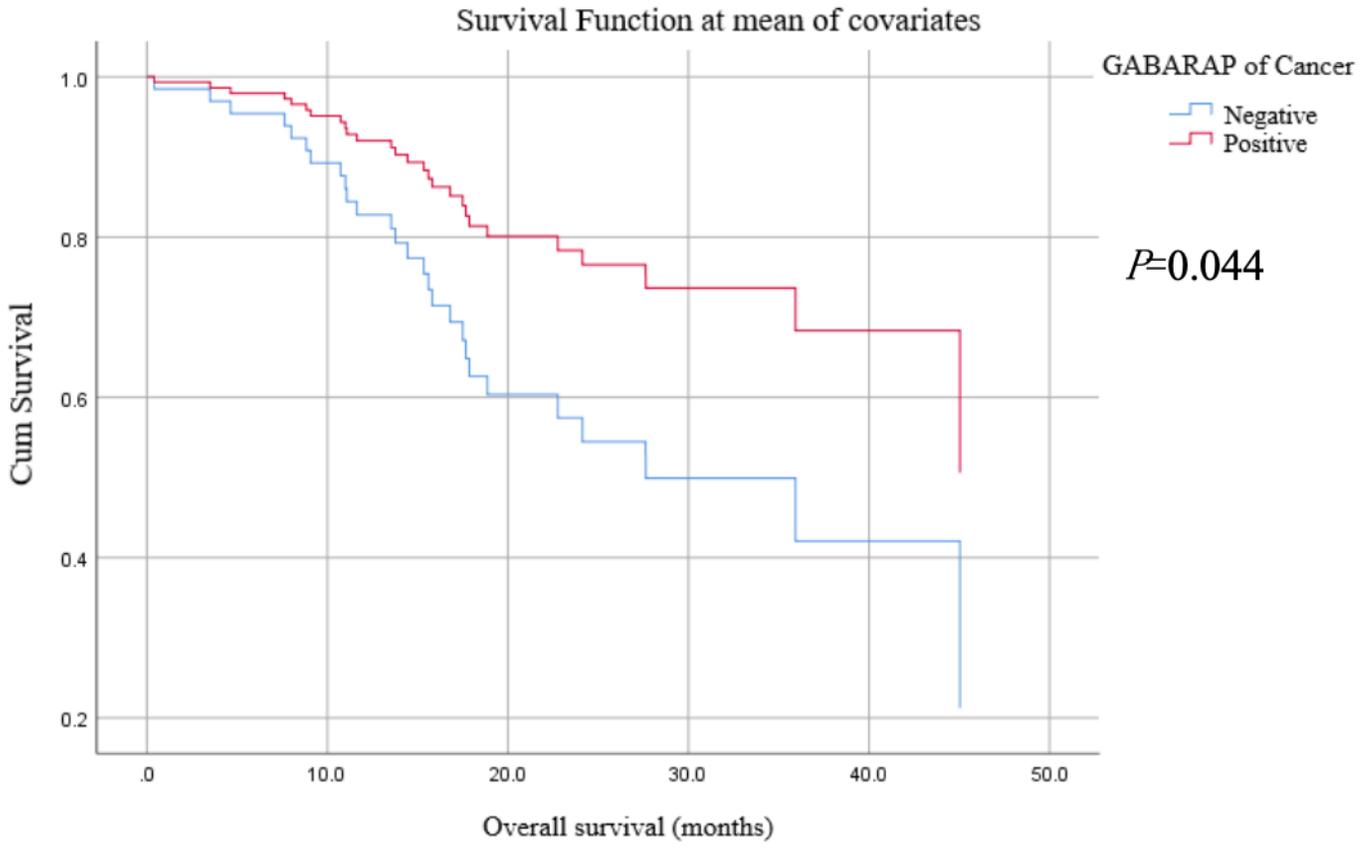


Figure 11

Cox regression multifactorial analysis of GABARAP in relation to total survival

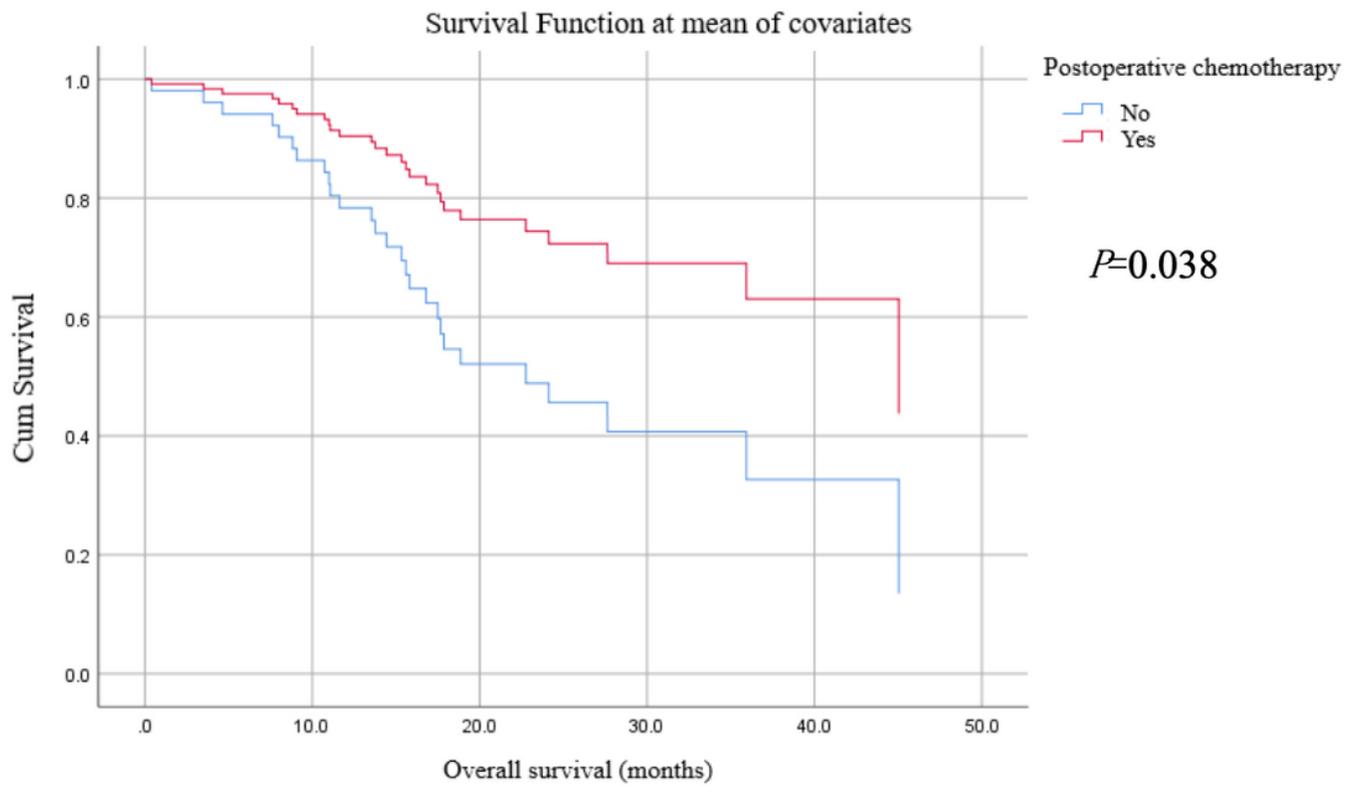


Figure 12

Cox regression multifactorial analysis of the relationship between adjuvant chemotherapy and overall survival

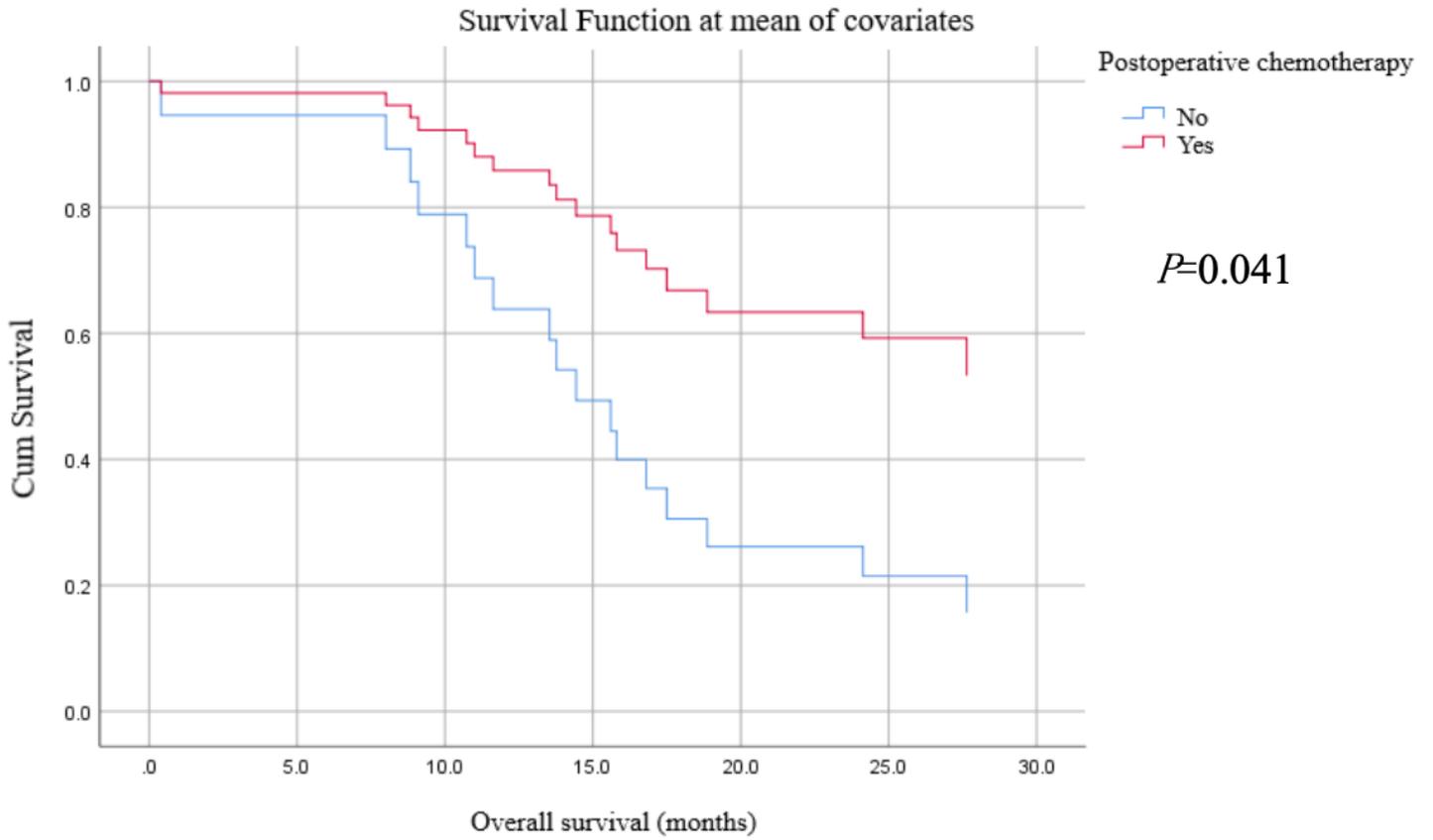


Figure 13

Cox regression multifactorial analysis of the relationship between GABARAP-negative adjuvant chemotherapy and overall survival in 34 cases of cancer tissue