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***Retrospective Study***

**TTK may serve as a prognostic biomarker for gallbladder cancer**

Xie Y *et al*. TTK expressed in gallbladder cancer

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**Abstract**

***AIM***

To investigate the expressions of TTK in gallbladder cancer (GBC) specimens and the associations between TTK expressions and clinicopathological parameters and clinical prognosis.

***METHODS***

A total of 68 patients with GBC who underwent surgical resections were enrolled in this study. The expressions of TTK were detected in paraffin-embedded tissues by immunohistochemistry (IHC). The assessment of TTK expressions were conducted by means of the H-score system, which was calculated by the multiplication of the overall staining intensity with the percentage of positive cells. The cytoplasm and nucleus were scored separately to achieve respective H-score values. The correlations between TTK expressions and clinicopathological parameters and clinical prognosis were analyzed using Chi-square test, Kaplan–Meier method and Cox regression.

***RESULTS***

In both nucleus and cytoplasm, the expressions of TTK in tumor tissues were significantly lower than that in normal tissues (*P* < 0.001, *P* = 0.026). For patients with GBC, with a median H-score as the cutoff value, it was discovered that, patients with higher levels of TTK expressions in neucleus had favourable overall survival (*P* < 0.001), but not in cytoplasm, and it was still stastically meaningful in Cox regression analysis. Further investigation indicated that, there were close negative correlations between TTK expressions and tumor differentiation (*P* = 0.041), Ca19-9levels (*P* = 0.016), T stage (*P* < 0.001), nodal involvement (*P* < 0.001), distant metastasis (*P* = 0.024) and TNM stage (*P* < 0.001).

***CONCLUSION***

The expressions of TTK in gallbladder cancer are lower than normal tissues, for cancer *per se*, higher levels of TTK expressions are concomitant with longer overall survival. TTK is a favorable prognostic biomarker for the patients with GBC.

**Keywords:** TTK; Biomarker; Prognosis; Gallbladder cancer; Gallbladder cancer

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**Core tip:** Numerous studies demonstrate that high levels of threonine and tyrosine kinase (TTK) are present in many types of human malignancies, and their overexpressions closely correlate with early recurrence and poor survival. However, no prior studies have attempted to concentrate on the expressions of TTK in the patients with GBC. In this study, we explored the expressions of TTK in GBC specimens and the associations between TTK expressions and clinicopathological parameters and clinical prognosis.

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**INTRODUCTION**

Gallbladder cancer (GBC) is the most common malignancy and a potentially lethal disease of the biliary tract. It has a dismal prognosis, with median survival of 3–11 mo and 5-year survival of 3%–13%, respectively[[1](#_ENREF_1)]. Prolonged survival and better prognosis could be primarily seen in a small group of patients with incidental gallbladder cancer (IGBC), but this fortunate scenario exists in only 0.3%-2% of all performed cholecystectomies due to benign conditions or after cholecystectomy[[2](#_ENREF_2),[3](#_ENREF_3)]. The incidence of GBC is characterized by remarkable geographical variations and ethnic disparities, with an extraordinarily high occurrence in Chile, Japan, and northern India[[4](#_ENREF_4)]. The incidence of GBC is quite low in most western countries and thus it is referred to as an orphan disease in the United States[[5](#_ENREF_5)]. However, with increasing global migration, the incidence of GBC in the west is on the rise, making it a global disease and afflicting thousands of individuals worldwide. Currently, a complete surgical resection remains to be the mainstay treatment to extend the life expectancy for the eligible patients. There are few chemotherapeutic agents for the patients with GBC and there are low response rates for the adjuvant treatments.

The spindle assembly checkpoint (SAC) is a safeguard mechanism that functions to monitor improperly oriented chromosomes, generate correct bipolar attachments to the spindle and minimize chromosome missegregation errors prior to anaphase onset[[6](#_ENREF_6),[7](#_ENREF_7)]. TTK is a dual-specificity protein kinase capable of phosphorylating threonines/serines and tyrosines[[8](#_ENREF_8)]. It is the core component and major regulator of the SAC, which is able to recruit and orchestrate other SAC protein kinases to the kinetochore, thereby ensuring faithful chromosome segregation and maintaining genome stability[[9](#_ENREF_9),[10](#_ENREF_10)]. Increased TTK levels are readily discovered in many types of human tumors, involving glioblastoma, thyroid carcinoma, breast cancer, hepatocellular carcinoma, pancreatic cancer as well as prostate cancer, and their overexpressions closely correlate with early recurrence and poor survival[[11-23](#_ENREF_11)]. We reviewed relevant clinical research and trials concerning TTK in several human cancers[[24](#_ENREF_24)], however, no prior studies were found regarding the expressions of TTK in patients with GBC. In this study, we first investigate the expressions of TTK in GBC specimens and the associations between TTK expressions and clinicopathological parameters and clinical prognosis.

**MATERIALS AND METHODS**

***Patients and tissue specimens***

Sixty eight cases were selected retrospectively from the patients with GBC who underwent surgical resections at the department of liver surgery of Peking Union Medical College Hospital (PUMCH) between June 2004 and January 2014 (Figure 1). Sixty eight pairs of GBC specimens and adjacent normal tissue specimens were acquired from these patients. Written informed consent was obtained from each patient before surgery, and the type of surgical procedure was performed according to the approved guidelines. Surgery types were categorized as curative and noncurative resections. Curative resection (R0) was referred to *en bloc* resection with a negative surgical margin, while the presence of microscopic (R1) or macroscopic (R2) residual cancer was considered noncurative. The protocol of tissue specimens was approved by Peking Union Medical College Hospital Ethics Committee. The clinicopathological data were collected from the history medical records and the follow-up were made from the date of surgery till October 2016. Patients with GBC were staged according to the 7th edition of American Joint Committee on Cancer (AJCC) system. The assays for liver function and serum tumor marker were considered positive when concentrations were beyond the normal upper limits. The follow-up data were obtained via outpatient records, phone visits and personal emails. The endpoint was overall survival (OS), defined as the time interval from the date of surgery to the cancer-related death. The study above was approved by the Ethics Committee of PUMCH.

***Immunohistochemistry and H-score for TTK***

All fresh tissue specimens were collected and immersed into 10% neutral-buffered formalin solution after immediate surgical resections and then embedded with paraffin. The paraffin-embedded tissues were sectioned with a thickness of 5 µm and stained with immunohistochemistry technique. Immunohistochemical staining was conducted manually and each slide was strictly processed in accordance with the immunohistochemical protocols. TTK polyclonal antibody (HPA016834, Sigma, USA, 1:100), produced in rabbit, was used for biomarker expression analysis. High pressure induced antigen retrieval was performed in the PBS buffer solution (pH 7.3), subsequent TTK staining was carried out for 90 min at the room temperature. Small intestine tissue was recommended by the manufacturer as positive control, and staining without the primary antibody was used as negative control.

The immunohistochemical slides were evaluated independently by two experienced pathologists in a blinded fashion. The assessment of TTK staining was conducted by means of the H-score system[[25-28](#_ENREF_25)], which was calculated by the multiplication of the overall staining intensity with the percentage of positive cells. The staining intensity graded from 0 to 3 (0 = negative, 1 = weak, 2 = medium, 3 = strong) and the positive percentage increased from 0 to 100. Theoretically, the final H-score values were obtained with a range from 0 to 300. The cytoplasm and nucleus were scored separately to achieve respective values.

***Statistical analysis***

Statistical analysis was performed using SPSS 17.0 software (Chicago, IL, USA). Kolmogorov‑Smirnov test was used to assess the distribution of the data and to decide the selection of statistical method. Chi-square test was used to compare qualitative variables and Mann-Whitney *U* test was used to compare the abnormally distributed variables. Kaplan–Meier method and log-rank test were used to compare OS. All potential prognostic factors on univariate analyses were entered into the Cox regression model. Cox regression multivariate analysis was performed further to identify the independent prognostic factors of significance. All *P* values were two sided and considered statistically significant when less than 0.05.

**RESULTS**

***Clinicopathological characteristics and survival data***

The mean and median age of patients at surgery were 65 and 66 years (range, 35-79 years), separately. 57.4% of patients were females and the female:male ratio was 1.3:1. Gallstones were present in 60.3% of cases. 51.5% of patients underwent curative resections and intermediate- to well-differentiated adenocarcinoma were found in 77.9% cases. Nodal involvement and distant metastasis occurred in 44.1% and 11.8% populations, respectively. Three cases were completely lost to follow-up after surgery. The median overall follow-up periods are 55 mo (range, 27-159 mo). The 1-year and 2-year survival rate were 66.2% and 41.2%, separately. More information about the cohort is listed inTable 1.

***Staining and H-score for TTK expressions***

TTK Staining was counted and analyzed in the patients (Table 2 and Figure 2). In tumor tissues, all specimens displayed cytoplasm staining, of which 1 case was strong positive (3+); most specimens (88.2%, 60/68) displayed nucleus staining, 8 cases exhibited negative nuclear staining and no strong positive cases were found among the specimens. While in normal tissues, overall specimens displayed cytoplasm staining, of which 1 case was strong positive (3+); nearly all specimens (97.1%, 66/68) displayed nucleus staining, 2 cases exhibited negative nuclear staining, and no strong positive cases were found within the specimens.

We calculated the H-score in both tumor and normal tissues (Table 3 and Figure 3**)**, it was found that, in tumor tissues, TTK exhibited a median H-score of 170 (range, 10-220) and 12.5 (range, 0-196) in cytoplasm and nucleus separately. While in normal tissues, the median H-score observed for TTK were 190 (range, 40-270) and 60 (range, 0-180), respectively.

***Various comparisons and cutoff value for TTK expressions***

Indicated by Kolmogorov-Smirnov test, the values of H-score were distributed abnormally. So Mann-Whitney *U* test was selected to make comparisons for them (Table 4). In both tumor and normal tissues, the expressions of TTK in nucleus were dramatically lower than that in cytoplasm (*P* < 0.001, *P* < 0.001, respectively). Moreover, we found that, in both nucleus and cytoplasm, there were significantly lower expressions of TTK in tumor tissues, compared with normal tissues (*P* < 0.001, *P* = 0.026, respectively).

For patients with GBC, the population was divided into two groups according to the median H-score value of neucleus and cytoplasm respectively. Surprisingly, we found that, patients with higher H-score values in neucleus had favourable OS (Figure 4A), but not in cytoplasm, and it was still stastically meaningful in Cox regression multivariate analysis (Table 5). Thus, the median neucleus H-score was used as the discriminating threshold[[29](#_ENREF_29)] and the cutoff value was set at 12.5.

***Correlations between TTK expressions and clinicopathological parameters***

The correlations between TTK expressions and clinicopathological parameters are detailed in Table 6. There were no significant associations between TTK expressions and age, gender, tumor size and CEA levels. However, TTK expressions exhibited close negative correlations with tumor differentiation (*P* = 0.041), Ca19-9 levels (*P* = 0.016), T stage (*P* < 0.001), nodal involvement ( *P*< 0.001), distant metastasis(p=0.024) and TNM stage (*P* < 0.001). Higher TTK expression rates were observed in patients with normal Ca19-9 levels, intermediate-well differentiation, T1+2 stage, negative nodal involvement, free distant metastasis and TNM stage I+II, in contrast to the ones with elevated Ca19-9 levels, low-undifferentiation, T3stage, positive nodal involvement, distant metastasis and TNM stage III+IV, respectively.

***Survival analysis***

A total of 65 clinical follow-up data were available among the 68 patients. Univariate survival analysis (Table 5) revealed that TTK expressions (*P* < 0.001); jaundice (*P* = 0.024); concentrations of ALT (*P* = 0.005), AST (*P* = 0.017), ALP (*P* = 0.043), DBil (*P* = 0.010), Ca19-9 (*P* < 0.001); surgery type (*P* < 0.001); tumor differentiation (*P* < 0.001); T (*P* < 0.001), N (*P* < 0.001), M (*P* < 0.001) and TNM stage (*P* < 0.001) were associated with OS in the patients with GBC. While age, gender, fever, cholecystolithiasis, diabetes, concentrations of TBil, GGT and CEA had no significant influence on the survival in our study.

Cox regression multivariate analysis revealed that surgery type, T stage and TTK expressions were independent prognostic factors for OS (Table 5). Further, subgroup analysis by log-rank test indicated that patients with higher levels of TTK expressions had longer OS and better prognosis, no matter in T, N, M, or TNM stage, in comparison with the ones with lower TTK expressions (Figure 4 B-H).

**DISCUSSION**

On account of its critical role in maintaining chromosome stability, an increasing number of researchers have concerntrated on the relationship between TTK and cancer. While TTK has been studied in many types of malignancies, no prior research has been found regarding its expression and clinical prognosis, in the gallbladder cancer.

In our present study, it was discovered that, the overall expressions of TTK in tumor tissues were significantly lower than that in normal tissues; but for cancer per se, patients with higher TTK expressions were associated with better prognosis. By contrast, TTK overexpressions were found in numerous neoplasms, where they were concominant with worse prognosis. Parallel with our results, Xu *et al*[[30](#_ENREF_30)] also found increased TTK expressions were related with prolonged disease free survival and OS in triple-negative breast cancer, but without comparisons between tumor and normal tissues. It may be suggested that TTK play a certain role in the initiation of gallbladder cancer. TTK is required for the execution of the SAC machinery during mitosis and conductive to the fidelity of chromosome segregations at the kinetochores. The inhibition of TTK activity could therefore compromise the function of the SAC and culminate in undesirable effects, including chromosomal instability (CIN), aneuploidy formation, cell death or carcinogenesis [[31-35](#_ENREF_31)]. It is widely accepted that chromosomal instability is correlative with intratumour heterogeneity, chromosome aberrations and aneuploidy formations, in fact, aneuploidy has been found at the earliest stages of carcinogenesis and CIN is considered as a fundamental process for cancer development[[36](#_ENREF_36)]. So far, a premature termination of TTK synthesis, due to cancer-associated frameshift mutations, has been frequently found in gastric and colorectal cancers with microsatellite instability[[37](#_ENREF_37)]. Whether the same process take place in gallbladder cancer remains to be explored afterwards.

Apart from the variances between tumor and normal tissues, it was revealed in our current study that there were close negative correlations between TTK expressions and tumor differentiation, T, N, M as well as TNM stages, furthermore, lower levels of TTK expressions were more helpful for cancer infiltration, nodal involvement and distant metastasis. Patients with higher levels of TTK expressions at the same stage had a longer OS than the lower ones. Thus, TTK may serve as a positive biomarker indicative of prognosis and make a strategic choice for the doctors. For instance, patients with higher levels of TTK expressions, perhaps they could benefit better from the adjuvant chemo- or radio-therapy, in contrast to the ones with lower expressions. The use of TTK expressions may be more important for the patients with IGBC. Currently, the management of IGBC is primarily dictated by T-stage alone, with a re-resection recommendation for T1b, T2, or T3 disease[[38](#_ENREF_38)].In terms of T1a patients, simple laparoscopic cholecystectomy is sufficient, with a 5-year survival rate of 95.5%[[39](#_ENREF_39)]. If lower levels of TTK expressions are detected in the cancer specimens, then more attentions should be paid to these patients, due to a larger likelihood of nodal involvement and distant metastasis in the future. The survival of the remaining 4.5% of patients may be improved, with a combination of routine pathology and TTK expressions.

Of note, our study may provide theoretical support for the immunotherapy and targeted therapy in different tumors concerning TTK. TTK has been utilized as an immunogenic epitope to elicit potent and peptide-specific cytotoxic T lymphocyte activity against cancer cells. Its safety, immunogenicity and clinical response have been validated in some clinical trials, including lung cancer, esophageal cancer and biliary tract cancer[[40-46](#_ENREF_40)]. Even, in a variety of human tumors, TTK has been used as a therapeutic target for innovative approaches in combating the malignancies, involving glioblastoma, breast cancer, hepatocellular carcinoma and pancreatic cancer[[11](#_ENREF_11), [12](#_ENREF_12), [14-16](#_ENREF_14), [18-21](#_ENREF_18)].TTK inhibitors could give rise to attenuated aggressiveness, reduced viability, augmented autophagy and increased apoptosis in these cancers. Further, the first phase I clinical trials of oral TTK inhibitors (BAY1161909, BAY1217389) has been performed in breast cancer (ClinicalTrials.gov ID: NCT02138812, NCT02366949). In terms of these tumors above, TTK overexpressions are exploited and TTK-targeted therapies are available. Nevertheless, it should be cautious for these therapies applied in gallbladder cancer, due to a low TTK expression.

To date, some mechanisms have been revealed in several tumors, concerning TTK and neoplasia. It was unveiled that, in hepatocellular carcinoma, demethylations of TTK promoters were conducive to its overexpressions and highly expressed TTK could activate the Akt/mTOR pathway in a p53 dependent fashion[[18](#_ENREF_18)]. Moreover, in melanoma, TTK/AKT and B-RafWT/ERK signaling constituted an auto-regulatory negative feedback loop together, continuous phosphorylations of TTK through oncogenic B-RafV600E signaling were able to abrogate the negative feedback loop, leading to aberrant SAC function and tumorigenesis[[47](#_ENREF_47),[48](#_ENREF_48)]. It was discovered that, in breast cancer, high levels of TTK expressions were protections for aneuploidy and enabled these cells to tolerate aneuploidy[[49](#_ENREF_49)]. While in colon cancer, overexpressed TTK could increase aneuploidy, owing to a weakened SAC function, and contribute to carcinogenesis[[50](#_ENREF_50)]. Among studies available in the literature, it was demonstrated that overexpressions of TTK consisted positively with tumor grades and poor survival. Conversely, it was indicated in our study that, lower expressions of TTK corresponded closely with tumor grade and dismal prognosis. Taken together, aberrant TTK expressions, either increased or decreased expressions, are clearly correlated with tumorigenesis, which may result from chromosomal instabilities and aneuploidy formations, as a result of compromised SAC functions (Figure 5). Therefore, it is different roles that TTK may play in disparate malignancies, more thorough research works are still encouraged to verify the exact relationships between TTK and cancers, as well as the subtle mechanisms.

Recently, it has been found that TP53, KRAS and ERBB3 are the most frequent somatic mutations in the GBC spectrum[[51](#_ENREF_51)]. Additionally, striking progress has been made in the clarification of emergent intracellular signaling pathways, such as Hedgehog, PI3K/AKT/mTOR, Notch, *etc*., which are activated in GBC[[52](#_ENREF_52)]. These promising studies may provide valuable clues for the deep insight into the roles which TTK plays in GBC.

Our study has several limitations. Primarily, the study was retrospective, all the information of each patient was collected from history medical records, which may contribute to the selection bias. Next, it was a single-institutional investigation and the number of cases was relatively small, restricting the power of statistical analysis. Finally, some patients received postoperative chemotherapy or radiotherapy, the effects of these adjuvant therapies on prognosis were not considered, in spite of limited survival benefits brought by them. For these reasons above, multi-institutional investigations and prospective studies are required to explore the expressions of TTK in the gallbladder cancer, and further to evaluate the clinical significance in a larger cohort of patients.

In conclusion, our data suggest that the expressions of TTK in gallbladder cancer are lower than normal tissues, for cancer *per se*, higher levels of TTK expressions are concomitant with longer overall survival. TTK is a favorable prognostic biomarker for the patients with GBC.

**comments**

***Background***

TTK is a dual-specificity protein kinase capable of phosphorylating threonines/serines and tyrosines. It is the core component and major regulator of the spindle assembly checkpoint (SAC), which functions to ensure faithful chromosome segregation and maintaining genome stability. Increased TTK levels could be readily discovered in many types of human tumors, including glioblastoma, thyroid carcinoma, breast cancer, *etc.*, and their overexpressions closely correlate with early recurrence and poor survival. However, no prior studies were found regarding the expressions of TTK in patients with gallbladder cancer (GBC). In this study, we first investigate the expressions of TTK in GBC specimens and the associations between TTK expressions and clinicopathological parameters and clinical prognosis.

***Research frontiers***

Numerous studies demonstrate that high levels of TTK are present in many types of human malignancies, and their overexpressions closely correlate with early recurrence and poor survival. Several TTK inhibitors have been developed to combat the malignancies and they exhibit demonstrable survival benefits. Moreover, TTK has been used as an immunogenic epitope to elicit potent and peptide-specific cytotoxic T lymphocyte activity against cancer cells. Its safety, immunogenicity and clinical response have been validated in several clinical trials.

***Innovations and breakthroughs***

The results demonstrate that the expressions of TTK in gallbladder cancer are lower than normal tissues, for cancer *per se*, higher levels of TTK expressions are concomitant with longer overall survival. TTK is a favorable prognostic biomarker for the patients with GBC.

***Applications***

This study suggests that TTK may serve as a positive biomarker indicative of prognosis, meanwhile, it may also provide theoretical support for the immunotherapy and targeted therapy in different tumors concerning TTK.

***Terminology***

The spindle assembly checkpoint (SAC) is a safeguard mechanism that functions to monitor improperly oriented chromosomes, generate correct bipolar attachments to the spindle and minimize chromosome missegregation errors prior to anaphase onset. TTK is a dual-specificity protein kinase that phosphorylates threonines/serines and tyrosines. TTK acts as the core component and major regulator of the SAC, which functions recruit and orchestrate other SAC protein kinases to the kinetochore, thereby ensuring faithful chromosome segregation and maintaining genome stability.

***Peer-review***

This is an interesting study about the TTK in GBC. In this study, the authors investigated the expressions of TTK in GBC specimens and the associations between TTK expressions and clinicopathological parameters and clinical prognosis. The author found that for patients with GBC, with a median H-score as the cutoff value, patients with higher levels of TTK expressions in neucleus had favourable overall survival. This study is overall well designed and the manuscript is very well written.

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All initial screening GBC cases between June 2004 and January 2014 at the department of liver surgery of PUMCH

Exclusion criteria:

(1) Non-surgical resections

(2) Papillary adenocarcinomas

(3) Deaths within 1 month due to post-operative complications

Eligible GBC cases (*n*=68)

Data collection:

Clinicopathological and follow-up data collected

Expression assesment:

Immunohistochemical staining and H-score

Data analyses:

(1) Staining and H-score for TTK expressions

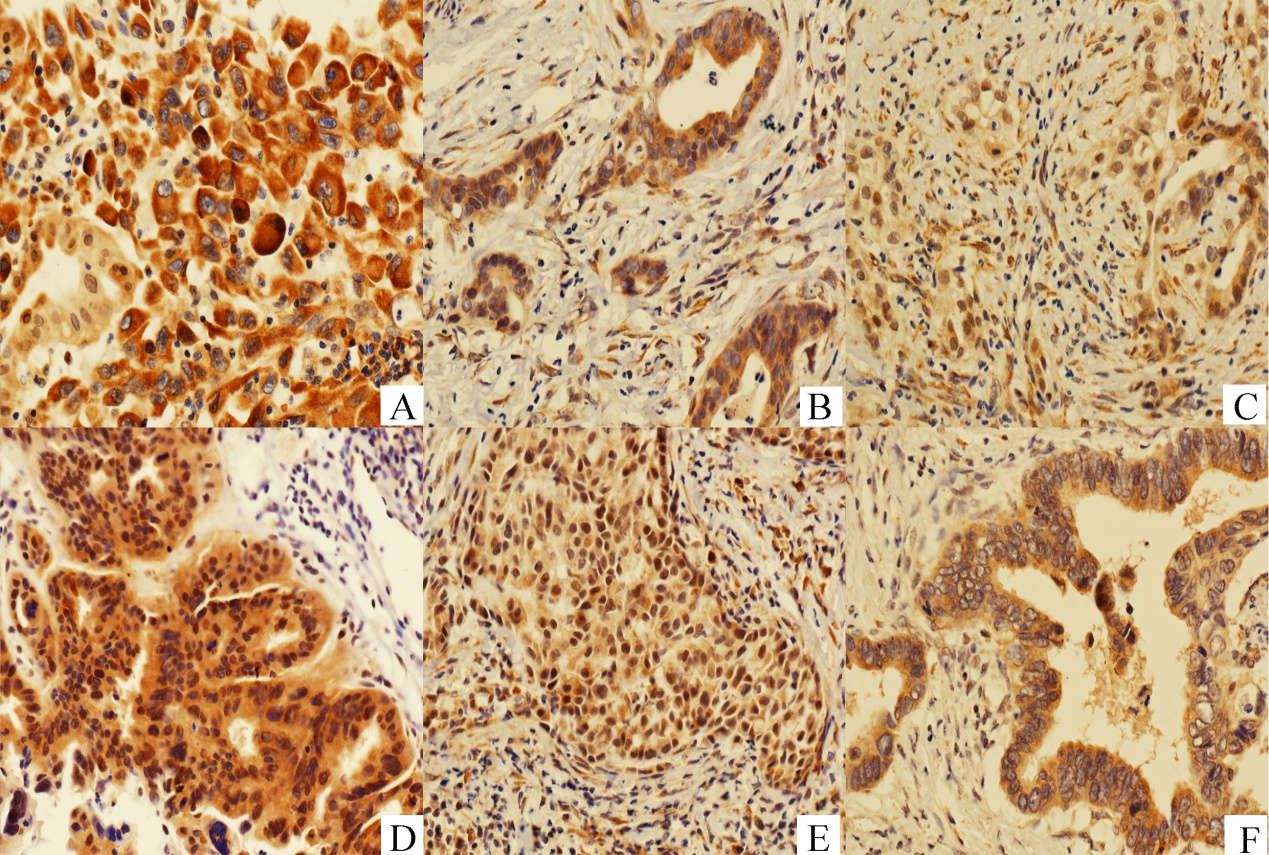
(2) Various comparisons and cutoff value for

TTK expressions

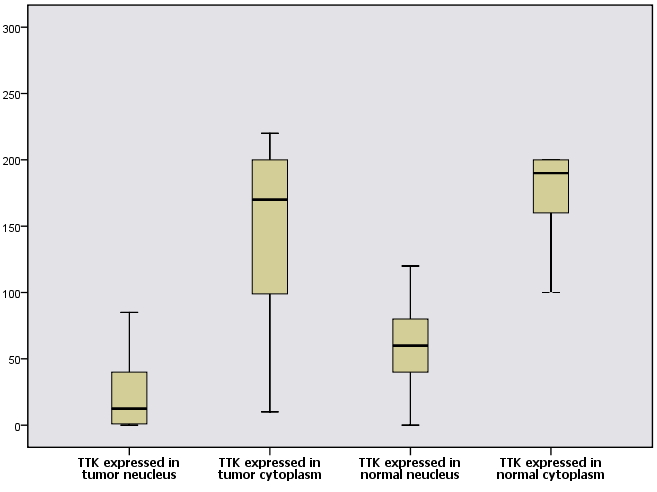
(3) Associations between TTK expressions and clinicopathological parameters

(4) Correlations between TTK expressions and survival prognosis

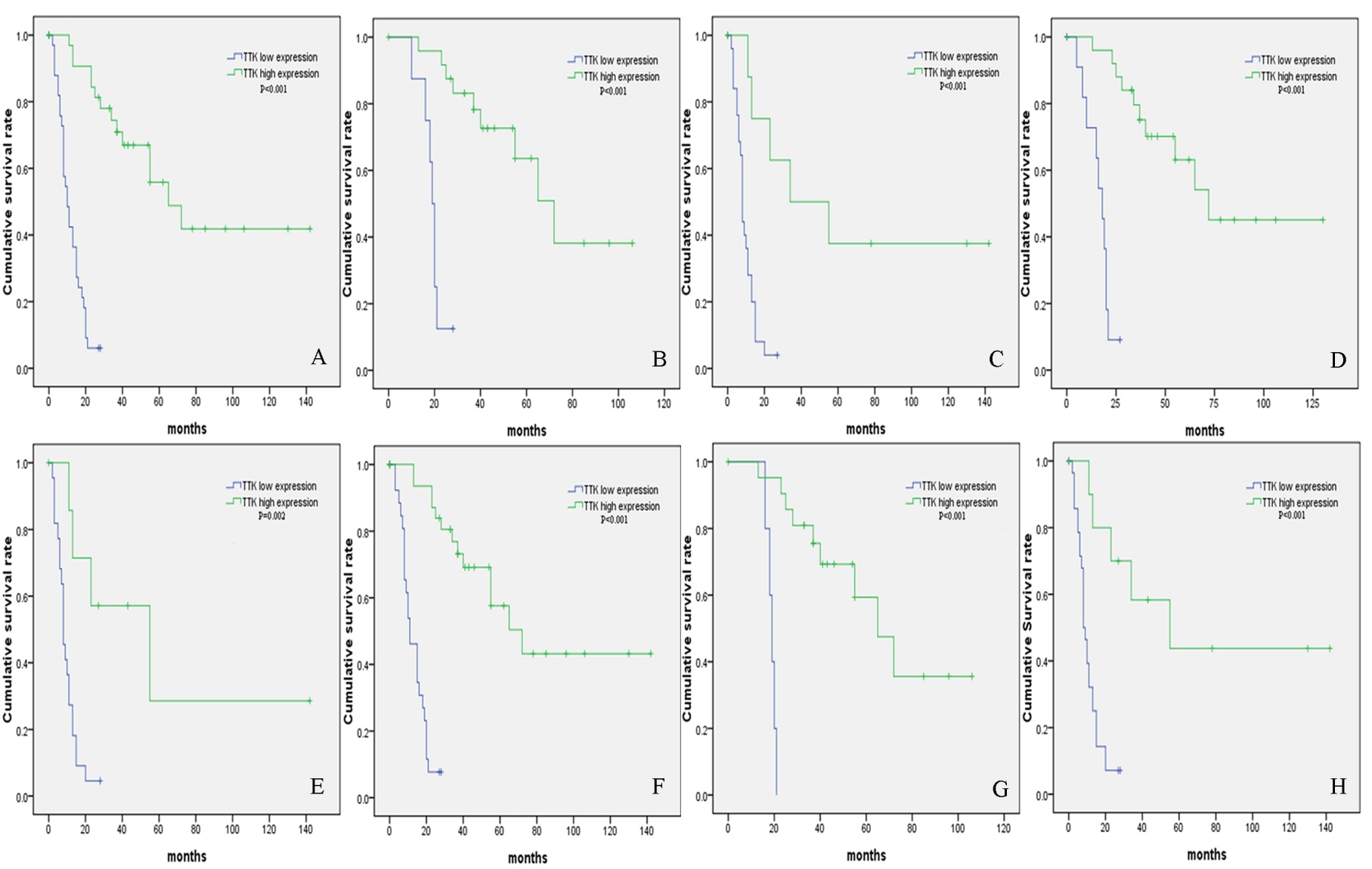
**Figure 1 Flow diagram of the study.** A total of 68 cases were enrolled in the study, with explicit exclusion criteria. After collected clinicopathological and follow-up data, and conducted immunohistochemistry staining, correlations between TTK expressions and clinicopathological parameters and survival prognosis were analyzed then. GBC:Gallbladder cancer.



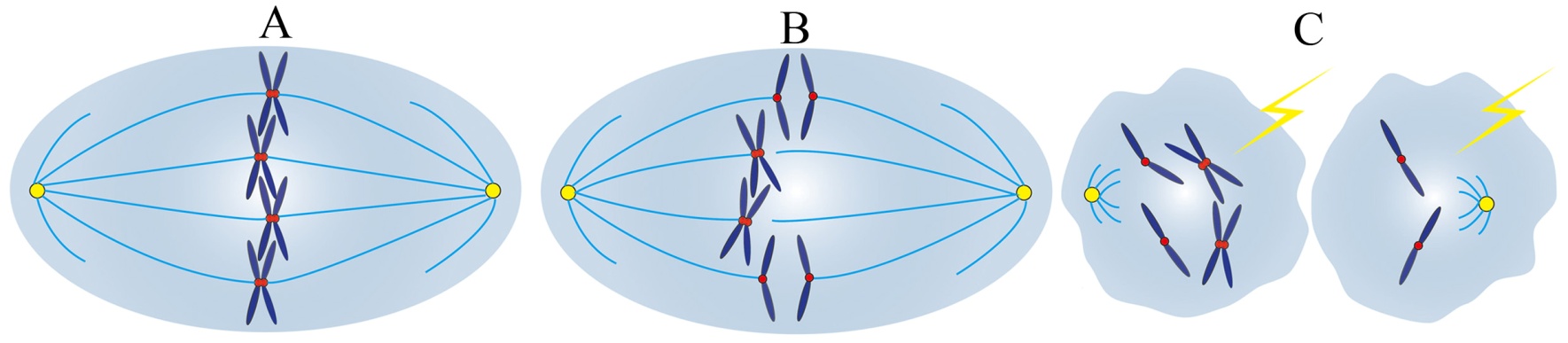
**Figure 2 Immunohistochemical staining of tumour tissues at magnification X 200.** A-C: Cytoplasm staining with 3+, 2+ and 1+ intensity respectively; D-F: Neucleus staining with 2+, 1+ and 0+ intensity respectively**.**

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**Figure 3 Boxplot for H-score in tumor and normal tissues.**

****

**Figure 4 Kaplan-Meier survival analyses in different subgroups, according to TTK expressions.** A: The whole cohort; B: T1 + 2 group; C: T3 group; D: Negative nodal involvement group; E: Positive nodal involvement group; F: Free distant metastasis group; G: Stage I + II group; H: Stage III + IV group.



**Figure 5 Diagram for possible mechanism concerning TTK and carcinogenesis.** A: Sister chromatids are properly arrayed in equatorial plate at metaphase, with correct attachments to microtubules by kinetochores, attributed to the normal SAC safeguard mechanism; B: Aberrant TTK expressions, either increased or decreased expressions, could definitely compromise the functions of the SAC, with frequent chromosome missegregation errors; C: Severe chromosome missegregation errors, due to the override of SAC function, could result in chromosomal instabilities, aneuploidy formations, cell deaths and carcinogenesis.

**Table 1 Clinicopathological characteristics of the cohort**

|  |  |
| --- | --- |
| **Characteristics** | ***n* (%)** |
| **Age (yr)** |  |
| ≤ 65 | 33 (48.5) |
| > 65 | 35 (51.5) |
| **Gender** |  |
| Female | 39 (57.4) |
| Male | 29 (42.6) |
| **Cholecystolithiasis** |  |
| With | 41 (60.3) |
| Without | 27 (39.7) |
| **Diabetes** |  |
| With | 17 (25.0) |
| Without | 51 (75.0) |
| **Fever** |  |
| With | 9 (13.2) |
| Without | 59 (86.8) |
| **Jaundice** |  |
| With | 15 (22.1) |
| Without | 53 (77.9) |
| **ALT** |  |
| Normal | 53 (77.9) |
| Elevated | 15 (22.1) |
| **AST** |  |
| Normal | 50 (75.8) |
| Elevated | 16 (24.2) |
| **TBil** |  |
| Normal | 49 (72.1) |
| Elevated | 19 (27.9) |
| **DBil** |  |
| Normal | 50 (73.5) |
| Elevated | 18 (26.5) |
| **GGT** |  |
| Normal | 43 (69.4) |
| Elevated | 19 (30.6) |
| **ALP** |  |
| Normal | 45 (72.6) |
| Elevated | 17 (27.4) |
| **CEA** |  |
| Normal | 42 (72.4) |
| Elevated | 16 (27.6) |
| **CA19-9** |  |
| Normal | 26 (44.1) |
| Elevated | 33 (55.9) |
| **Surgery type** |  |
| curative | 35 (51.5) |
| noncurative | 33 (48.5) |
| **Tumor size (cm)** |  |
| ≤ 3 | 43 (63.2) |
| > 3 | 25 (36.8) |
| **Differentiation** |  |
| Low-undifferentiated | 15 (22.1) |
| Intermediate-well | 53 (77.9) |
| **T stage** |  |
| Tis | 1 (1.5) |
| T1 | 3 (4.4) |
| T2 | 29 (42.6) |
| T3 | 35 (51.5) |
| **N stage** |  |
| N0 | 38 (55.9) |
| N1 | 22 (32.4) |
| N2 | 8 (11.7) |
| **M stage** |  |
| M0 | 60 (88.2%) |
| M1 | 8 (11.8%) |
| **TNM stage** |  |
| I | 4 (5.9) |
| II | 24 (35.3) |
| IIIA | 9 (13.2) |
| IIIB | 17 (25.0) |
| IVA | 0 (0) |
| IVB | 14 (20.6) |

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase;TBil: Total bilirubin; DBil: Direct bilirubin; GGT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; CEA: Carcinoembryonic antigen; Ca19-9: Carbohydrate antigen 19-9.

**Table 2 TTK staining results in tumor and normal tissues**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Localization** | **Positive cell rates (%)**  **Median (range)** | | ***n* (%)** | |
| **Tumor tissues** | **Normal tissues** | **Tumor tissues** | **Normal tissues** |
| Cytoplasm staining | | | | |
| Negative | - | - | 0 | 0 |
| Positive | 100 (10-100) | 99 (40-100) | 68 (100) | 68 (100) |
| 1+ | 55 (10-100) | 85 (18-100) | 41 (60.3) | 14 (20.6) |
| 2+ | 90 (5-100) | 100 (80-100) | 49 (72.1) | 54 (79.4) |
| 3+ | 20 | 90 | 1 (1.5) | 1 (1.5) |
| Nucleus staining | | | | |
| Negative | - | - | 8 (11.8) | 2 (2.9) |
| Positive | 15 (1-98) | 60 (1-90) | 60 (88.2) | 66 (97.1) |
| 1+ | 15 (1-85) | 60 (1-90) | 57 (83.8) | 59(86.8) |
| 2+ | 45 (5-98) | 90 (60-90) | 8 (11.8) | 7 (10.3) |
| 3+ | - | - | 0 | 0 |

**Table 3 H-score values in tumor and normal tissues**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Group** | ***N*** | **localization** | **Minimum** | **Maximun** | **Median** |
| Tumor | 68 | Nucleus | 0 | 196 | 12.5 |
| 68 | Cytoplasm | 10 | 220 | 170 |
| Normal | 68 | Nucleus | 0 | 180 | 60 |
| 68 | Cytoplasm | 40 | 270 | 190 |

**Table 4 Various comparisons in tumor and normal tissues for TTK expressions**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Group** | ***N*** | **Minimum** | **Maximum** | **Median** | **mean rank** | ***P*** |
| Comparison between nucleus and cytoplasm in normal tissues | | | | | | |
| Nucleus | 68 | 0 | 180 | 60 | 39.65 | <0.0011 |
| Cytoplasm | 68 | 40 | 270 | 190 | 97.35 |
| Comparison between nucleus and cytoplasm in tumor tissues | | | | | | |
| Nucleus | 68 | 0 | 196 | 12.5 | 38.98 | <0.0011 |
| Cytoplasm | 68 | 10 | 220 | 170 | 98.02 |
| Comparison between tumor and normal tissues in nucleus | | | | | | |
| Tumor | 68 | 0 | 196 | 12.5 | 49.54 | <0.0011 |
| Normal | 68 | 0 | 180 | 60 | 87.46 |
| Comparison between tumor and normal tissues in cytoplasm | | | | | | |
| Tumor | 68 | 10 | 220 | 170 | 61.19 | 0.0261 |
| Normal | 68 | 40 | 270 | 190 | 75.81 |

1Statistically significant.

**Table 5 Univariate and multivariate survival analyses for gallbladder cancer**

|  |  |  |
| --- | --- | --- |
| **Univariate** | **χ2** | ***P* value** |
| Age | 2.221 | 0.136 |
| Gender | 0.167 | 0.683 |
| Cholecystolithiasis | 0.346 | 0.558 |
| Diabetes | 0.165 | 0.685 |
| Fever | 0.001 | 0.989 |
| Jaundice1 | 5.110 | 0.0241 |
| ALT1 | 7.781 | 0.0051 |
| AST1 | 5.708 | 0.0171 |
| TBil | 0.241 | 0.516 |
| DBil1 | 6.645 | 0.0101 |
| GGT | 0.899 | 0.343 |
| ALP1 | 4.099 | 0.0431 |
| CEA | 3.137 | 0.077 |
| CA19-91 | 12.385 | <0.0011 |
| Surgery type1 | 20.715 | <0.0011 |
| Tumor size | 0.099 | 0.754 |
| Differentiation1 | 12.385 | <0.0011 |
| T stage1 | 21.594 | <0.0011 |
| N stage1 | 19.887 | <0.0011 |
| M stage1 | 29.503 | <0.0011 |
| TNM stage1 | 33.062 | <0.0011 |
| TTK1 | 21.226 | <0.0011 |
| **Multivariate** | **OR (95% CI)** |  |
| Surgery type1 | 4.250(1.867-9.674) | 0.0011 |
| T stage1 | 2.927(1.258-6.808) | 0.0131 |
| TTK1 | 0.076(0.024-0.241) | 0.0011 |

1Statistically significant.

**Table 6 Correlations between TTK expressions and clinicopathological parameters**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Parameters** | **TTK expression** | | **χ2** | ***P* value** |
| **Low expression** | **High expression** |
| Age (yr) |  | | | |
| ≤ 65 | 16 | 17 | 0.059 | 0.808 |
| ＞65 | 18 | 17 |
| Gender |  | | | |
| Female | 20 | 19 | 0.277 | 0.598 |
| Male | 13 | 16 |
| CEA |  | | | |
| Normal | 20 | 22 | 3.512 | 0.061 |
| Elevated | 12 | 4 |
| Ca19-91 |  | | | |
| Normal | 10 | 16 | 5.756 | 0.0161 |
| Elevated | 23 | 10 |
| Tumor size |  | | | |
| ≤3 | 22 | 21 | 0.063 | 0.801 |
| ＞3 | 12 | 13 |
| Differentiation1 |  | | | |
| Low-undifferentiated | 11 | 4 | 4.191 | 0.0411 |
| Intermediate-well | 23 | 30 |
| T stage1 |  | | | |
| T1+2 | 8 | 25 | 17.015 | <0.0011 |
| T3 | 26 | 9 |
| Nodal involvement1 |  | | | |
| Negative | 12 | 26 | 11.691 | <0.0011 |
| Positive | 22 | 8 |
| Metastasis1 |  | | | |
| M0 | 27 | 33 | 5.100 | 0.0241 |
| M1 | 7 | 1 |
| TNM stage1 |  | | | |
| I+II | 5 | 23 | 19.671 | <0.0011 |
| III+IV | 29 | 11 |

1Statistically significant.