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Original Article

Hydrogen peroxide and cisplatin regulate the ROS/PKM2 pathway to affect the growth of cancer

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Abstract: Aberrant production of reactive oxygen species (ROS) cause DNA damage which led to the chronic diseases and cancer. During glycolysis, the enzyme pyruvate kinase M2 (PKM2) is responsible for energy metabolism and its overexpression can be found in various malignancies. To investigate the impact of PKM2 and ROS, hydrogen peroxide (H_2O_2) and cisplatin were used. This study showed that H_2O_2 and cisplatin induced ROS production and apoptosis in these four tumor cells: pancreatic cancer, oral cancer, gastric cancer, and hepatocellular carcinoma. In addition, H_2O_2- and cisplatin-increased apoptosis was partially reduced by pre-treatment with an antioxidant *N*-acetylcysteine (NAC) in SC-M1 gastric cancer and HSC-3 oral cancer cells. Interestingly, the levels of p-PKM2 in the nucleus were downregulated after treatment with H_2O_2 and cisplatin. This phenomenon was reversed with the combination of NAC. These findings provide PKM2 may be a potential target for anticancer therapy.

Keywords: Apoptosis, cisplatin, hydrogen peroxide, PKM2, reactive oxygen species

Introduction

According to the statistically estimation from the American Cancer Society, the cancer-related deaths are more than 0.6 million in America in 2024 [1]. Current cancer treatments include surgery, chemotherapy, molecular-targeted therapy, and radiotherapy. However, there are some limitations that compromise the efficacy of conventional antitumor therapy [2]. In advanced gastric cancer, for example, combination chemotherapy (cisplatin, bevacizumab, fluorouracil, and capecitabine) applied for the efficacy results in the median overall survival is 12.1-13.1 months [3]. Cao et al. reported that the high re-occurrence of oral squamous cell carcinoma was related to non-specific cell death after chemotherapy treatment [4]. In addition, chemoresistance is another critical obstacle that may affect the treatment of solid tumors. New strategies or approaches are urgently needed for the therapy of cancer.

Aberrant reactive oxygen species (ROS) is part of the oxidative stress, which promotes the progression of inflammatory diseases and the pathogenesis of cancer, aging, and diabetes mellitus [5]. Free radicals, including hydroxyl radicals, superoxide anions, peroxyl radicals, and non-radical molecules like hydrogen peroxide (H_2O_2), are secondary messengers involved in the initiation of free radical chains causing extreme damage to the cells [6]. Several studies showed that ROS were oncogenic, promoted tumorigenesis, angiogenesis, and metastasis [6, 7]. Increased levels of angiotensin-converting enzyme 1 by H_2O_2 causes trophoblast cell damage *in vitro* [8]. A previous study revealed that H_2O_2 is released from metal peroxides, alleviates tumor hypoxia in the tumor microenvironment [9]. However, it is worth nothing that ROS represents a double-edged way in tumor cells. Imbalance production of ROS could initiate oxidative stress-induced tumor cell death [10]. Certain anti-tumor agents, including molecular-

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targeted drugs and chemotherapeutic agents, inhibit tumor growth by increasing ROS production [11]. For example, the gemcitabine prodrug was activated in the presence of H_2O_2, leading to apoptosis in pancreatic cancer cells [12]. The combination therapy of cisplatin and pitavastatin, a HMG-CoA inhibiter suppressed the growth of cervical cancer through increasing ROS production and DNA damage [13]. In addition to DNA damage, ROS alters several signaling pathways, including NF-κB [14], MAPK kinase [15], PI3K/Akt, NRF2 [16], and mitochondrial oxidative metabolism [17].

Pyruvate kinase M2 (PKM2) is an enzyme involved in the final step of glycolysis, catalyzing the conversion of phosphoenolpyruvate to pyruvate with the concomitant production of ATP, and it plays a central role in the Warburg effect [18]. PKM2 may either be an dimer or an tetramer, depending on its physiological conditions [18]. Numerous studies have reported that PKM2 contributes to tumor growth and cell proliferation by supplying the anabolic conditions required [19, 20]. Over-expression of PKM2 is involved in the progression of numerous cancers, including pancreatic cancer, oral cancer, and hepatocellular carcinoma (HCC) [19, 20]. In addition, PKM2 activation was responsible for the resistance of the chemotherapeutic drugs [21]. A previous report showed that the transcription factors including p53, HIF-1α, and c-Myc modulated the activity/expression of PKM2 through Warburg effect which are critical for the metabolism of tumor cells [22]. Therefore, multiple evidences revealed that PKM2 can serve as a potential target for cancer therapy [23-25]. For example, the combination of PKM2 knockdown and estrogen enhanced apoptosis in colon cancer cells [26]. Polyphyllin II, a saponin, induces apoptosis by decreasing nuclear PKM2 and its downstream genes such *Glut1* and *MYC* in fibrosarcoma [27]. Wang et al. reported that shikonin, a PKM2 inhibitor used in treatment of bladder cancer, enhanced the sensitivity to cisplatin by inducing necroptosis [24]. Notably, PKM2 knockdown decreased the intracellular ROS generation, inhibited cell migration and invasion of tongue tumor *in vitro* and *in vivo* [28]. A recent study reported that lambertianic acid, a diterpene derivative, suppresses prostate cancer cell growth through modulation of ROS/PKM2/STAT3 signaling [29], highlighting the contribution of ROS signaling to PKM2 regulation in tumor progression [28, 29]. Cisplatin, a well-known chemotherapeutic agent, has been found the anti-tumor effects though the interaction between PKM2 and mTOR in cervical cancer [30]. However, the role of PKM2 or the correlation with Warburg effect in H_2O_2- and cisplatin- treated cancers, including gastric cancer, oral cancer, and HCC, remains ambiguous. This study, we evaluated the effects of H_2O_2 and cisplatin against tumor cells and showed that the apoptotic mechanism of H_2O_2 and cisplatin were associated with ROS/PKM2/STAT3 signaling related to the Warburg effect.

Materials and methods

Cell lines and chemical reagents

SC-M1 gastric adenocarcinoma cell line was obtained from ATCC. Hep3B hepatocellular carcinoma cells and PANC-1 pancreatic ductal adenocarcinoma cells were gifts from Professor Po-Chen Chu. The above cells were maintained in DMEM (Invitrogen) medium. The HSC-3 oral cancer cells were purchased from JCRB cell Bank and cultured in DMEM/F12 medium. All of the cell lines were cultured in the environment containing 10% fetal bovine serum at 37°C. Antibodies used were as follows: p-PKM2 (Tyr105), PKM2, fibrillarin, LC3B, p-STAT3 (Tyr705), STAT3, and GADPH (Cell Signaling Technology).

Determination of cell viability

SC-M1, HSC-3, PANC-1, and Hep3B cells were plated in 96-well plates at a density of 5×10^3 per well for 24 h. Then, these cell lines were treated with DMSO or H_2O_2 (10, 25, 50, 75, and 100 μM) or cisplatin (5, 10, 20, 50, 75, and 100 μM). After 24-h treatment, 100 μL MTT reagent was added and maintained for 4 h, followed by detection of the optical density at 450 nm using a microplate reader. The percentage of cell survival was assessed using MTT colorimetric assay.

Flow cytometry analysis for apoptosis and ROS determination

SC-M1, HSC-3, PANC-1, and Hep3B cells were seeded in 6-well plates at 2×10^5/well. For apoptosis determination, after treatment with DMSO or drug for 24 h, the reagent of annexin

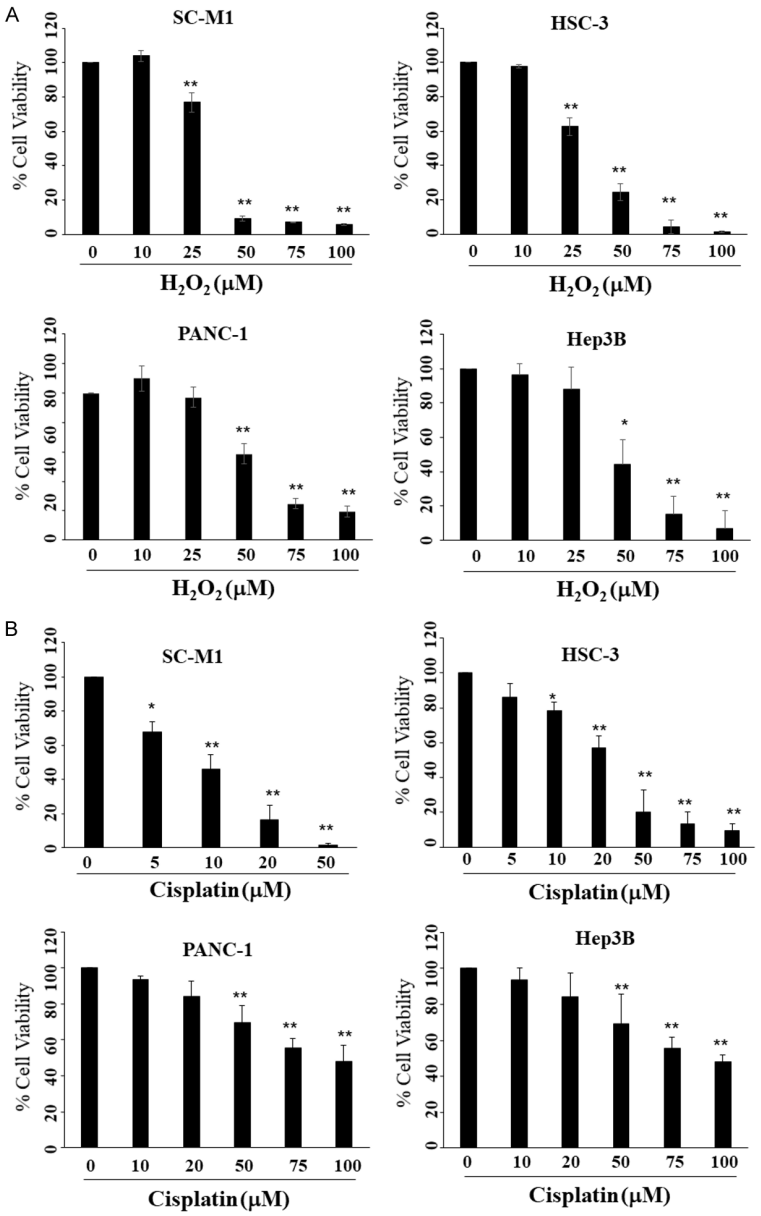

Figure 1. Anti-proliferative effects of hydrogen peroxide (H_2O_2) (A) and cisplatin (B) in four cell lines: SC-M1 gastric cancer, HSC-3 oral cancer, PANC-1 pancreatic cancer, and Hep3B hepatocellular carcinoma. Cells were treated with H_2O_2 or cisplatin for 24 h. Cell viability was determined using MTT assays. Points, means; bars, S.D. (n = 3). *P < 0.05, **P < 0.01.

V-FITC/PI (5 μl) were added for 20 min at room temperature. Then, the percentage of the apoptotic cells were measured by a flow cytometer (BD FACSAria, Franklin Lakes, NJ). To detect ROS generation, DMSO- or cisplatin- or H_2O_2-treated cells were collected and washed with PBS. Then, these cells were stained with the probe (DCFH-DA, 5 μM) in the dark condition. The fluorescence intensity of cells was detected by a flow cytometer.

Preparation of nuclear and cytosolic extract

SC-M1 and HSC-3 cell lines (1×10^6) were treated with DMSO or H_2O_2 or cisplatin. After 24 h, nuclear and cytosolic extracts were collected using Nuclear Protein Extraction Kit (Thermo Fisher Scientific, Maltham, MA).

Western blotting

The protein samples (5 μg) were separated by SDS-PAGE gel and transferred to nitrocellulose membranes. Nonspecific binding of the antibody was blocked with milk containing PBST for 40 min, and the membranes were maintained with primary antibodies overnight. Then, the appropriate secondary antibodies were added into the containers of these membranes for at least 1 h with shaking. The signals detected using an ECL kit (Amersham).

Statistical analysis

All experiments in the present study were conducted for three times (n = 3). The statistically analysis for these data were processed using Student's t tests. P values < 0.05 means significant.

Results

H_2O_2 and cisplatin inhibit growth of tumor cells

We first explored the viability of H_2O_2 and cisplatin against these four strains of tumor cells including gastric cancer (SC-M1), oral cancer (HSC-3), pancreatic cancer (PANC-1), and HCC (Hep3B) using the MTT assays. After the treatment of H_2O_2 (10, 25, 50, 75, and 100 μM) for 24 h, the results reveal that SC-M1 cells exhibited the highest sensitivity to H_2O_2 (**Figure 1A**). The IC_{50} values of H_2O_2 are between 30.2 μM and 60.3 μM for the four cancer cell lines evaluated (**Table 1**). Similar to the findings of H_2O_2 in

Table 1. Cytotoxicity of H_2O_2 and cisplatin in four tumor cell lines

Compound	IC_{50} (μM)			
	SC-M1[b]	HSC-3[b]	PANC-1[b]	Hep3B[b]
H_2O_2[a]	30.2 ± 1.0	31.6 ± 3.7	60.3 ± 6.1	48.5 ± 1.0
Cisplatin	9.7 ± 1.6	27.0 ± 5.4	94.7 ± 15.8	49.3 ± 19.5

[a]Data are presented as mean ± S.E.M. (n = 3). [b]Key to all cell lines: SC-M1, human stomach adenocarcinoma; HSC-3, human oral squamous cell carcinoma; PANC-1, human pancreatic adenocarcinoma; Hep3B, human hepatocellular carcinoma.

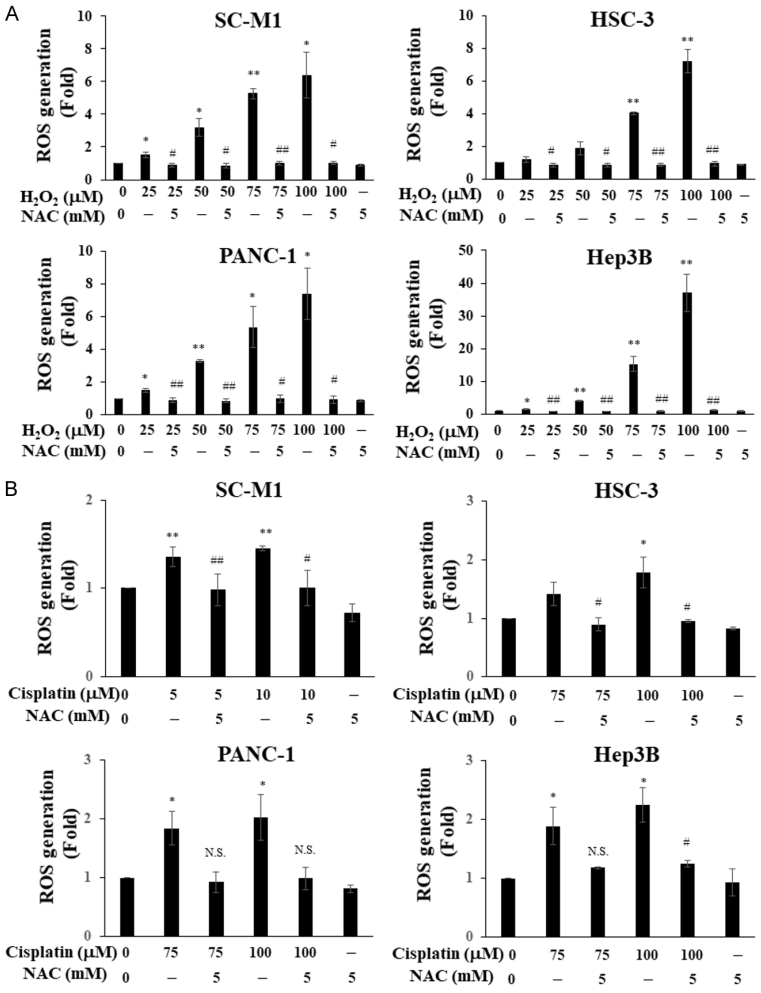

Figure 2. Analysis of reactive oxygen species in hydrogen peroxide (H_2O_2) (A) and cisplatin (B) in SC-M1 gastric cancer, HSC-3 oral cancer, PANC-1 pancreatic cancer, and Hep3B hepatocellular carcinoma cell lines. Cells were treated with H_2O_2 or cisplatin, either alone or in combination with *N*-acetylcysteine (NAC) and stained with carboxy-DCFDA. Data is presented as the mean ± S.D. (n = 3). *P < 0.05, **P < 0.01, as compared with the control group. #P < 0.05, ##P < 0.01, compared with H_2O_2- and cisplatin-treated group at the same concentration. NS, statistically not significant.

SC-M1 cells, cisplatin not only showed the extremely susceptibility with an IC_{50} value of 9.7 μM, but also inhibited the cell proliferation of the other cancer cell lines (**Figure 1B**; **Table 1**).

H_2O_2 and cisplatin increase ROS production

Multiple evidences have been shown that the imbalance of ROS production is related with tumor progression and carcinogenesis [10, 11]. In this study, the ROS generation after the treatment of H_2O_2 or cisplatin were also examined. The results revealed that the ROS production were increased in H_2O_2-treated cells (**Figure 2A**). *N*-acetylcysteine (NAC), a ROS scavenger, was used for the further investigation of the role of ROS. The combination of NAC diminished the production of ROS induced by H_2O_2 (**Figure 2A**). Meanwhile, cisplatin also increased the ROS generation (**Figure 2B**). Compared to that of cisplatin alone, the combination of NAC partly reversed the cisplatin-induced increase in ROS production in SC-M1 and HSC-3 cells (**Figure 2B**).

The ROS scavenger rescues H_2O_2- and cisplatin-mediated cytotoxicity

The SC-M1, HSC-3, PANC-1, and Hep3B cell lines were pretreated with 5 mM NAC in the presence of either H_2O_2 or cisplatin, to determine if ROS production contributed to H_2O_2- or cisplatin-mediated cytotoxicity. As shown in **Figure 3A**, the percentage viability increased with the combination of NAC and H_2O_2 compared with that of H_2O_2 alone. A similar phenomenon was observed with cisplatin treatment (**Figure 3B**).

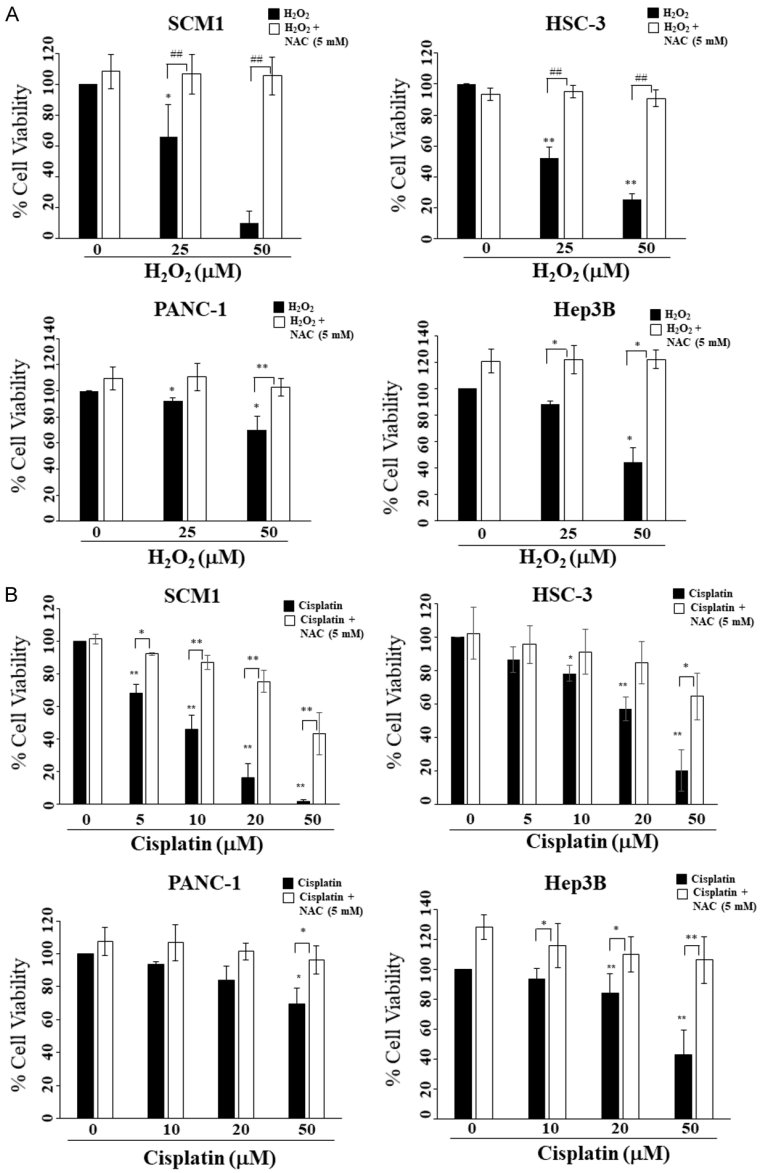

Figure 3. Hydrogen peroxide (H_2O_2)- (A) and cisplatin-mediated (B) cytotoxicity on reactive oxygen species generation in SC-M1 gastric cancer, HSC-3 oral cancer, PANC-1 pancreatic cancer, and Hep3B hepatocellular carcinoma cell lines. Cells were pre-treated with 5 mM NAC for 15 min, then incubated with H_2O_2 or cisplatin for 24 h. Cell viability was determined using the MTT assay. Points, means; bars, S.D. (n = 3). *P < 0.05, **P < 0.01.

ROS production is involved in H_2O_2- and cisplatin-increased apoptosis

To determine the impact of ROS on apoptosis, these drug-treated cancer cell lines were used and analyzed using flow cytometry. The results showed that H_2O_2 induce apoptosis in three cancer cell lines including SC-M1, HSC-3, and PANC-1, except for Hep3B cells (**Figure 4A**). In Hep3B cells (**Figure 4A**), there is no apoptotic cells were significantly changed after treatment of H_2O_2 at 100 μM (the highest concentrations). We further investigate whether the other cell death pathway is involved after treatment of H_2O_2 in Hep3B cells. As shown in Figure S1A, H_2O_2 increased the levels of LC3B-II, an autophagy biomarker, in a concentration-dependent manner in Hep3B cells. In the presence of an autophagic inhibitor chloroquine (CQ) partially reversed H_2O_2-induced cytotoxicity which suggested that H_2O_2 induces autophagy (Figure S1B). In the combination of NAC, the percentage of apoptosis were decreased in H_2O_2-treated cells (**Figure 4A**). Unlike H_2O_2, the treatment of cisplatin induced apoptosis in these four cancer cell lines (**Figure 4B**). For SC-M1, HSC-3, and PANC-1 cells, the occurrence of NAC cause the decreased on cisplatin-induced apoptosis (**Figure 4B**). We found that the percentage of apoptosis of cisplatin-treated Hep3B cells was not significantly affected by treatment with NAC, indicating that ROS might not play an essential role for cisplatin. Western blotting demonstrated that cisplatin increased the levels of LC3B-II in Hep3B cells (Figure S2A). In addition, the combination of cisplatin and CQ group showed the less anti-proliferative effect than cisplatin alone (Figure S2B). The above results showed that cisplatin induces autophagy in Hep3B cells.

Nuclear PKM2 is important for H_2O_2 and cisplatin in gastric and oral cancer cells

Several reports have shown that PKM2 is a key molecule in regulating cancer metabolism, providing a correlation with tumorigenesis and poor prognosis [19, 31]. Modulation of PKM2

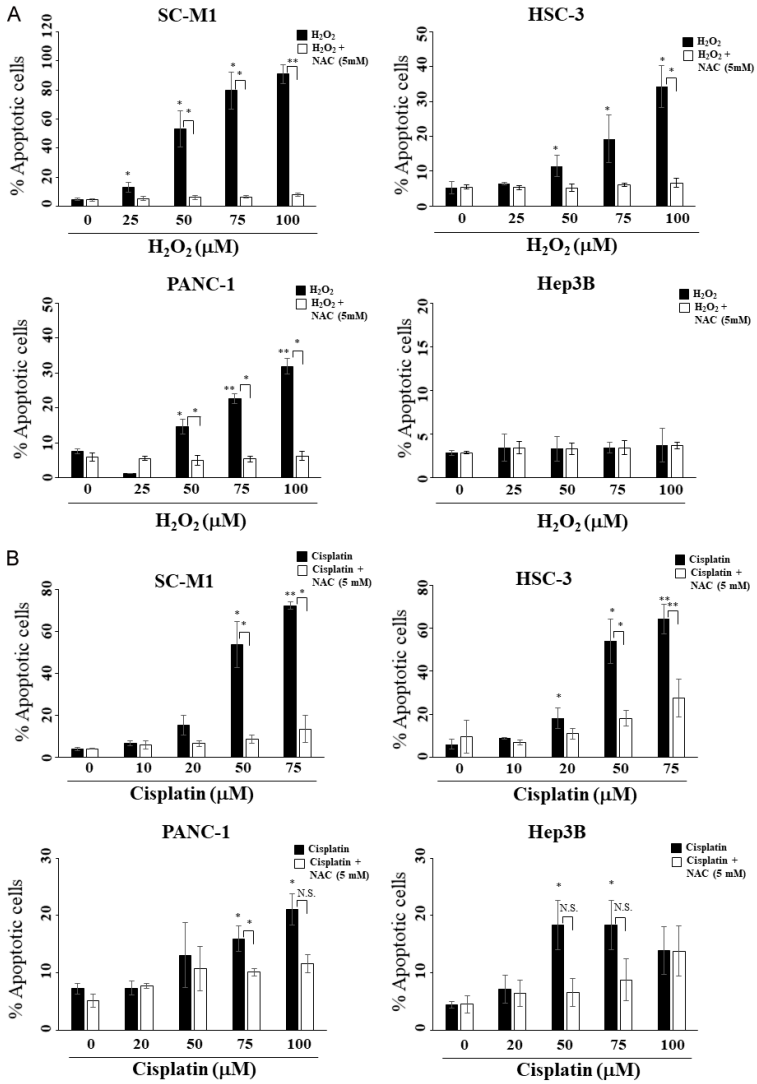

Figure 4. Hydrogen peroxide (H_2O_2)- (A) and cisplatin-induced (B) apoptosis was reversed by pre-treatment with NAC. Flow cytometric analysis was performed on apoptotic cells pre-treated for 15 min with 5 mM NAC, incubated for 24 h with H_2O_2 or cisplatin, and then stained with annexin V/PI. Points, means; bars, S.D. (n = 3). *P < 0.05, **P < 0.01.

has been successful in controlling cancer; thus, PKM2 could be a valuable antitumor biomarker [24, 32]. We tested the effects of H_2O_2 and cisplatin on the phosphorylation of PKM2 in both gastric and oral cancer cells. Our data showed that both compounds decreased the levels of p-PKM2 in the nucleus (**Figure 5A**, **5B**). To assess whether the ROS production is correlate with the nuclear translocation of PKM2. As shown in **Figure 5A** and **5B**, the treatment of NAC partially reversed the levels of p-PKM2 in H_2O_2- and cisplatin-treated nuclear extracts, suggesting that PKM2 is involved in the ROS production by these two compounds.

Demaria et al. reported that nucleus PKM2 regulates *in vivo* growth by directly activating STAT3 [33]. Western blotting revealed that the levels of p-STAT3 were decreased in H_2O_2- and cisplatin-treated Hep3B cells (Figure S3A). Fluorescence analysis showed that the intensity of p-STAT3 was reduced following treatment with H_2O_2 as well as cisplatin (Figure S3B).

Discussion

It is well known that ROS are one of the by-products of oxygen consumption during the cellular metabolism and have been associated with cancer for centuries [10]. Numerous studies have shown that PKM2 is regard as an oncogene and the deletion of PKM2 leads to the growth advantage to tumor progression [18, 19]. In this study, H_2O_2 and cisplatin were shown to mediate cytotoxicity through the production of ROS in gastric cancer, pancreatic cancer, oral cancer, and HCC cells. The inhibition of ROS generation prevented the cancer cells from apoptosis and PKM2 nuclear localization which induced by these two compounds.

Some evidences have revealed that excess ROS can trigger genetic mutations such as KRAS and boosts tumor formation [6, 34]. However, in some research reports, ROS plays an opposite role in inhibiting cell growth and preventing the tumor invasion [12, 35]. Pilco-Ferreto et al. reported that doxorubicin inhibited the growth of breast cancer through decreasing NF-κB and increasing H_2O_2 [36]. It has been reported that the free radical H_2O_2 induce apoptosis through the activation of the death receptor Fas and p53 in HCC cells [37]. Itoh et al. also reported both H_2O_2 and cisplatin cause the increased of intracellular ROS through increasing the activity of NADPH oxidase [38]. Our study showed that

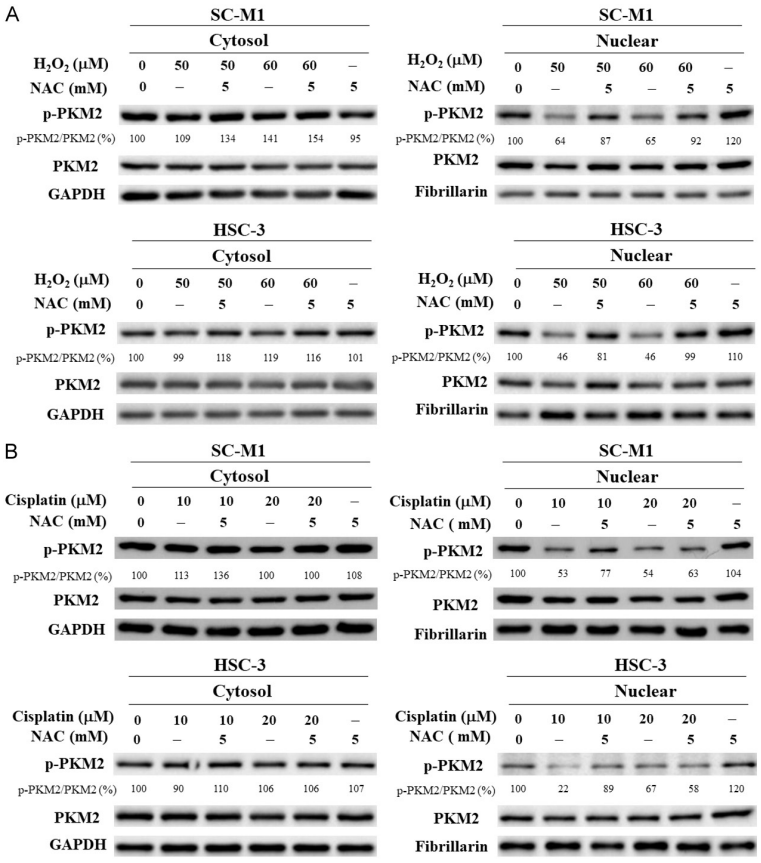

Figure 5. Effects of hydrogen peroxide (H_2O_2) (A) and cisplatin (B) on p-PKM2 and PKM2. Two cancer cell lines (SC-M1 gastric cancer and HSC-3 oral cancer) were pre-treated with 5 mM NAC for 15 min and incubated for 24 h with H_2O_2 or cisplatin. GAPDH or fibrillarin was used as a loading control. Cytosolic and nuclear extracts were isolated as described in the Materials and Methods section. Values are expressed as a percentage of the control.

both H_2O_2 and cisplatin increased ROS generation in all these four cancer cell lines (gastric cancer, oral cancer, PANC-1, and HCC). Compared with the H_2O_2 alone, the combination of the anti-oxidant NAC led to the inhibition of apoptosis in H_2O_2-treated tumor cell lines including SC-M1, HSC-3, and PANC-1 (**Figure 4**). The similar phenomenon was observed between cisplatin group and the combined group (NAC + cisplatin). Yazihan et al. reported that H_2O_2 induces apoptosis after 48 h treatment through caspase-3 activation in the Hep3B cell line [39]. However, our results showed that H_2O_2 did not induce apoptosis in Hep3B cells. Autophagy, one of cellular death, serves a tumor-suppressor function in hepatocellular carcinoma [40]. A previous study reported that H_2O_2 causes hepatocyte injury and suppress liver cancer cell growth through activation of autophagy [41]. Instead of inducing apoptosis, both H_2O_2 and cisplatin induce autophagy in Hep3B cells.

Previous studies showed that PKM2 can dimerize prior to entering the nucleus, thereby regulating gene expression in the tumor cell energy supply pathway [20, 31]. Zhang et al. reported that the phosphorylation of PKM2 increases its nuclear localization, protecting the mitochondria from oxidative stress which leads to cell growth [42]. For example, the phosphorylation at Try105 of PKM2 has been demonstrated to promote the nuclear translocation and lead to the tumor growth [43]. Furthermore, p-PKM2 (Tyr105) is particularly observed in several human cancer cell lines such as Detroit-562 oral cancer, SK-Hep1 liver cancer, and PC3 prostate cancer [29, 43]. After exposure to H_2O_2, the intracellular concentration of ROS was increased and PKM2 activity was decreased in lung cancer cells [44]. In our study, H_2O_2 and cisplatin were shown to reduce nuclear p-PKM2 levels in SC-M1 gastric cancer and HSC-3 oral cancer cells, implying their apoptotic effect may be mediated through regulation of p-PKM2 (Tyr105). Notably, the decreased p-PKM2 levels induced by H_2O_2 and cisplatin were reversed by pre-treatment with NAC, suggesting that PKM2 as a critical biomarker in ROS generation. Also, the data indicated that ROS decreased the p-PKM2 (Tyr105) as a downstream target of ROS in H_2O_2- and cisplatin-treated cells. PKM2 is an antitumor target that improves the prognosis of cancer. Zhou et al. reported that silencing PKM2 activates DNA damage proteins p-ATM and γH2AX and increases the susceptibility for the PARP inhibitor olaparib-treated ovarian cancer cells [45]. For image therapy, [^{18}F]DASA-23, a new tracer for monitoring PKM2-inducing glycolytic glioblastoma has been used clinically [46]. Regulation of p-PKM2 has been shown to promote ERK

activation and Myc transcription, resulting in an enhanced Warburg effect [42]. Bi et al. reported that STAT3 promotes the Warburg effect by enhancing p-PKM2 in transformed hepatic progenitor cells *in vivo* [47]. A previous study demonstrated that PKM2 can localized to the nucleus, where it directly activates STAT3 [33]. Consistent with this, our data showed decreased p-STAT3 protein expression in H_2O_2- and cisplatin-treated cells (Figure S3A). Furthermore, fluorescence analysis revealed that an interaction between p-PKM2 and STAT3 (Figure S3B), suggesting that nuclear PKM2 may regulate tumor growth through its interaction with STAT3. Taken together, these findings suggested that H_2O_2- and cisplatin inhibited cell growth, at least in part, by increasing ROS production and suppressing the PKM2/STAT3 signaling pathway.

Conclusions

Our results showed that H_2O_2 and cisplatin suppressed the growth of various cancer cell lines including SC-M1, HSC-3, PANC-1, and Hep3B. We demonstrated that the anti-tumor effects of these compounds were partially related to ROS generation and the inhibition of PKM2/STAT3 signaling. Therefore, the inhibition of nuclear PKM2 might be an important biomarker for cancer therapy.

Acknowledgements

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Disclosure of conflict of interest

None.

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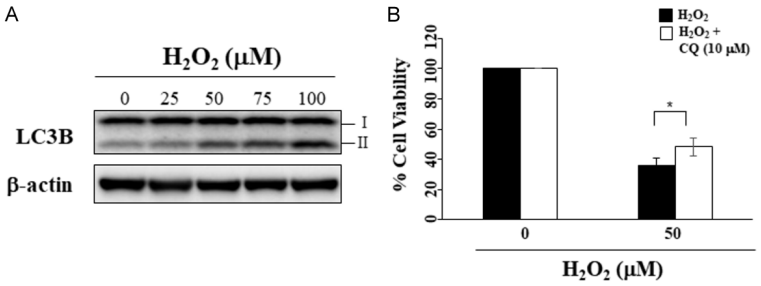

Figure S1. Effects of hydrogen peroxide (H_2O_2) on autophagy. A. Hep3B cells were treated with H_2O_2 for 24 h and western blot was performed. B. Cells were treated with H_2O_2 or in combination with chloroquine (CQ), and cell viability was determined using MTT assays. Points, mean; bars, S.D. (n = 3) $*P < 0.05$.

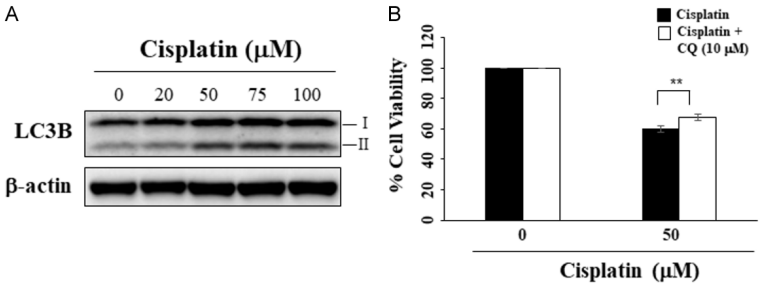

Figure S2. Effects of cisplatin on autophagy. A. Hep3B cells were treated with cisplatin for 24 h and western blot was performed. B. Cells were treated with cisplatin or in combination with chloroquine (CQ), and cell viability was determined using MTT assays. Points, mean; bars, S.D. (n = 3) $**P < 0.01$.

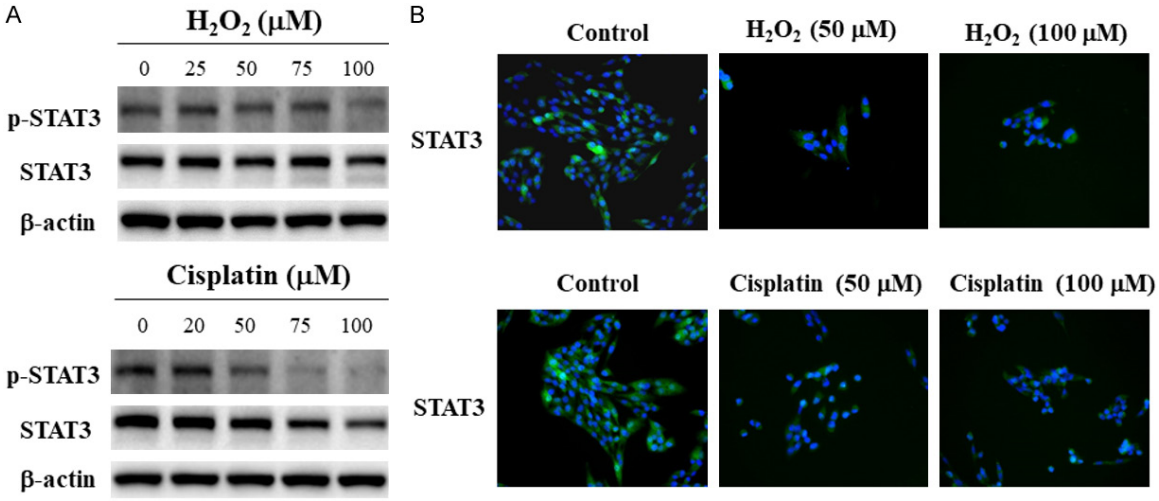

Figure S3. Expression/phosphorylation of STAT3 in hydrogen peroxide (H_2O_2)- and cisplatin-treated Hep3B cells. A. Western blotting of p-STAT3 and STAT3 protein levels after treatment of H_2O_2 or cisplatin for 24 h. B. Fluorescence analysis of STAT3 in Hep3B cells in response to H_2O_2 or cisplatin.