Kaempferol induces ROS-dependent apoptosis in pancreatic cancer cells via TGM2-mediated Akt/mTOR signaling

Wang et al. BMC Cancer (2021) 21:396
https://doi.org/10.1186/s12885-021-08158-z

Abstract

Background: Kaempferol, a natural flavonoid, exhibits anticancer properties by scavenging reactive oxygen species (ROS). However, increasing evidence has demonstrated that, under certain conditions, kaempferol can inhibit tumor growth by upregulating ROS levels. In this study, we aimed to investigate whether kaempferol effectively suppresses pancreatic cancer through upregulation of ROS, and to explore the underlying molecular mechanism.

Methods: PANC-1 and Mia PaCa-2 cells were exposed to different concentrations of kaempferol. Cell proliferation and colony formation were evaluated by CCK-8 and colony formation assays. Flow cytometry was performed to assess the ROS levels and cell apoptosis. The mRNA sequencing and KEGG enrichment analysis were performed to identify differentially expressed genes and to reveal significantly enriched signaling pathways in response to kaempferol treatment. Based on biological analysis, we hypothesized that tissue transglutaminase (TGM2) gene was an essential target for kaempferol to induce ROS-related apoptosis in pancreatic cancer. TGM2 was overexpressed by lentivirus vector to verify the effect of TGM2 on the ROS-associated apoptotic signaling pathway. Western blot and qRT-PCR were used to determine the protein and mRNA levels, respectively. The prognostic value of TGM2 was analyzed by Gene Expression Profiling Interactive Analysis (GEPIA) tools based on public data from the TCGA database.

Results: Kaempferol effectively suppressed pancreatic cancer in vitro and in vivo. Kaempferol promoted apoptosis in vitro by increasing ROS generation, which was involved in Akt/mTOR signaling. TGM2 levels were significantly increased in PDAC tissues compared with normal tissues, and high TGM2 expression was positively correlated with poor prognosis in pancreatic cancer patients. Decreased TGM2 mRNA and protein levels were observed in the cells after treatment with kaempferol. Additionally, TGM2 overexpression downregulated ROS production and inhibited the abovementioned apoptotic signaling pathway.

Conclusions: Kaempferol induces ROS-dependent apoptosis in pancreatic cancer cells via TGM2-mediated Akt/mTOR signaling, and TGM2 may represent a promising prognostic biomarker for pancreatic cancer.

Keywords: Pancreatic cancer, Kaempferol, Tissue transglutaminase, ROS, Apoptosis, Biomarker
Background
Pancreatic cancer is the third most lethal malignancy, with associated mortality rates that continue to increase [1]. To date, surgery remains the only curative therapy for patients with pancreatic cancer. However, only 10–20% of patients have access to curative resection due to the notoriously difficult diagnosis [2]. Moreover, there have been few improvements in first- and second-line palliative therapies or progress in increasing survival with adjuvant treatment in pancreatic cancer [3]. Therefore, it is imperative to identify biomarkers for the early identification of novel, effective therapies for pancreatic cancer.

Numerous factors are involved in the instability and pathogenesis of pancreatic cancer, such as proinflammatory cytokines, oxidative stress and microenvironment-associated proteins. Among them, oxidative stress is commonly referred to as a process in which reactive oxygen species (ROS) produced by cellular metabolism are coupled with ROS scavenging by antioxidative mechanisms that prevent potential ROS-induced cytotoxicity and maintain cell survival [4]. In recent years, it has become apparent that ROS are implicated in the regulation of biological and physiological processes [5, 6]. Elevated ROS production is generally thought to be protumorigenic, leading to DNA, protein and lipid damage, increasing adaptations to hypoxia and promoting genetic instability and tumorigenesis [7, 8]. However, the roles of ROS in pancreatic cancer were shown to function as a double-edged sword that either facilitates cancer progression or dramatically promotes cell death [9, 10]. Evidence has demonstrated that cell apoptosis and senescence occur at toxic levels of ROS [11]. Given the dual roles of ROS, eliminating or increasing ROS levels may be a potentially effective cancer therapy, although realizing the potential of this approach has remained challenging.

Flavonoids are secondary plant metabolites that include kaempferol, luteolin and apigenin and are widely known to attenuate tissue damage or fibrosis through their antioxidant and anti-inflammatory activities [12, 13]. Kaempferol was recently reported to have ROS-mediated anticancer properties, including the inhibition of cell proliferation, cell cycle arrest, the induction of apoptosis and the suppression of cell invasion and migration, in diverse cancers, such as colorectal cancer, breast cancer, melanoma and hepatoma [14–17]. For example, kaempferol triggers ROS-induced apoptosis in human breast cancer through the JNK signaling pathway [16] and potentiates apoptosis in human hepatoma cells through the p53-inducible gene 3 (PIG3)-regulated mitochondrial apoptotic pathway [17]. However, it remains unclear how kaempferol alters ROS levels (upregulation or downregulation), as do the underlying mechanisms by which kaempferol inhibits pancreatic cancer.

In the present study, we investigated the anticancer activities of kaempferol on pancreatic cancer, the results of which showed that kaempferol promotes the potentiation of apoptosis and the inhibition of cell proliferation. Moreover, tissue transglutaminase (TGM2) overexpression via lentiviral vector-mediated transfection reversed the kaempferol-mediated induction of apoptosis. Collectively, our results demonstrated that kaempferol induces apoptosis in pancreatic cancer cells via ROS-mediated Akt/mTOR pathway inactivation, and that pharmacological inhibition of TGM2 by kaempferol in vitro and in vivo may provide a novel treatment option for the treatment of pancreatic cancer.

Materials and methods
Cells lines and reagents
The human pancreatic cancer cell lines Mia PaCa-2 and PANC-1 were obtained from the American Type Culture Collection (ATCC, USA). Cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37 °C under an atmosphere with 5% CO₂. Kaempferol powder (C₁₅H₁₀O₆, molecular weight: 286.24, purity > 99.0%) was purchased from Sigma-Aldrich (Shanghai, China). Kaempferol is a lipophilic polyphenol that is soluble in organic solvents. A 150 mM kaempferol stock solution was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. N-Acetyl-L-cysteine (NAC) was obtained from Beyotime (Shanghai, China).

Establishment of TGM2-overexpressing cell lines
We used a lentiviral vector-mediated transfection method to overexpress TGM2 in Mia PaCa-2 and PANC-1 cells. The coding sequence of TGM2 was inserted into the UbimCS-3FLAG-SV40-EGFP-IRES-puromycin lentiviral vector (GV358; GENE, Shanghai, China). The lentivirus TGM2-overexpressing constructs were co-transfected with pHelper 1.0 and pHelper 2.0 vectors at a ratio of 4:3:2 into HEK293T cells.

Cell viability assay
A Cell Counting Kit-8 (CCK-8; DOJINDO, Shanghai, China) was used to measure cell viability. In brief, Mia PaCa-2 and PANC-1 cell suspensions (1 × 10⁴ cells/ml) were seeded in 96-well plates with 200 μl of DMEM and preincubated for 24 h. After adherence, the cells were treated with different doses of kaempferol (0, 2.5, 10, 25, 100, 250 and 1000 μmol/L) and then incubated at 37 °C for an additional 48 h. After the indicated incubation times, the cells were incubated with a CCK-8 DMEM solution (10 μl of CCK-8 in 100 μl of DMEM) for 2 h. Then, the absorbance at 450 nm was measured using a microplate reader. The half maximal inhibitory
concentration (IC50) values, which indicate 50% inhibition of cell viability, were determined by generating proliferation inhibition curves. Concentrations lower than the IC50 values were used in subsequent experiments.

**Cell apoptosis assay**

Cell apoptosis was evaluated by flow cytometry by staining cells with FITC Annexin V in combination with propidium iodide (PI) or 7-amino-actinomycin (7-AAD) (BD Bioscience, San Diego, CA, USA) according to the manufacturer's recommendations.

**Colony formation assay**

Suspended pancreatic cells were seeded in 6-well plates at a density of 500 cells/well. Then, the cells were treated with 0, 25 or 50 μM kaempferol after attachment for 48 h. After 2 weeks of cultivation, cells that formed visible colonies were washed with phosphate-buffered saline (PBS) three times and fixed with 4% paraformaldehyde for 20 min for further staining with 0.1% crystal violet. Cell colonies containing more than 50 cells were counted using a light microscope.

**Western blotting**

Cells were treated with kaempferol, washed with ice-cold PBS and then lysed with RIPA buffer supplemented with phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitor. Cell lysates were centrifuged at 15,000 rpm and 4°C for 15 min, and protein concentrations were determined with a BCA protein assay kit (Beyotime, Shanghai, China). Subsequently, equal amounts of protein samples were separated by electrophoresis using 6, 10 or 12.5% SDS-PAGE Gel Fast Preparation Kits (Epizyme, Shanghai, China) and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with antibodies against the following proteins: β-actin (1:1000; Proteintech), Akt (1:1000; Proteintech), phospho-Akt (Ser473) (1:1000; CST), TGM2 (1:1000; CST), Kelch-like ECH-associated protein 1 (KEAP1) (1:1000; Proteintech), NF-E2-related factor 2 (NRF2) (1:1000; Proteintech), mTOR (1:1000; Proteintech), phospho-mTOR (S2448) (1:1000; Abcam), Bax (1:1000; Proteintech), Bcl2 (1:1000; Proteintech), Caspase 3 (1:500; Proteintech), PARP1 (1:1000; Proteintech), and cleaved PARP1 (1:1000; Abcam).

**RNA preparation and quantitative real-time PCR**

Total RNA was extracted with RNAiso Plus (Takara, Tokyo, Japan). Reverse transcription was conducted using Takara PrimeScript™ RT Master Mix to obtain cDNA. Quantitative RT-PCR was performed with TB Green® Premix Ex Taq™ to determine the expression of candidate genes using an ABI 7900HT Real-Time PCR system (Applied Biosystems, CA, USA). The primer sequences are listed in Table 1.

**mRNA sequencing**

Total RNA was isolated from pancreatic cells treated with kaempferol (50 μM) and control cells were isolated with TRIzol reagent (Invitrogen, China). Both cell lines were analyzed in triplicate by mRNA sequencing, which was conducted by Sangon Bitotech (Shanghai, China) using the Dual-mode mRNA Library Prep Kit for Illumina. The fragments per kilobase of exon per million fragments mapped reads (FPKM) for each gene were calculated after data processing [18].

**Reactive oxygen species detection**

The accumulation of intracellular ROS was assessed using an ROS assay kit (Beyotime, Shanghai, China). Cells were incubated with 10 μmol/L DCFH-DA at 37°C for 30 min before being collected and suspended in ice-cold PBS. DCFH-DA is further oxidized by ROS after deacetylation to form 2,7-dichlorofluorescein (DCF). The DCF fluorescence intensities were then measured by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. ROS levels in TGM2-overexpressing cell lines were detected using dihydroethidium (DHE), which avoids the effects of EGFP fluorescence.

**Immunohistochemistry staining**

Immunohistochemistry (IHC) staining was performed as previously described [19]. In brief, the paraffin-embedded tumor sections were dewaxed in xylene and then rehydrated with a graded alcohol series. A 3% H2O2 methanol buffer was used to block endogenous peroxidase activity, and citrate buffer (pH 6.0) was used for antigen retrieval. Tumor sections were incubated with a primary anti-TGM2 antibody (1:300, CST) at 4°C overnight and then incubated with a secondary antibody at room temperature. TGM2 expression levels were assessed by determining the immunoreactive scores (IRS), which were determined by multiplying the percentage of positive cells ((0, < 5%; 1, 5~25%; 2, 25~50%; 3, 50~75%; and 4, 75%~100%) by the intensity scores (0, negative expression; 1, weak; 2, moderate; 3, strong). A score ≥ 4 was defined as high TGM2 expression, with lower scores indicating low expression.

**In vivo tumor Xenograft models**

Twelve male BALB/c mice (4–6 weeks old, 18–20 g) were purchased from Shanghai Jihui Experimental...
Animal Breeding Co., Ltd. and housed under specific pathogen-free conditions with free access to food and water. Approximately $1 \times 10^7$ PANC-1 cells in 200 μl of PBS were subcutaneously injected into the right flank of each mouse to establish the tumor model. The mice with tumor xenographs were then randomly assigned to the kaempferol and control groups ($n = 6$ in each group). Treatment was initiated when a tumor reached approximately 100 mm$^3$ in size. Many previous experiments have confirmed that an appropriate high dose of kaempferol does not produce cytotoxicity but plays a protective role. For instance, Cheng X et al. suggested that the pre-treatment with kaempferol (25, 50 and 100 mg/kg) by gavage for 7 days significantly alleviated inflammatory responses and blood-brain barrier dysfunction [20]. Luo C et al. showed that kaempferol at doses of 50 and 150 mg/kg dose-dependently improved blood lipids in the treatment of diabetic rats [21]. Based on the abovementioned dose safety report, we chose a dose of 100 mg/kg for intragastric administration. The mice in the control and kaempferol-treated groups were orally administered PBS or 100 mg/kg kaempferol for 2 weeks daily, respectively. The mice were closely monitored starting from the first treatment, and the tumor sizes were calculated twice every week using the following formula: tumor volume = length × (width)$^2$/2. The mice were sacrificed 6 weeks after injection, and paraffin-embedded tumor tissue sections were generated for subsequent analysis.

**Statistical analysis**
Experiments were repeated at least three times, and the results are presented as the means ± SD. Two-tailed unpaired Student’s t tests or one-way ANOVA were used to evaluate the data. GraphPad Prism (version 7.0a) was used for the data analysis. Differences were considered statistically significant at $P < 0.05$.

**Results**

**Cytotoxic effects of kaempferol on pancreatic cancer**

Kaempferol is a flavonoid compound that is primarily found in the Chinese herbal medicine Zingiberaceae, and its structure is shown in Fig. 1a. Kaempferol exhibited anticancer effects on PANC-1 and Mia PaCa-2 cells in a concentration-dependent manner, with decreased cell viability observed at high concentrations. The IC50 values of kaempferol in PANC-1 and Mia PaCa-2 cells were 78.75 μM and 79.07 μM, respectively (Fig. 1b). Based on the observed IC50 values, kaempferol was used at concentrations of 0, 25 and 50 μM in subsequent analyses. The CCK-8 assay results showed that the rates of cell proliferation were inhibited after 24 h, and significant dose-dependent inhibitory effects between 25 μM and 50 μM were detected after 96 h in both PANC-1 and Mia PaCa-2 cells ($P < 0.05$) (Fig. 1c). Furthermore, colony formation assay results indicated that colony formation was suppressed by treatment with increasing concentrations of kaempferol (Fig. 1d, e).

**Kaempferol induces apoptosis through the Akt/mTOR signaling pathway**

RNA sequencing was performed to investigate the underlying mechanisms by which kaempferol inhibits pancreatic cancer. KEGG enrichment analysis based on significantly differentially expressed genes revealed a series of enriched signaling pathways, including apoptosis signaling (Fig. 2a). To further confirm these results, cells were examined using flow cytometry after incubation with 0, 25 and 50 μM kaempferol for 48 h. Our results showed a significant increase in PANC-1 and Mia PaCa-2 cell apoptosis in response to 50 μM of kaempferol (13.93 ± 1.474% and 21.50 ± 3.195%, respectively), and the rate of apoptosis was positively correlated with kaempferol concentration (Fig. 2b, c). Moreover, this finding is consistent with the observed levels of important proteins involved in cell apoptosis, such as Bcl-2, Bax, Caspase 3, and PARP1 (Fig. 2d). The Akt/mTOR signaling pathway, which is widely acknowledged to have crucial functions in cells, is activated in many cancers and inhibits apoptosis, contributing to drug resistance in the clinic [22]. Consistent with these previous findings, in our present study, Akt and mTOR phosphorylation were downregulated after 48 h of kaempferol treatment (Fig. 2e). Collectively, our results indicate that Akt/mTOR signaling inactivation is involved in kaempferol-induced apoptosis of pancreatic cells.

**Kaempferol-mediated Akt/mTOR signaling occurs in an ROS-dependent manner**

Kaempferol, a flavonoid phytochemical, has a potential role in regulating cellular processes by mediating oxidative stress [23, 24]. Therefore, we hypothesized that kaempferol disrupts Akt/mTOR signaling by inducing alterations in ROS levels. Therefore, we assessed the production of ROS after treatment with various concentrations of kaempferol using an ROS assay kit. ROS levels increased in cancer cells concomitantly with increases in the kaempferol concentration (Fig. 3a). The KEAP1-NRF2 system is the major node that senses and responds to redox disturbances resulting from intrinsic and extrinsic factors [25, 26]. The dual roles of the KEAP1-NRF2 pathway in preventing or initiating pancreatic cancer primarily depend on the stages of cancer and the states of KEAP1 and NRF2 [27]. Therefore, in our present study, we subsequently evaluated KEAP1 and NRF2 expression to confirm the occurrence of redox imbalance. Immunoblotting results indicated that KEAP1 levels were downregulated, while NRF2 levels were increased to some extent in pancreatic cancer cells.
treated with kaempferol (Fig. 3b). Subsequently, we examined the effects of NAC, which scavenges ROS, on Akt and mTOR phosphorylation, and diminished ROS levels are shown in Fig. 3c. The decrease in Akt Ser473 and mTOR S2448 phosphorylation in PANC-1 and MiaPaCa-2 cells was reversed by NAC treatment (Fig. 3d). In summary, our results demonstrated that kaempferol increases ROS levels, which in turn suppresses the Akt/mTOR signaling pathway.

Kaempferol downregulates TGM2 in vitro and in vivo, and TGM2 expression is negatively correlated with PDAC prognosis

The results of a heat map clustering analysis revealed notable differences in gene expression between the control and kaempferol-treated groups, with significant TGM2 downregulation detected in the kaempferol-treated groups (Fig. 4a). Decreased TGM2 mRNA and protein levels were observed in the cells after treatment with kaempferol (Fig. 4b, c). In addition, we assessed the effect of kaempferol on tumor growth in vivo using a subcutaneous xenograft model. Mice treated with 100 mg/kg of kaempferol exhibited significantly suppressed the tumor growth, including tumor weight and tumor volume, compared to that observed in the control group (Fig. 4d, e). Furthermore, kaempferol had no significant inhibitory effect on the weights of mice (Fig. 4f). To further investigate the role of TGM2 in pancreatic cancer, we performed IHC staining to examine TGM2 expression in tumor tissues (Fig. 4g). The results showed that
TGM2 levels were significantly decreased in the group treated with kaempferol, which was consistent with the in vitro findings. Subsequently, we analyzed publicly assessable data using the gene expression profiling interactive analysis (GEPIA) tools to assess TGM2 gene expression in PDAC and normal tissues (number (T) = 179 and number (N) = 171, respectively). The results showed higher TGM2 transcription in PDAC tissues than in normal tissues, while differences were not observed between the different stages (Fig. 4h, i). Furthermore, higher TGM2 mRNA expression was correlated with shorter overall survival (OS) (\(P = 0.011\)) and disease-free survival (DFS) (\(P = 0.000\)) (Fig. 4j, k). These results indicated that TGM2 may be a potential target of kaempferol to inhibit pancreatic cancer and could serve as a promising prognostic biomarker for this disease.

TGM2 functions as a target of kaempferol to promote pancreatic cancer cell apoptosis via ROS-dependent Akt/mTOR signaling

Given the crucial role of TGM2 in responding to oxidative stress, which involves various cellular processes, including apoptosis, we next investigated the potential functions of TGM2 in pancreatic cancer. We hypothesized that TGM2 serves as a target of kaempferol to facilitate apoptosis, which may be correlated with ROS-dependent Akt/mTOR signaling in pancreatic cancer. To assess this possibility, we stably overexpressed TGM2 in PANC-1 and Mia PaCa-2 cells, and the overexpression efficiency was confirmed by western blotting and quantitative RT-PCR (Fig. 5a, b). ROS production was significantly decreased upon overexpression of TGM2 in both the pancreatic cancer cell lines (Fig. 5c). The rate of cell apoptosis was accordingly reversed in TGM2-overexpressing
PANC-1 and Mia PaCa-2 cells in response to 50 μM kaempferol treatment when compared to the scramble group (2.733 ± 0.285% vs 16.8 ± 1.044% and 7.733 ± 0.328% vs 30.03 ± 1.749%, respectively) (Fig. 5d, e), suggesting that TGM2 plays a crucial role in pancreatic cancer cell apoptosis. Moreover, the observed levels of KEAP1, NRF2, p-AKT and p-mTOR in TGM2-overexpressing cells following kaempferol treatment were consistent with the aforementioned results, indicating that kaempferol promotes apoptosis via the TGM2-mediated ROS-dependent Akt/mTOR signaling pathway (Fig. 5f).

Discussion
Redox biology is ubiquitous among different cell types and biological systems and is important in many different fields of study, such as neuroscience, inflammation and cancer [28]. Redox homeostasis is essential for aerobic systems and is dependent on a sustained balance between ROS formation and detoxification [29]. It has long been postulated that tumor cells exhibit continuous and elevated ROS production as a consequence of genetic, metabolic and microenvironment-associated alterations [29]. Interestingly, it seems paradoxical that cancer cells reprogram metabolic mechanisms toward glutamine utilization in the TCA cycle to attenuate ROS-induced cell damage and maintain cellular redox homeostasis [30]. Considering their abovementioned dualistic nature, ROS act as harmful or beneficial molecules to regulate cellular physiology or trigger cytotoxicity depending on their duration and concentration [31]. Therefore, the development of strategies aimed at leveraging cellular redox signaling events in cancer to
develop effective anticancer therapeutic treatments or targeting ROS regulators is important for cancer treatment.

As one of the processes associated with cancer initiation and development, apoptosis is a major type of programmed cell death (PCD) that invariably determines the fate of malignant cells to some extent [32]. Furthermore, the Akt/mTOR cascade has emerged as a major regulator in cancer cell apoptosis [33]. Fiorini, C. et al. demonstrated that elevated ROS can act as adaptors to promote pancreatic cancer chemosensitivity and reduce autophagy by inducing the Akt/mTOR pathway [34]. Of note, whether the upregulation or scavenging of ROS in pancreatic cancer involves intricate interrelationships among factors involved in Akt/mTOR-induced apoptosis remains controversial.

Traditional Chinese medicine (TCM) treatments have drawn close attention because of their diverse pharmacological activities, such as anti-inflammatory, antitumor and antioxidant activities, high efficacy and low toxicity [35]. Kaempferol, a typical flavonol-type flavonoid that is abundantly found in a variety of Chinese herbs, exhibits strong inhibitory activity toward cancers, such as gastric cancer and lung cancer [36, 37]. In recent years, kaempferol has become known for its potent ability to scavenge free radicals and superoxide radicals as well as its ability to activate antioxidant enzymes [38]. However, an increasing number of studies have recently focused on its ability to
inhibit cancer by triggering ROS production [14, 39]. In the present study, we observed that when cell apoptosis occurred, ROS levels increased, NRF2 proteins were activated, and KEAP1 expression was downregulated. In the past, NRF2 was proven to contribute to tumor progression and was associated with poor prognosis [40, 41]. However, accumulating evidence consistent with our work suggests that the activation of NRF2 is able to inhibit tumorigenesis. For instance, multiple compounds, such as sulforaphane and curcumin, have been discovered to exert...
anticancer activities by activating NRF2/ARE-regulated genes, such as hemeoxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1) [42]. However, the precise mechanism by which kaempferol activates NRF2 to affect apoptosis in pancreatic cancer needs to be further investigated in our subsequent work.

Mammalian transglutaminases (TGs) are a family of structurally and functionally related proteins with enzymatic and scaffolding functions that participate in the regulation of cell processes and are implicated in the development of disease [43, 44]. TGM2 was the first TG member to be discovered and is a multifunctional protein involved in the pathogenesis of multiple types of cancers [45, 46]. It has been reported to inhibit apoptosis and is associated with chemotherapy resistance in colorectal cancer (CRC) via activation of Wnt/β-catenin signaling [47]. In our present study, we showed that the level of TGM2 mRNA transcription was significantly higher in PDAC tissues than in normal tissues. Furthermore, increased TGM2 mRNA expression was correlated with shorter OS and DFS, suggesting that TGM2 may serve as a prognostic biomarker for pancreatic cancer. Intriguingly, TGM2 is known to be a stress-response gene that can be activated by various stressors, including inflammatory cytokines and ROS [48]. In the present study, our findings showed that kaempferol treatment caused the pharmacological inhibition of TGM2 in vitro and in vivo, and a negative correlation was observed between TGM2 expression and the regulation of ROS-dependent apoptosis, indicating that TGM2 may be a potential anticancer target of kaempferol for pancreatic cancer treatment.

Conclusions
In summary, the results of our present study indicate that kaempferol induces ROS-dependent apoptosis in pancreatic cancer cells via TGM2-mediated Akt/mTOR signaling (Fig. 5g), and TGM2 may represent a promising prognostic biomarker for pancreatic cancer.

Abbreviations
ROS: reactive oxygen species; GEPIA: Gene Expression Profiling Interactive Analysis; TG2: tissue transglutaminase; NAC: N-acetyl-L-cysteine; PCR: polymerase chain reaction; DCF: 2,7-dichlorofluorescein; DHE: dihydroethidium; CCK-8: Cell Counting Kit-8; IC50: half maximal inhibitory concentration; PI: propidium iodide; PBS: phosphate-buffered saline; DMSO: Dimethyl sulfoxide; CCR: Cell Counting Kit-8; IC50: the half maximal inhibitory concentration; PI: Propidium iodide; PBS: Phosphate-buffered saline; PMSF: Phenylmethylsulfonyl fluoride; PVD/POD: Polyvinylidene difluoride; KEAP1: Kelch-like ECH-associated protein 1; NRF2: NF-E2-related factor 2; RT-PCR: Real-Time PCR; DCF: 2,7-dichlorofluorescein; DHE: Dihydroethidium; LSCM: Laser scanning confocal microscopy; TCM: Traditional Chinese medicine; PCD: Programmed cell death

Acknowledgements
We thank the American Journal Experts (AJE) for their help in polishing the manuscript.

Authors’ contributions
Z. Chen and F. Wang conceived and designed the experiments. F. Wang, L. Wang, C. Qu, L. Chen, Y. Geng, C. Cheng, S. Yu, D. Wang, L. Yang and Z. Meng contributed materials and methods. F. Wang and L. Wang performed the experiments and analyzed the data. All authors approved the final manuscript.

Funding
The study was supported by the National Natural Science Foundation of China (grant no. 81930115 to Z. Chen and 81973629 to L. Chen).

Availability of data and materials
All data generated or analyzed during this study are included in this published article. RNA-sequencing data have been deposited into the NCBI Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA699118.

Declarations
Ethics approval and consent to participate
All animal experiments were conducted in accordance with the National Guidelines for the Care and Use of Laboratory Animals. This study was approved by the Laboratory Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine. The study was carried out in compliance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1Department of Integrative Oncology, Fudan University Shanghai Cancer Center, 270 Dong An Road, Shanghai 200032, China. 2Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China. 3Cancer Center, Tenth People’s Hospital of Tongji University, Shanghai 200072, China. 4Cancer Institutes, Fudan University, Shanghai 200032, China. 5Department of Genetics and Cell Biology, Qingdao University Medical College, Qingdao 266071, China.

Received: 29 December 2020 Accepted: 6 April 2021
Published online: 12 April 2021

References
1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2018;68(1):7–30. https://doi.org/10.3322/caac.21442.
2. The Lancet Gastroenterology H. Pancreatic cancer: how can we tackle the lack of progress? Lancet Gastroenterol Hepatol. 2017;2(2):73.
3. Neoptolemos JP, Kleeff J, Michl P, Costello E, Greenhalf W, Palmer DH. Therapeutic developments in pancreatic cancer: current and future perspectives. Nat Rev Gastroenterol Hepatol. 2018;15(6):333–48. https://doi.org/10.1038/s41575-018-0005-x.
4. Mittler R. ROS are good. Trends Plant Sci. 2017;22(1):11–9. https://doi.org/10.1016/j.tplants.2016.08.002.
5. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Curr Biol. 2014;24(10):R53–62. https://doi.org/10.1016/j.cub.2014.03.034.
6. Le Belle JE, Orozco NM, Pauca AA, Saxe JP, Mottahedeh J, Pyle AD, et al. Proliferative neural stem cells have high endogenous ROS levels that regulate self-renewal and neurogenesis in a PI3K/Akt-depandent manner. Cell Stem Cell. 2011;8(1):59–71. https://doi.org/10.1016/j.stem.2010.11.028.
7. Moloney JN, Cotter TG. ROS signalling in the biology of cancer. Semin Cell Dev Biol. 2018;80:50–64. https://doi.org/10.1016/j.semcdb.2017.05.023.
8. Sabharwal SS, Schumacker PT. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles’ heel? Nat Rev Cancer. 2014;14(11):709–21. https://doi.org/10.1038/nrc3803.
9. Martinez-Useros J, Li W, Cabeza-Morales M, Garcia-Foncillas J. Oxidative Stress: A New Target for Pancreatic Cancer Prognosis and Treatment. J Clin Med. 2017;6(3):29. https://doi.org/10.3390/jcm6030029.
23. Du W, Dando I, Zaniboni T, Costanzo C, Dalla Pozza E, Scupoli MT, et al. Gemicatbine/cannabinoid combination triggers autophagy in pancreatic cancer cells through a ROS-mediated mechanism. Cell Death Dis. 2011;2(4)152. https://doi.org/10.1038/cddis.2011.36.

24. Moon DO, Kim MO, Choi YH, Hyun JW, Chang WT, Kim GY. Butein induces G2/M phase arrest and apoptosis in human hepatoma cancer cells through ROS generation. Cancer Lett. 2010;288(2):204–13. https://doi.org/10.1016/j.canlet.2009.07.002.

25. Maleki SJ, Crespo JF, Cabanillas B. Anti-inflammatory effects of flavonoids. Food Chem. 2019;299:125124. https://doi.org/10.1016/j.foodchem.2019.125124.

26. Yamamoto M, Kensler TW, Motohashi H. The KEAP1-NRF2 system: a Thiol-dependent oxidoreductase activity on human cancer cells. Eur J Med Chem. 2015;104:1518–28. https://doi.org/10.1016/j.ejmech.2014.08.011.

27. Luo C, Yang H, Tang C, Yao G, Kong L, He H, et al. Kaempferol alleviates DSS-induced colitis by inhibiting Txnip-dependent NLRP3 inflammasome activation in macrophages via reducing oxidative stress. Micruloc Immun. 2019;12(1):1150–63. https://doi.org/10.1016/j.micim.2019.01.077.

28. Choo JB, Kim JH, Lee H, Pak JN, Shim BS, Kim SH. Reactive oxygen species production and Akt/mTOR pathway in a ROS-dependent manner. Biochim Biophys Acta. 2015;1853(3):549–60. https://doi.org/10.1016/j.bbamcr.2014.12.016.

29. Jan J, Xian F, Qin H, Huang R. The antioxidant, anti-inflammatory and anti-apoptotic activities of the Buthinia Championii flavone are connected with protection against myocardial ischemia/reperfusion injury. Cell Physiol Biochem. 2016;38(3):1365–75. https://doi.org/10.1159/000443080.

30. Zhang X, Kow-Kong Ng P, Kucherlapati M, Chen F, Liu Y, Tsang YH, et al. A Pan-Cancer Proteogenomic Atlas of PI3K/AKT/mTOR Pathway Alterations. Cancer Cell. 2017;31(6):823–32.e823.

31. Piskounova E, Agathocleous M, Murphy MM, Hu Z, Huddlestun SE, Zhao Z, et al. Oxidative stress inhibits distant metastasis by human melanoma cells. Nature. 2015;527(7577):186–91. https://doi.org/10.1038/nature15726.

32. Liu X, Zhao P, Wang X, Wang L, Zhu Y, Gao W. Triptolide induces Glioma cell autophagy and apoptosis via Upregulating the ROS/NJNK and Downregulating the Akt/mTOR signaling pathways. Front Oncol. 2019;9:387. https://doi.org/10.3389/fonc.2019.00387.

33. Zhang Y, Kow-Kong Ng P, Kucherlapati M, Chen F, Liu Y, Tsang YH, et al. A Pan-Cancer Proteogenomic Atlas of PI3K/AKT/mTOR Pathway Alterations. Cancer Cell. 2017;31(6):823–32.e823.

34. Fiocini C, Cordani M, Gotte G, Picone D, Donadelli M. Onconase induces autophagy sensitizing pancreatic cancer cells to gemcitabine and activates Akt/mTOR pathway in a ROS-dependent manner. Biochim Biophys Acta. 2015;1853(3):549–60. https://doi.org/10.1016/j.bbamcr.2014.12.016.

35. Tsuchiya S, Sugimoto K, Kanno H, Okabe K, Kuroda K, Okido M, et al. Kaempferol-immobilized titanium dioxide promotes formation of new bone: effects of loading methods on bone marrow stromal cell differentiation in vivo and in vitro. Int J Nanomedicine. 2018;13:1665–76. https://doi.org/10.2147/IJN.S150786.

36. Luo W, Chen L, Ma X, Jiao R, Li X, Wang Y. Protective effects of kaempferol against reactive oxygen species-induced hemolysis and its antiproliferative activity on human cancer cells. Eur J Med Chem. 2016;114:2432–34. https://doi.org/10.1016/j.ejmech.2016.02.045.

37. Zhao Y, Guo Q, Zhu Q, Tan R, Bai D, X, et al. Flavonoid VI-16 protects against myocardial ischemia/reperfusion injury. Cell Physiol Biochem. 2015;203(1):101–16. https://doi.org/10.1152/physrev.00023.2017.

38. Zhang Q, Cheng G, Qiu H, Zhu L, Ren Z, Zhao W, et al. The p53-inducible gene 3 involved in flavonoid-induced cytotoxicity through the reactive oxygen species-mediated mitochondrial apoptotic pathway in human hepatoma cells. Food Funct. 2015;6(5):1518–28. https://doi.org/10.1039/C5FO00142K.

39. Mortazavi A, Williams BA, McCue K, Schueffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods. 2008;5(7):621–8. https://doi.org/10.1038/nmeth.1226.

40. Dong JL, Wu J, Guo Q, Qu H, Zhu L, Ren Z, et al. The p53-inducible gene 3 involved in flavonoid-induced cytotoxicity through the reactive oxygen species-mediated mitochondrial apoptotic pathway in human hepatoma cells. Food Funct. 2015;6(5):1518–28. https://doi.org/10.1039/C5FO00142K.

41. Cheng X, Yang YL, Yang H, Wang YH, Du GH. Kaempferol alleviates LPS-induced neuroinflammation and BBB dysfunction in mice via inhibiting HMGB1 release and down-regulating TLR4/MyD88 pathway. Int Immunopharmacol. 2018;65:29–35. https://doi.org/10.1016/j.intimm.2018.04.002.

42. Choo JB, Kim JH, Lee H, Pak JN, Shin BS, Kim SH. Reactive oxygen species production and Akt/mTOR pathway in a ROS-dependent manner. Biochim Biophys Acta. 2015;203(1):101–16. https://doi.org/10.1152/physrev.00023.2017.

43. Dong JL, Wu J, Guo Q, Qu H, Zhu L, Ren Z, et al. The p53-inducible gene 3 involved in flavonoid-induced cytotoxicity through the reactive oxygen species-mediated mitochondrial apoptotic pathway in human hepatoma cells. Food Funct. 2015;6(5):1518–28. https://doi.org/10.1039/C5FO00142K.

44. Hernandez-Fernaud JR, Ruengeler E, Casazza A, Neilson LJ, Pulleine E, Santi SK. Downregulating the Akt/mTOR signaling pathways. Front Oncol. 2019;9:387. https://doi.org/10.3389/fonc.2019.00387.

45. Singh A, Daeren M, Nickels D, Jeon SM, Foreman O, Sudini K, et al. Nrf2 activation promotes aggressive lung Cancer and associates with poor clinical outcomes. Clin Cancer Res. 2021;27(3):877–88. https://doi.org/10.1158/1078-0432.CCR-20-1985.

46. Xu Y, Yang Y, Huang Y, Ma Q, Shang J, Guo J, et al. Inhibition of Nrf2/HO-1 signaling pathway by dextran sulfate suppresses angiogenesis of gastric Cancer. J Cancer. 2021;12(4):1042–60. https://doi.org/10.7150/jca.50605.

47. Wu S, Lu H, Bai Y. Nrf2 in cancers: a double-edged sword. Cancer Med. 2019;8(5):2252–67. https://doi.org/10.1002/cam4.2101.

48. Eckert RL, Kaartinen MT, Nummikoski M, Belkin AM, Colak G, Johnson GW, et al. Transglutaminase regulation of cell function. Physiol Rev. 2014;94(2):383–417. https://doi.org/10.1152/physrev.00019.2013.

49. Hernandez-Fernaud JR, Ruengeler E, Casazza A, Neilson LJ, Pulleine E, Santi SK, et al. Secreted CLIC3 drives cancer progression through its glutathione-dependent oxidoreductase activity. Nat Commun. 2017;8(1):1426. https://doi.org/10.1038/ncomms1426.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.