INTRODUCTION

PC is often diagnosed at an advanced stage leaving no effective therapies. At present, the 5-year relative survival rate of PC is about 8%, ranking lowest amongst all cancers. The reasons for the poor survival and high mortality of PC are multi-factorial including the close proximity of surrounding important tissues and its special tumour microenvironment. Although surgical resection remains the
only chance for cure, less than 20% of patients are even surgical candidates.\textsuperscript{5} In addition, despite the completion of surgical resection and adjuvant chemotherapy, nearly 60% of patients relapse within 2 years after surgery.\textsuperscript{6}

CAMKs are serine/threonine kinases that are activated by increased intracellular calcium concentration and can mediate subsequent cell activity. Ca\textsuperscript{2+} binding greatly changes the conformation of CaM and increases its affinity for some CaMKs including CaMKK, CaMKI, CaMKII and CaMKIV. These CaM kinases are widely expressed and can participate in a variety of cancer-related functions.\textsuperscript{7} Their potential in anti-cancer treatment interventions has gradually begun to receive attention. It was reported that targeting Ca\textsuperscript{2+} signalling may provide therapeutically useful options, such as inducing epigenetic reactivation of tumour suppressor genes in cancer patients.\textsuperscript{8} CaMKI family consists of 4 members including CaMKI\textsubscript{α}, CaMKI\textsubscript{β}/Pnck, CaMKI\textsubscript{γ}/CLK3 or CaMKI\textsubscript{δ}/CLK-I, which are coded for by CAMK1, PNCK, CAMK1G and CAMK1D, respectively.\textsuperscript{7} CAMK1 is known to play important roles in Ca\textsuperscript{2+} signalling pathways and it is also involved in multiple cell functions, including ATP binding, signal transduction, cell differentiation, et al.\textsuperscript{9} Despite the importance of CAMK1 in cell functions, it is also faced with some intriguing questions and challenges in tumour field. In this present study, we plan to illustrate the presence and importance of CAMK1 in PC through bioinformatic mining analysis and the samples presented in TMAs.

2 | MATERIALS AND METHODS

2.1 | Bioinformatics mining methods

The GEPIA 2 database (http://gepia2.cancer-pku.cn) could analyse the gene expression profiles from the Cancer Genome Atlas (TCGA) dataset and the Genotype-Tissue Expression (GTEx) projects. The expression level of one gene in different types of cancer could be achieved by Boxplot.\textsuperscript{10} We identified the expression levels of CAMK1 in PC based on TCGA normal and GTEx data. The cut-off value of log2FC was set as 1, and P value was set to 0.01. Next, Oncomine (www.oncomine.org), a cancer microarray database and integrated data-mining platform,\textsuperscript{11,12} was used to compare CAMK1 expression in PC tissues with that in normal tissues. In this study, we chose mRNA levels of cancer vs. normal patient datasets, 1.5-fold change and P value = 0.01 as threshold. We also retrieved the data from the HPA database (http://www.proteinatlas.org). The HPA database was made available freely to provide the expression profiles at protein levels, as well as IHC images for a wide variety of cancer tissues. In the HPA database, genome-wide transcriptomics data and clinical metadata of almost 8000 patients were used in order to analyse the proteome of 17 major cancer types. The IHC analysis in the HPA database is also presented for many protein-coding genes in respective cancer patients, the antibody information used for each IHC analysis can also be obtained in the HPA database. The IHC score is mainly classified into strong, moderate, weak and negative based on the staining intensity and fraction of stained cells.\textsuperscript{13,14} Furthermore, we also used KM Plotter database (http://kmplot.com/analysis), an online database is capable to assess the effect of any gene on survival in cancer patients\textsuperscript{15} and GEPIA 2 database to evaluate the OS and DFS of PC patients. In order to assess the prognostic values of CAMK1, the patient samples were divided into two cohorts based on the median expression (high expression and low expression) of CAMK1. CAMK1 was uploaded respectively to obtain the survival plots, in which Logrank P value and hazard ratio (HR) with 95% confidence intervals(CI) were calculated and showed on the webpage.

2.2 | Tissue microarray construction

TMA is a high throughput tool that allows hundreds of tissue samples to be analysed quickly, and conveniently, this method allows all tissue samples in an experiment to be analysed under standardized conditions. In our study, each TMA was constructed in the way described many times before.\textsuperscript{16} The sections were placed on slides coated with 3-aminopropyltriethoxysilane. The non-cancer tissue samples were taken at a distance of > 3 cm from the tumour margin. For TMAs detection, 90 cases of PC and matched non-tumour tissues were obtained between January 2001 and December 2006 from Shanghai Outdo Biotech Co, LTD (TMA number: HPanA150Su01). All these human tissue samples were obtained with appropriate bioethics approvals and informed consents. Diagnoses of PC were confirmed on the basis of pathological evidence. These PC patients had not received any preoperative anti-cancer therapy before surgery. All clinicopathological features of these PC patients were provided (Table 2), and tumour differentiation grades and clinical stages were classified based on the 7th American Joint Committee on Cancer (AJCC) TNM classification. A pathologist participated in reviewing the process.

2.3 | Immunohistochemistry

Immunohistochemistry technology can detect antigens in tissue sections through immunological and chemical reactions, and this technique has high sensitivity and specificity and can detect a variety of antigens in tissue.\textsuperscript{17} We placed the paraaffin-coated microarray sections on a 60°C heating block for 30 min and continuously washed with xylene. The slides were rehydrated in different concentrations of alcohols and boiled in a pressure cooker containing 6.5 mm sodium citrate buffer to restore the antigen.\textsuperscript{18} Then, we used 3% hydrogen peroxide to block the endogenous peroxidase activity for about 30 min at room temperature. Pre-incubate the slides with bovine serum albumin (BSA) in 0.1-mM Tris-buffered saline (TBS) for 2 hour to reduce non-specific background. Then we used rabbit monoclonal CAMKI antibody (ab68234, abcam) diluted 1:1000 in BSA to incubate slides at 4°C overnight. After incubation
with antibodies and BSA, we rinsed the slides with 0.05% Tween-20 three times, 5 min each time and secondary antibody was used to incubate with the slides for 2 h at room temperature. The slides were developed in dianinobenzidine solution and stained with haematoxylin. 3 representative fields of each case were collected by Leica Aperio Image Scope software to ensure homogeneity and representativeness. The immunoreactivity score (IRS) assessments of CAMKI were performed by two independent pathologists without knowing the clinical pathological data. The immunohistochemical staining results were considered both the intensity of staining and the score for positive area. The scoring criteria for staining intensity were as follows: 0(negative), 1(weak), 2 (moderate) and 3 (strong). The criteria for the score for positive area were 0 (<10%), 1 (11-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). Then the final expression score was calculated as the staining intensity score × positive area score, ranging from 0 to 12. A total score of 6 or higher were grouped as high expression group, and less than 6 was grouped as low expression group. The above criteria for the score were performed according to a previously described published literature.19

2.4 | PPI network construction and KEGG pathway analysis

STRING database (https://string-db.org/cgi/input.pl) can collect and integrate known and predicted protein-protein association data. The associations in STRING database include direct (physical) interactions and indirect (functional) interactions, as long as both are specific and biologically meaningful.20 The identification and characterization of protein-protein interactions will be necessary to better understand the functions and efficacy of CAMK1. In this study, we used STRING database to construct PPI network of CAMK1 with minimum required interaction score 0.7 and the interaction predictions were mainly derived from textmining, experiments, databases, co-expression and co-occurrence, et al. The KEGG pathway analysis was also constructed by STRING database.

2.5 | Statistical analysis

The CAMK1 expression levels between PC tissue and normal tissue were evaluated by the GEPIA 2 and the Oncomine database. The expression score in the HPA database describes a knowledge-based best estimate of the true protein expression. The survival analyses were estimated by GEPIA 2 and KM Plotter database. The overall survival was estimated using the Kaplan-Meier method with a logrank test. Furthermore, the variables with statistical significance in univariate analysis were included in multivariate analysis to identify independent prognostic factors by Cox proportional hazard regression model. The correlation between

![FIGURE 1](image)

**FIGURE 1** The expression of CAMK1 analysed by GEPIA 2. CAMK1 had significant expression level in pancreatic cancer specimen compared to normal specimen.*P < .01. Red colour means pancreatic cancer tissues and grey colour means normal tissues.

| Group                  | The number of samples | Percentage (%) |
|------------------------|-----------------------|----------------|
| CAMK1 staining         |                       |                |
| High                   | 1                     | 9 (1 of 11)    |
| Medium                 | 6                     | 55 (6 of 11)   |
| Low                    | 2                     | 18 (2 of 11)   |
| Not detected           | 2                     | 18 (2 of 11)   |
| CAMK1 intensity        |                       |                |
| Strong                 | 1                     | 9 (1 of 11)    |
| Moderate               | 7                     | 64 (7 of 11)   |
| Weak                   | 2                     | 18 (2 of 11)   |
| Negative               | 1                     | 9 (1 of 11)    |
| CAMK1 quantity         |                       |                |
| >75%                   | 5                     | 45 (5 of 11)   |
| 75% -25%               | 3                     | 27 (3 of 11)   |
| <25%                   | 2                     | 18 (2 of 11)   |
| None                   | 1                     | 9 (1 of 11)    |
| CAMK1 location         |                       |                |
| Nuclear                | 0                     | 0              |
| Cytoplasmic/membranous | 10                    | 91 (10 of 11)  |
| Cytoplasmic/membranous, nuclear | 0 | 0 |
| None                   | 1                     | 9 (1 of 11)    |
CAMK1 expression and clinicopathological characteristics was estimated by Chi-square test. The $P$ value $<0.05$ was considered as a statistical significance.

3 | RESULTS

3.1 | CAMK1 was highly expressed in pancreatic cancer in bioinformatics database

The GEPIA 2 database was used to determine CAMK1 expression in PC and normal tissues. This results showed that CAMK1 expression was higher in PC tissue (red box) than normal tissue (grey box) ($P < .01$, Figure 1). Next, the expression of CAMK1 was further validated with Oncomine database. The findings in the Oncomine database showed that CAMK1 mRNA expression was elevated in PC tissues when compared to normal tissues in the Logsdon Pancreas's dataset with the reporter L41816 (Figure S1A), the Ishikawa Pancreas's dataset with the reporter 204 392 (Figure S1B) and the Iacobuzio-Donahue Pancreas 2's dataset with the reporter 52 629(1) (Figure S1C). We also yielded and analysed IHC data for PC tissues from the HPA database, and the CAMK1 staining showed moderate to strong cytoplasmic immunoreactivity in most PC tissues (Antibody HPA051409) (Table 1).

3.2 | Predicting the prognostic values of CAMK1 in pancreatic cancer based on GEPIA 2 and KM Plotter database

To better understand the relevance of CAMK1 expression in PC patients, we investigated the relationship between CAMK1 expression and clinical characteristics of PC patients in GEPIA 2 and KM Plotter database. It was found that high expression of CAMK1 was associated with better OS and DFS for PC patients in GEPIA 2 database ($HR = 0.57$, Logrank $P = .0064$; $HR = 0.58$, Logrank $P = .014$) (Figure 2A,B). To further investigated the prognostic potential of CAMK1 in PC, KM Plotter database was used to evaluate the CAMK1 prognostic value. Again, we found that high CAMK1 expression was correlated with better OS and DFS ($HR = 0.5$, Logrank $P = .0014$; $HR = 0.41$, Logrank $P = .029$) (Figure 2C,D).

3.3 | Independent validation of prognostic value of CAMK1 by TMA-based IHC

Considering the results of prognostic value of CAMK1 in database, we further validated the prognostic value of CAMK1 expression by using TMA-based IHC in 90 paired PC tissues and
corresponding adjacent non-tumour tissues. Eventually, eliminating 8 ineffective tissues, 82 PC tissues included, with 50 male and 32 female. The median age of the patients was 60 years, ranging from 83 years to 34 years. In TMA-based IHC, CAMK1 was mainly located in the cytoplasm of PC cells, and the different staining intensities of CAMK1 were displayed in Figure 3. CAMK1 protein levels were up-regulated in PC tissues compared to the adjacent tissues (Figure 4). Higher expression of CAMK1 was associated with a better OS of PC patients (median OS 15 vs. 8 months, \( P = .0047 \), Figure 4B). The univariate analyses indicated that CAMK1 expression, grade and TNM stage played important roles in the prognosis of PC (\( P = .007 \), \( P < .001 \) and \( P = .011 \), respectively). These variables with statistical significance in the univariate analyses were included in a multivariate regression analysis.
The results showed that grade and TNM stage were the significant independent prognostic factors of PC (P < .001 and P = .002, respectively), but not CAMK1 (Figure 4C). Furthermore, the association between clinicopathological variables and CAMK1 immunostaining was also analysed using Pearson’s chi-square test (Table 2). The results showed that CAMK1 expression in PC may be associated with TNM stage and N stage (P = .013 and 0.038, respectively).

### 3.4 PPI network and KEGG pathway analysis

The PPI information about CAMK1 can be evaluated by STRING database. A PPI network consisted of 11 nodes and 28 edges. Each node represented all the proteins produced by a single, protein-coding gene locus and each edge represented the predicted functional associations. The predicted functional genes with CAMK1 mainly included CALM1, CALM3, CREB1, CALM2, SYN1, NOS3, ATF1, GAPDH, PPM1F and FBXL12 (Figure 5). The PPI information and pathway data enrichment analysis indicated that CAMK1 was mainly enriched in several KEGG pathways associated with aldosterone synthesis and secretion, oxytocin signalling pathway, et al (Supplementary Table 1). The candidate genes in these pathways included CALM1, CREB1, ATF1 and NOS3, and they were all up-regulated in PC (P < .05, Figure S2).

### 4 DISCUSSION

Calcium is a widespread second messenger which controls various mechanisms required for cell motility. In human body, CaM transmits information to many interaction partners by sensing local changes in Ca2+ concentration. Ca2+/CaM complex can modulate the activities of enzymes, channels, signals, adaptor and structural proteins, thereby regulating the functions of related signalling pathways that control various cell functions.21-23 It is reported that CaM can regulate cell growth and its function may change in malignant tumours.24,25 Changes in CaM-dependent cell
cycle and proliferation have been observed in many tumour cells.\textsuperscript{26} Targeting CaM and CaM-dependent systems are considered useful strategies for potential cancer treatment interventions. It has achieved modest success by using chemical antagonists to inhibit CaM function or its targets, or by using interfering RNA to down-regulate its expression alone or in combination with different chemotherapy drugs. CAMK1 is involved in multiple cell functions, including calmodulin binding, ATP binding, signal transduction, development and cell differentiation (GO database). Based on the GEPIA 2 database, Oncomine database and the HPA database, we demonstrated that compared to adjacent non-cancer tissues, CAMK1 was highly expressed in PC tissues. The IHC data from the HPA database also revealed that the CAMK1 staining showed moderate to strong cytoplasmic immunoreactivity in most of PC tissues. Moreover, the IHC score showed CAMK1 protein levels were up-regulated in PC tissues compared to the corresponding non-cancer tissues. The patients with CAMK1 higher expression also showed a superior OS compared to patients with CAMK1 lower expression.

The PPI information and pathway data enrichment analysis indicated that CAMK1 was mainly enriched in several KEGG pathways associated with aldosterone synthesis and secretion, oxytocin signalling pathway, et al, the candidate genes in these pathways included CALM1, CREB1, ATF1 and NOS3. Notably, these candidate genes were significantly up-regulated in PC.

NOS3 locates on chromosome 7q36 and can encode endothelial nitric oxide synthase (eNOS), which produce nitric oxide (NO).\textsuperscript{27} NO is one of the smallest molecules in nature which plays key roles in cancer formation and progression.\textsuperscript{28-30} The generation of NO gradients around the blood vessels can normalize the tumour blood vessels and improve the response to anti-cancer therapy.\textsuperscript{31} Recent research showed that the NanoNO, a nanoscale carrier that enables sustained NO release, can suppress tumour progression in combination with small-molecule chemotherapy, macromolecular therapeutic agents.\textsuperscript{32} Evidence supporting that allosteric

| variables                        | CAMK1 |          | Total | $\chi^2$ | p value |
|----------------------------------|-------|----------|-------|----------|---------|
| Age (year)                       |       |          |       |          |         |
| ≤ 60                             | 18    | 21       | 39    | 1.589    | 0.208   |
| > 60                             | 14    | 29       | 43    |          |         |
| Sex                              |       |          |       |          |         |
| Female                           | 9     | 23       | 32    | 2.62     | 0.106   |
| Male                             | 23    | 27       | 50    |          |         |
| Grade                            |       |          |       |          |         |
| I/II                             | 20    | 35       | 55    |          |         |
| III                              | 12    | 15       | 27    |          |         |
| T stage                          |       |          |       |          |         |
| T1/T2                            | 25    | 45       | 70    | 1.355    | 0.244   |
| T3                               | 7     | 5        | 12    |          |         |
| Tumour sizes (cm)                |       |          |       |          |         |
| ≤ 5                              | 19    | 32       | 51    | 0.178    | 0.674   |
| > 5                              | 13    | 18       | 31    |          |         |
| N stage                          |       |          |       |          |         |
| N0                               | 13    | 32       | 45    | 4.305    | 0.038   |
| N1                               | 19    | 18       | 37    |          |         |
| TNM stage                        |       |          |       |          |         |
| I                                | 9     | 28       | 37    | 6.123    | 0.013   |
| II                               | 23    | 22       | 45    |          |         |
| Invasion of nerve, lymph or blood vessels | No  | 20  | 34 | 54 | 0.262 | 0.608 |
|                                  | Yes   | 12 | 16 | 28 | | |

TABLE 2: Correlation between CAMK1 expression and clinicopathological characteristics of pancreatic cancer patients in the TMA-IHC cohort. $P < .05$ was considered statistically significant
interaction of Ca2+/CaM complex with NOS is essential in NOS activation and NO release. The Ca2+-dependent pathway involving Ca2+-binding protein CaM can activate NOS3. Together these findings, we hypothesized that CAMK1 may also play important roles in regulation of NOS3 expression, although the precise mechanism underlying the association between CAMK1 and NOS3 requires further study. CREB is a nuclear transcription factor activated by phosphorylation at Ser133 by multiple serine/threonine (Ser/Thr) kinases. CREB can bind CREB-binding protein (CBP) to initiate creb-dependent gene transcription through phosphorylation. Present studies showed that CREB plays important roles in tumour initiation, progression and metastasis. Targeting CREB-CBP interaction to inhibit CREB-mediated gene transcription has become a hot spot in cancer treatment research. ATF1 plays a key role in tumour progression in a tumour-specific manner. Overexpression of ATF1 has been found in various cancer. In lung cancer, ATF1 expression was associated with metastasis, tumour stage and poor prognosis, and in oesophageal cancer, ATF1 expression was correlated with poor differentiation, lymph node metastasis and early tumour invasion.

In conclusion, it seemed that CAMK1 might be a promising biomarker for a better prognosis in PC patients, although the potential effect of CAMK1 expression on the biological function of PC and the reason for better prognosis remains to be further investigated. The PPI data only provided potential probabilities for interactions between genes based on different sources of information, and the underlying molecular mechanisms of CAMK1 in PC would be further explored.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that would influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

Yangyang Lei: Formal analysis (lead); Conceptualization (equal); Data curation (equal); Writing-original draft (equal). Tianzhu Yu: Methodology (supporting); Project administration (equal); Supervision (equal); Validation (equal); Writing-original draft (supporting). Changyu Li: Resources (equal); Writing-original draft (supporting). Jianke Li: Validation (equal); Writing-original draft (supporting). Yicheng Liang: Supervision (equal); Validation (equal). Xinyuan Wang: Writing-original draft (supporting). Yi Chen: Project administration (equal); Supervision (equal). Xiaolin Wang: Funding acquisition; Project administration; Writing-review & editing.

DATA AVAILABILITY STATEMENT

Some publicly available datasets were analysed in this study. The authors confirm that these data can be found here: http://gepia2.cancer-pku.cn; www.oncomine.org; https://kmplot.com/analysis; https://string-db.org/cgi/input.pl; http://www.proteinatlas.org. These analyses of protein expression data of CAMK1 in the HPA database can be directly obtained from https://www.proteinatlas.org/ENSG00000134072-CAMK1/pathology/pancreatic+cancer#ihc.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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