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# Critical Role of $H_2O_2$ Generated by NOX4 during Cellular Response under Glucose Deprivation

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## Abstract

Glucose is the most efficient energy source, and various cancer cells depend on glycolysis for energy production. For maintenance of survival and proliferation, glucose sensing and adaptation to poor nutritional circumstances must be well organized in cancer cells. While the glucose sensing machinery has been well studied in yeasts, the molecular mechanism of glucose sensing in mammalian cells remains to be elucidated. We have reported glucose deprivation rapidly induces AKT phosphorylation through PI3K activation. We assumed that regulation of AKT is relevant to glucose sensing and further investigated the underlying mechanisms. In this study, AKT phosphorylation under glucose deprivation was inhibited by galactose and fructose, but induced by 2-deoxyglucose (2-DG). Both 2-DG treatment and glucose deprivation were found to induce AKT phosphorylation in HepG2 cells. These findings suggested that glucose transporter may not be involved in the sensing of glucose and induction of AKT phosphorylation, and that downstream metabolic events may have important roles. A variety of metabolic stresses reportedly induce the production of reactive oxygen species (ROS). In the present study, glucose deprivation was found to induce intracellular hydrogen peroxide ($H_2O_2$) production in HepG2 cells. N-acetylcysteine (NAC), an antioxidant reagent, reduced both the increase in cellular $H_2O_2$ levels and AKT phosphorylation induced by glucose deprivation. These results strongly suggest that the glucose deprivation-induced increase of $H_2O_2$ in the cells mediated the AKT phosphorylation. RNA interference of NOX4, but not of NOX5, completely suppressed the glucose deprivation-induced AKT phosphorylation as well as increase of the intracellular levels of ROS, whereas exogenous $H_2O_2$ could still induce AKT phosphorylation in the NOX4-knockdown cells. In this study, we demonstrated that the ROS generated by NOX4 are involved in the intracellular adaptive responses by recognizing metabolic flux.

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## Introduction

The supply of nutrients and oxygen is pivotal for cell survival and function, because of the large energy requirements of cells. This need is especially critical during cell proliferation. Proliferation is a process during which the numbers of cells successively double; therefore, the synthesis of nucleic acids, lipids, proteins and sugars is obligatory for successful proliferation. Glucose serves as a carbon source for the synthesis of nucleic acids, non-essential amino acids, lipids, and sugar. The intermediate metabolites in the glycolytic system are indispensable for non-essential amino acid synthesis, and intermediate metabolites and coenzymes in the pentose-5-phosphate pathway are required for the synthesis of nucleic acids and lipids. In addition, glucose is also needed for energy production in all cells.

Because of the pivotal role of glucose in the maintenance of the cellular functions, survival, and proliferation, elaborate mechanisms for detecting glucose availability in the cellular microenvironment exist in cells. The molecular mechanisms involved in the sensing of extracellular glucose concentrations have been extensively studied in yeasts. Yeasts detect the extracellular glucose concentrations using Snf3/Rtg2 (a glucose transporter homolog that has no capability as a transporter). Extracellular glucose causes this sensor to generate an intracellular signal that induces the expressions of several HXT genes encoding hexose transporters. The glucose signal induces HXT gene expression by influencing the function of the Rgt1 transcriptional repressor. In the absence of glucose, Rgt1 is functional and binds to the promoters of the HXT genes, repressing their functions [1,2,3]. In contrast, the biochemical basis of the glucose sensing mechanism in mammalian cells is largely unknown.

Meanwhile, most of human cancer tissues are known to be hypoxic, the hypoxia being caused mainly by a poor and heterogeneous blood supply [4,5,6,7]. Glucose as well as oxygen is supplied to cancer tissues via the blood stream, and we assumed that the glucose supply might be limited in human cancer tissues. In fact, the glucose concentrations in human colon cancer and gastric cancer tissues were found to be significantly lower than those in surrounding non-cancerous tissues [8]. In the cancer cells that exist in such environments, the monitoring of and adaptation to extracellular glucose concentrations are assumed to be important for the survival/proliferation of the tumor cells. We previously reported that AKT phosphorylation is immediately enhanced by the absence of glucose and plays a critical role in

cellular survival under such condition in various cell lines [9,10]. AKT can also be activated in response to a variety of cellular stresses, such as heat shock, ultraviolet light irradiation, ischemia, hypoxia, hyperglycemia, and oxidative stress. AKT is a serine and threonine kinase that mediates cell survival under these aforementioned conditions [11,12,13,14,15].

In the present study, we attempted to elucidate the molecular and biochemical mechanisms involved in the sensing of mammalian cells of the extracellular glucose concentrations, using AKT phosphorylation as an index of the cellular responses to glucose deprivation. We demonstrate the contribution of the $H_2O_2$ generated by NOX4 in the cellular sensing of and adaptation to poor glucose supply.

## Materials and Methods

### Cell cultures

Human fibroblasts derived from the subserosa of the stomach used for this study were kindly gifted to us by Dr Atsushi Ochiai (Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East). Human pancreatic cancer cells (PANC-1), human hepatocellular carcinoma cells (HepG2) and human fibroblasts derived from subserosa of the stomach were cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum (Biowest). All the cells were purchased from ATCC. The glucose-deprived condition was created as described previously [16].

### Reagents

2′, 7′- Dichlorodihydrofluorescin diacetate (DCFDA) was purchased from Invitrogen. 3′-O-Acetyl-6′-O-pentafluorobenzenesulfonyl-2′,7′-difluorofluorescein (Bes-$H_2O_2$), galactose and fructose were purchased from Wako Pure Chemical Industries. N-acetyl-L-cysteine (NAC) and 2-deoxy-D-glucose (2-DG) were purchased from Sigma Aldrich. LY294002 and PP2 were purchased from Calbiochem.

### Immunoblot analyses

Cells were homogenized in lysis buffer containing 10% SDS (sodium dodecyl sulfate), 10 mM Tris-HCl (pH 7.5) and 1 mM sodium orthovanadate, as described previously [17], and subjected to SDS-PAGE (SDS polyaclylamide gel electrophoresis). The proteins were transferred to a polyvinylidene fluoride microporous membrane (Millipore). The primary antibodies used were: anti-phospho-AKT Ser-473, anti-phospho-SRC Family Tyr-416, and anti-AKT, all obtained from Cell Signaling Technologies, and anti-actin (sc-1615), and c-SRC antibody (SRC2), obtained from Santa Cruz Biotechnology. The anti-OSSA antibody was a kind gift from Dr. Ryuuichi Sakai, National Cancer Center Research Institute. The following secondary antibodies were purchased from Santa Cruz Biotechnology: goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP. The immunoblots were scanned using a CanoScan LiDE60 image scanner (Canon).

### siRNA transfection

OSSA, NOX4, NOX5, and non-targeting siRNA were purchased from Invitrogen. For the siRNA experiments, the cells were transfected separately using a non-targeting siRNA or two separate specific siRNAs using Lipofectamine 2000 (Invitrogen).

### RT-PCR

Total RNAs were prepared from the cells using ISOGEN (Nippon Gene), and reverse transcription was performed using superscript VILO (Invitrogen). PCR for human NOX family genes was carried out using the following primers: forward 5′-CTCAGCGGAATCAATCAGCTGTG-3′ and reverse 5′-AGAGGAACACGACAATCAGCCTTAG-3′ for Nox4; forward 5′-ATCAAGCGGCCCCCTTTTTTTCAC-3′ and reverse 5′-CTCATTGTCACACTCCTCGACAGC-3′ for Nox5.

### Measurement of intracellular ROS levels

The cells were treated under various conditions and then incubated in DMEM or glucose-deprived medium containing 5 μM of DCFDA or 5 μM BES-$H_2O_2$-Ac at 37°C for 30 min. Then, the cells were detached from the plate with trypsin/EDTA, washed with PBS, resuspended in 500 μL of PBS, and placed on ice, protected from light. The intensity of the fluorescence of each cell was immediately measured using a FACS CANTO (Becton Dickinson) equipped with an argon ion laser (488 nm excitation). Each experiment was conducted in triplicate, and 10,000 cells per sample were measured. The histogram was analyzed using the software program BD FACS DIVA (Becton Dickinson).

## Results

### AKT activation by glucose deprivation

Within 30 minutes, and still after 3 hours, of transferring the HepG2 cells from ordinary DMEM to glucose-deprived medium, AKT was strongly phosphorylated at Ser 473; furthermore, AKT phosphorylation was significantly inhibited by treatment with LY294002 [18], an inhibitor of PI3K (Fig. 1A). Similarly, PI3K-dependent AKT activation was also observed in the pancreatic PANC-1 cells (Fig. S1) in a previous study [10]. Furthermore, increase of AKT phosphorylation induced by glucose deprivation was also observed in human fibroblasts derived from the subserosa of the stomach (Fig. S2).

To examine how glucose deprivation is recognized in these cells, concentration-dependent AKT activation in response to glucose deprivation was examined. When the HepG2 cells were exposed to media containing less than 1.38 mM of glucose, corresponding to one-quarter of the blood glucose level, AKT activation was clearly observed (Fig. 1B). Similarly, an increase in AKT phosphorylation was also observed in PANC-1 cells cultured in the presence of glucose at concentrations of less than 0.69 mM (Fig. S3). To elucidate the glucose sensing mechanism of the cells, the effect of glucose analogues on the AKT activation in response to glucose deprivation was examined. AKT activation was completely inhibited by the addition of either galactose or fructose at a final concentration of 5.5 mM (Fig. 1C). Similar results were observed in the PANC-1 cells (Fig. S4). These observations indicate that AKT is activated by a decrease of some metabolites of glycolysis or metabolic stress, rather than by the decrease of glucose itself. In yeast, the extracellular glucose concentration is sensed by a glucose transporter [1,2,3]. To examine whether a similar mechanism may also prevail in mammalian cells, the influence of 2-DG [19,20] on the AKT phosphorylation induced by glucose deprivation was examined. As shown in Fig. 1D, AKT phosphorylation in the HepG2 cells in response to glucose deprivation was not inhibited by 2-DG. Rather, AKT phosphorylation was clearly induced by the addition of 5.5 mM 2-DG, even in the presence of glucose. This observation indicates that glucose is not sensed by binding to a receptor or transporter, nor is it sensed by hexokinase, because 2-DG can be phosphorylated as efficiently by mammalian hexokinase as glucose. It is possible that the inhibition of binding of some sensors to glucose, if such an interaction occurs, might evoke the same cellular responses as glucose deprivation.

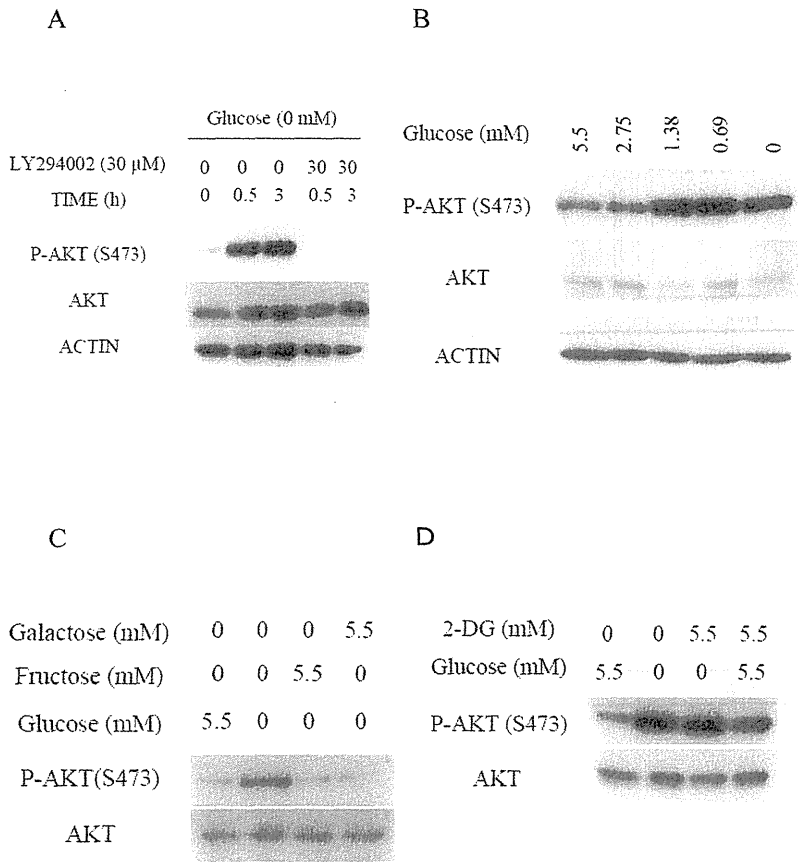


**Figure 1. AKT phosphorylation was induced under glucose deprivation.** (A) Immunoblotting analyses after incubation of HepG2 cells in the absence or presence of 5.5 mM of glucose and absence or presence of 30 μM of LY294002 for the indicated times. (B) HepG2 cells treated or not treated with various concentrations of glucose for 0.5 h were subjected to immunoblotting. (C) Immunoblotting analyses of HepG2 cells treated or not treated with 5.5 mM of glucose, 5.5 mM of galactose, or 5.5 mM of fructose for 0.5 h. (D) Immunoblotting analyses of HepG2 cells treated or not treated with 5.5 mM of glucose, 5.5 mM of 2-DG, or 5.5 mM of glucose plus 5.5 mM of 2-DG for 0.5 h.
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## Role of hydrogen peroxide in the activation of AKT in response to glucose deprivation

Since AKT phosphorylation in response to glucose deprivation was attenuated by galactose, we assumed that changes in the metabolism might be the cause of the increase in AKT activation. Reactive oxygen species (ROS) are reportedly produced in cells under metabolic stresses [21,22]. We evaluated the intracellular levels of ROS using dichlorofluorescein diacetate (DCFDA), which measures hydroxyl and peroxyl radicals and other ROS. A significant increase in the intracellular ROS production was observed in the HepG2 cells cultured in glucose-deprived medium treated with DCFDA for 30 minutes (Fig. 2A). 3′-O-acetyl-6′-O-pentafluorobenzenesulfonyl- 2′,7′-difluorofluorescein (BES-$H_2O_2$) specifically detects an increase in the amounts of hydrogen peroxide ($H_2O_2$) [23] in cells treated under the same conditions (Fig. 2B). An increase in the production of ROS induced by glucose deprivation was also observed in the PANC-1 cells and human fibroblasts derived from the subserosa of the stomach (Fig. S5,S6). Addition of galactose or fructose completely prevented the $H_2O_2$ increase (Fig. S7). These results clearly showed that $H_2O_2$ production is induced by glucose deprivation. To elucidate the causal relationship between $H_2O_2$ production and AKT phosphorylation, the effect of addition of exogenous $H_2O_2$ on AKT phosphorylation was examined. Exogenous $H_2O_2$ addition to the culture medium induced PI3K-dependent AKT phosphorylation in a manner similar to glucose deprivation (Fig. 2C). To confirm the causal relation further, the influence of N-acetylcysteine (NAC), an antioxidant reagent, on the AKT phosphorylation induced in the absence of glucose was examined. The addition of NAC to the culture medium at a final concentration of 12.5 mM markedly reduced the ROS levels even under glucose-deprived conditions (Fig. 2A and 2B). Furthermore, the NAC treatment also suppressed the AKT phosphorylation induced by glucose deprivation (Fig. 2D).

## SRC and OSSA are indispensable for AKT phosphorylation induced by glucose deprivation

SRC is involved in an alternate PI3K-activating pathway, and OSSA, a scaffold protein also known as FAM120A, reportedly activates the SRC-PI3K pathway in the presence of oxidative stress [24]. Thus, the involvements of SRC and OSSA in the glucose deprivation-induced phosphorylation of AKT were examined. PP2, a specific SRC family inhibitor [25], clearly inhibited the AKT phosphorylation induced by glucose deprivation (Fig. 3A). PP2 also inhibited AKT phosphorylation induced by exogenous $H_2O_2$ (Fig. 3B). Consistent with these findings, PP2

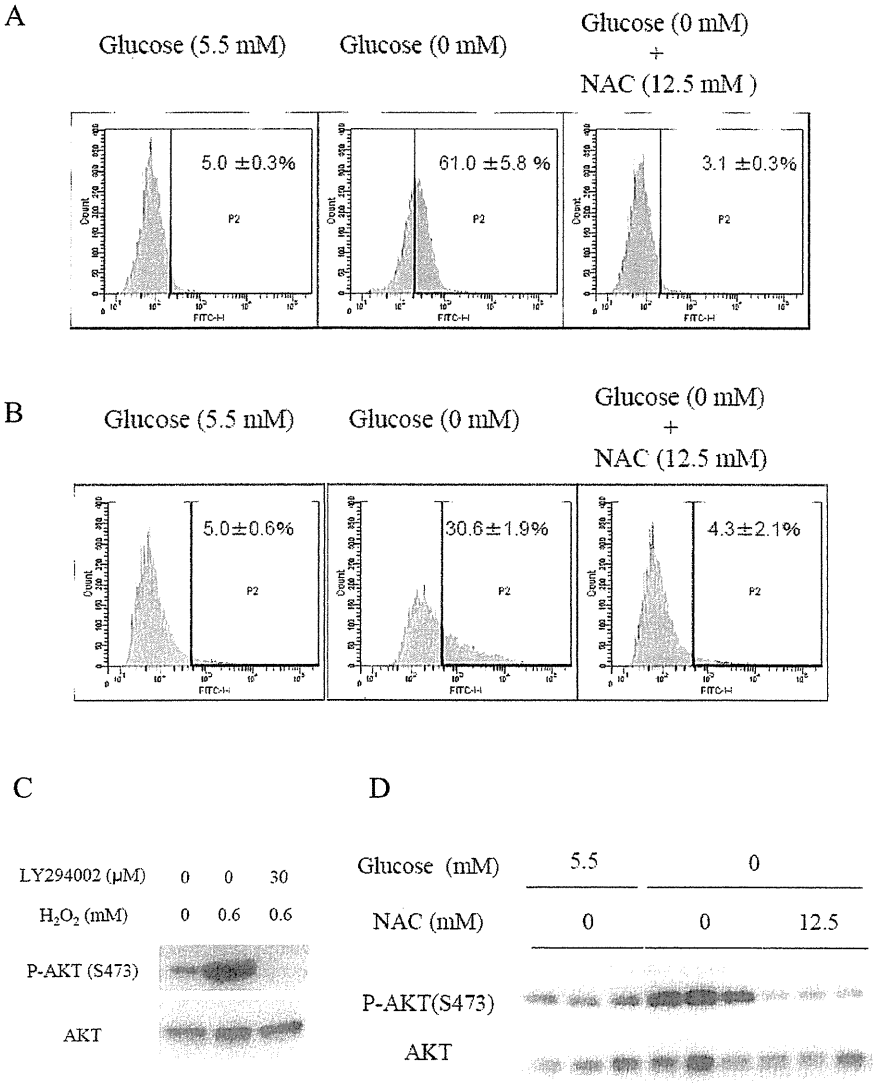


**Figure 2. ROS mediates AKT phosphorylation under glucose deprivation.** (A)(B)(D) HepG2 cells were cultured in either glucose-containing medium or glucose-deprived medium in the absence or presence of 12.5 mM of NAC for 0.5 h. ROS production was measured using flow cytometry. Cells were stained with (A) 5 μM of DCFDA or (B) 5 μM of BES-$H_2O_2$. Cells were gated within a range contained in the upper 5% of the total cell count under the glucose replete condition. (D) The AKT phosphorylation level was evaluated by immunoblotting. (C) Addition of $H_2O_2$ to media containing 5.5 mM of glucose in the absence or presence of 30 μM of LY294002 for 0.5 h, followed by immunoblotting.
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also suppressed the phosphorylation of SRC induced by glucose deprivation and exogenous $H_2O_2$ (Fig. S8). PP2 treatment did not alter the increased ROS levels in HepG2 cells cultured under glucose-deprived conditions (Fig. 3C). Similarly, LY294002 treatment inhibited AKT phosphorylation, but did not alter the ROS production (Fig. 1A, 3C). Suppression of OSSA expression by RNA interference inhibited the AKT phosphorylation induced by glucose deprivation and exogenous $H_2O_2$ (Fig. 3D, 3E and 3F). Thus, SRC and OSSA were concluded as being mediators of the $H_2O_2$ signals induced by glucose deprivation that activate the PI3K-AKT axis.

## NOX4 knockdown inhibits hydrogen peroxide generation under glucose-deprived conditions

NOX4, one of the members of the NADPH oxidase family, is known to be closely involved in the production of ROS in response to growth factor stimuli [26]. Thus, its involvement also in glucose deprivation-induced AKT phosphorylation was examined. RNA interference selectively reduced the expression of NOX4 in HepG2 cells (Fig. 4A). Increase of intracellular ROS levels by glucose deprivation was suppressed by NOX4 knockdown (Fig. 4B). Consistent with this finding, AKT phosphorylation was also not induced in the NOX4 knockdown cells, while exogenous $H_2O_2$ clearly induced AKT phosphorylation in the cells (Fig. 4C). Similar results were obtained in the PANC-1 cells (Fig. S9A, B). PANC-1 cells express NOX5 as well as NOX4, however,

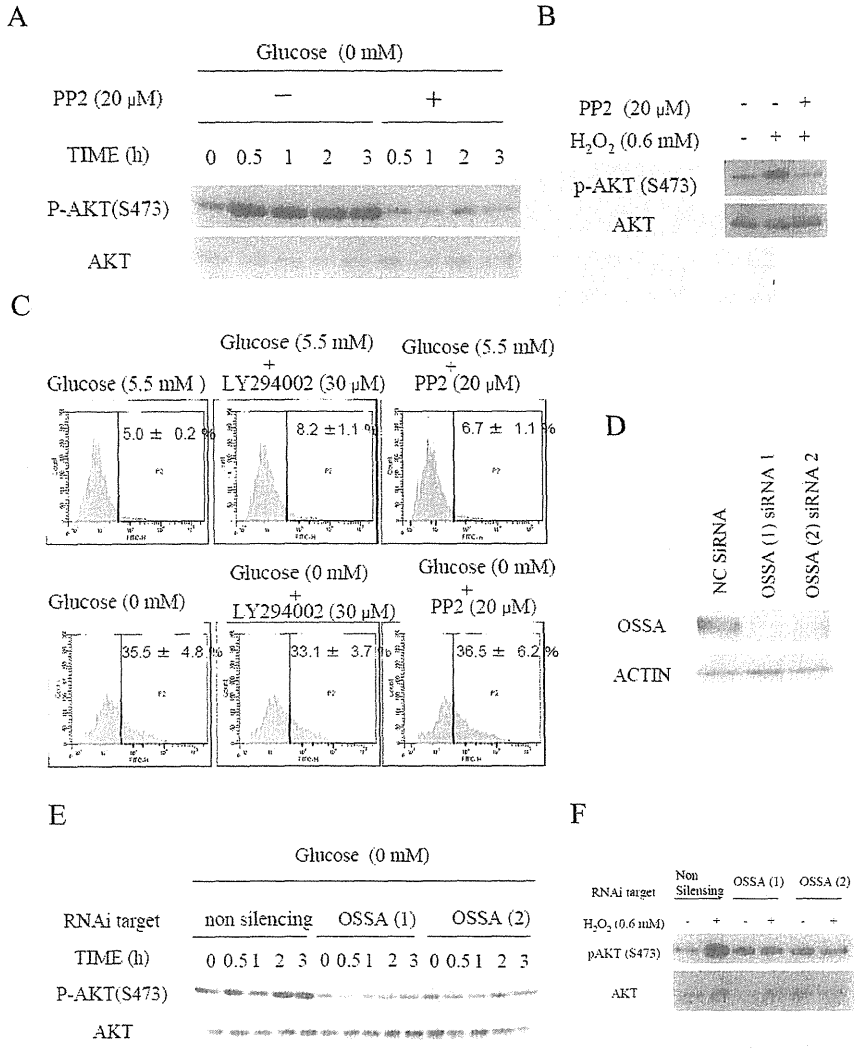


**Figure 3. SRC and OSSA are indispensable for the AKT phosphorylation induced by glucose deprivation.** (A) Immunoblotting analyses of HepG2 cells in the absence or presence of 5.5 mM of glucose in the and absence or presence of 20 μM of PP2 for the indicated times. (B) Addition of $H_2O_2$ to the culture medium containing 5.5 mM glucosein the absence or presence of 20 μM of PP2 for 0.5 h, followed by immunoblotting. (C) HepG2 cells were cultured in medium containing or not containing (glucose-deprived) 5.5 mM of glucose in the absence or presence of 30 μM of LY204002 or 20 μM of PP2 for 0.5 h. The cells were stained with 5 μM of BES-$H_2O_2$. ROS production was measured using flow cytometry. (D) siRNA-treated HepG2 cells were subjected to immunoblotting analyses using OSSA antibody. (E) Immunoblotting analyses of HepG2 cells transfected with a non-targeting siRNA or two separate OSSA siRNAs in the absence or presence of 5.5 mM of glucose for the indicated times. (F) Addition of $H_2O_2$ to the medium of OSSA-knockdown cells containing 5.5 mM glucose for 0.5 h, followed by immunoblotting.
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knockdown of NOX5 did not alter the AKT phosphorylation level (Fig. S10A, B).

## Discussion

In this study, we tried to elucidate the mechanism of sensing of the extracellular glucose concentration by cells, using AKT phosphorylation as a marker. As reported previously, AKT phosphorylation is induced by glucose deprivation [9,10]. In addition, increase in AKT phosphorylation has also been confirmed in HepG2 cells cultured in media containing one-quarter of the normal physiological glucose concentration. This fact suggests that cells have sophisticated mechanisms for monitoring extracellular glucose levels. In another study, increase in AKT phosphorylation was confirmed in PANC-1 cells cultured in the presence of glucose levels that are one-eighth of the normal physiological condition. The difference in the minimal trigger concentration of glucose between the HepG2 cells and PANC-1 cells could be related to differences in the origins of the cells or differences in the microenvironments of the tumors the cells were derived from.

In the present study, increase in ROS production was observed by 30 minutes after glucose deprivation, both in cancer cells and human fibroblasts. Thus, it became evident that the mechanism of ROS production under glucose deprivation is preserved in not only cancer cells, but also human fibroblasts. ROS was strongly suspected to mediate the AKT phosphorylation, because AKT

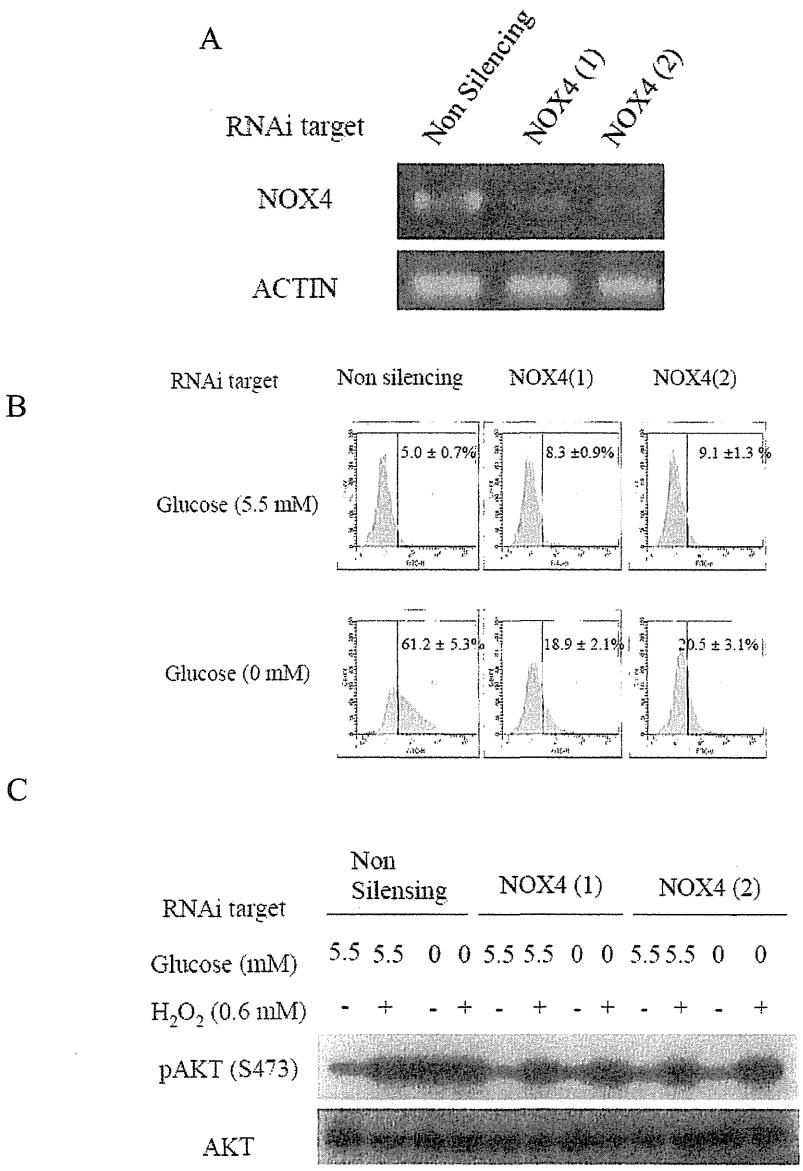


**Figure 4. Induction of AKT phosphorylation under glucose deprivation is mediated by ROS generated by NOX4.** (A) siRNA-treated HepG2 cells were subjected to reverse-transcriptase PCR (RT-PCR) to confirm NOX4 knockdown. (B) NOX4 knockdown HepG2 Cells were stained with 5 μM of BES-$H_2O_2$ in the absence or presence of 5.5 mM of glucose for 0.5 h. ROS production was measured using flow cytometry. (C) Immunoblotting analyses of HepG2 cells transfected with a non-targeting siRNA or two separate NOX4 siRNAs in the absence or presence of 5.5 mM of glucose or treatment with exogenous $H_2O_2$ for 0.5 h.
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phosphorylation was inhibited by treatment with NAC. As $H_2O_2$ has a low selectivity for downstream molecules, it may be involved in the regulation of numerous signaling pathways [27,28,29]. Among them, the regulation of AKT phosphorylation, as reported here, is particularly intriguing. AKT mediates cell proliferation and survival [30,31]. In our previous work, Akt activation was found to play a critical role in cell survival under glucose deprivation [10]. Furthermore, OSSA knockdown and the inhibition of SRC by PP2 suggests that these two elements are fundamental to AKT phosphorylation induced by glucose deprivation. It has been reported that SRC family kinases as their redox sensitive cysteines are the targets of specific oxidation by various oxidants, including $H_2O_2$ [32]. In this study, we clarified that PP2, a specific SRC inhibitor, inhibited AKT phosphorylation induced by glucose deprivation and exogeneous hydrogen peroxide. Thus, SRC is a strong candidate as a hydrogen peroxide sensor. Since PP2 inhibits SRC and other members of the SRC family, we should be careful before denying the relevance of other SRC family kinases [25]. Further investigations, such as by knockdown of individual SRC family kinases will be needed to identify the relevant Src-family kinase.

As with most intracellular signaling cascades, cross-talk and feedback interactions contribute to the overall regulation of PI3K/AKT signaling. S6 kinase-1, a downstream effector of mTORC1,

is known to be involved in a negative feedback loop of AKT activation. S6 kinase phosphorylates and inhibits upstream insulin receptor substrate proteins, which diminishes signaling through the PI3K/AKT pathway [33]. We observed that S6 kinase-1 phosphorylation was suppressed in PANC-1 cells under glucose deprivation (unpublished data), suggesting that the negative feedback machinery could be another mechanism regulating AKT phophorylation in cells under glucose deprivation. Furthermore, it was considered that the NADPH/NADP and ATP/AMP ratios may possibly change under glucose deprivation. Therefore, we measured the NADPH/NADP and ATP/AMP ratios; however, no significant changes were observed in at least the first 30 minutes. We also examined the effect of AMPK activation induced by AICAR on AKT activation and the cellular levels of hydrogen peroxide level, but again no significant changes were observed (unpublished data).

AKT phosphorylation in response to glucose deprivation was also completely inhibited following the addition of galactose or fructose instead of glucose. Galactose and fructose enter the glycolytic pathway after they have been metabolized intracellularly to glucose-6-phosphate and fructose-1 or 6-phosphate, respectively. Therefore, the contribution of decrease in metabolites downstream of fructose-1 or 6-phosphate to the induction of AKT phosphorylation under glucose deprivation was hypothesized.

To examine the contribution of the mitochondria, which are the major loci of ROS production, PANC-1 $Rho^0$ cells depleted of mitochondrial DNA were produced. When the $Rho^0$ cells were exposed to glucose-deprived medium, a large amount of intracellular $H_2O_2$ was produced. As pyruvic acid alone did not inhibit the ROS production completely, we could not assess the contribution of the mitochondria to the induction of ROS production by glucose deprivation further by this method (Shimoda et al. unpublished data). We then studied the involvement of NOX4 as another major locus of ROS production. AKT phosphorylation induced by glucose deprivation was not observed after NOX4 knockdown; no increase in the intracellular ROS levels was observed either, indicating the involvement of NOX4 in the intracellular accumulation of ROS. The contribution of NOX4, but not NOX5, in the signaling triggered by glucose deprivation was rather unexpected. Interestingly, a previous study reported that NOX4 regulates the survival of PANC-1 cells via ROS/ASK1/AKT signaling [34]. It might also be involved in cell survival under glucose-deprived conditions. With respect to the regulation of their activities, there are fundamental differences among the NOX isoforms. Most NOX family members are reportedly switched on and off by their regulatory subunits. NOX4 also functions as a complex with p22phox on internal membranes to produce ROS [35,36]. NOX4, unlike other members of the NOX family, is known to constitutively induce the production of large amounts of $H_2O_2$, however, the possibility of growth factor signaling being mediated by NOX4 has also been suggested [37]. The results of the present study also suggested that the activity of NOX4 might be regulated. In the present study, glucose deprivation increased the cellular levels of $H_2O_2$, which was suppressed by frucotose and galactose, indicating that NOX4 might be activated by deprivation of some glycolytic intermediate or some downstream products, such as of the pentose phosphate shunt and/or TCA cycle. The results obtained with the use of 2-DG are consistent with this idea. Whether the ROS accumulation under glucose deprivation is caused by increased production of ROS as a result of enhanced activity of NOX4, or by decreased antioxidant capacity, such as that associated with deficient activities of catalase, glutathione peroxidase, and glutathione needs to be further investigated. The intracellular amount of ROS is determined by the activity of the enzymes and the amounts of the substrates available. Therefore, metabolomic analysis of the entire set of metabolites is desired.

In the present study, we found that cells sense and respond to metabolic flux rather than glucose itself, and NOX4 and its product, ROS, play important roles in the cellular adaptive responses.

## Supporting Information

**Figure S1 Immunoblotting analyses after incubating PANC-1 cells in the absence or presence of 5.5 mM of glucose in the absence or presence of 30 μM of LY294002 for the indicated times.**
(TIF)

**Figure S2 Immunoblotting analyses after incubating human fibroblasts derived from subserossa of stomach in the absence or presence of 5.5 mM of glucose for 0.5 h.**
(TIF)

**Figure S3 PANC-1 cells were treated with or without various concentrations of glucose for 0.5 h.**
(TIF)

**Figure S4 Immunoblotting analyses after incubating PANC-1 cells in the absence or presence of 5.5 mM of glucose, 5.5 mM of galactose, or 5.5 mM of fructose for 0.5 h.**
(TIF)

**Figure S5 PANC-1 cells were cultured in either glucose-containing medium or glucose-deprived medium for 0.5 h.** Cells were stained with 5 μM BES-$H_2O_2$. ROS production was measured using flow cytometry.
(TIF)

**Figure S6 Human fibroblasts derived from subserossa of stomach were cultured in either glucose-containing medium or glucose-deprived medium for 0.5 h.** Cells were stained with 5 μM BES-$H_2O_2$. ROS production was measured using flow cytometry.
(TIF)

**Figure S7 HepG2 cells were cultured in the absence or presence of 5.5 mM of glucose, 5.5 mM of galactose, or 5.5 mM of fructose for 0.5 h. ROS production was measured using flowcytometry.** Cells were stained with 5 μM of BES-$H_2O_2$.
(TIF)

**Figure S8 Immunoblotting analyses of HepG2 cells in the absence or presence of 5.5 mM of glucose or treatment with exogenous $H_2O_2$ for 0.5 h.**
(TIF)

**Figure S9** (A) siRNA-treated PANC-1 cells were subjected to reverse transcriptional PCR (RT-PCR) to confirm NOX4 knockdown. (B) Immunoblotting analyses after incubating PANC-1 cells transfected with a non-targeting siRNA or two separate NOX4 siRNA in the absence or presence of 5.5 mM of glucose for 0.5 h.
(TIF)

**Figure S10** (A) siRNA-treated PANC-1 cells were subjected to reverse transcriptional PCR (RT-PCR) to confirm NOX5 knockdown. (B) Immunoblotting analyses after incubating

PANC-1 cells transfected with a non-targeting siRNA or two separate NOX5 siRNA in the absence or presence of 5.5 mM of glucose for 0.5 h.
(TIF)

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## Author Contributions

Conceived and designed the experiments: SO HE KT. Performed the experiments: SO YS. Analyzed the data: SO YS. Wrote the paper: SO HE KT.

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Drug Design, Development and Therapy Dove

ORIGINAL RESEARCH

# (+)-Grandifloracin, an antiausterity agent, induces autophagic PANC-1 pancreatic cancer cell death

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**Abstract:** Human pancreatic tumors are known to be highly resistant to nutrient starvation, and this prolongs their survival in the hypovascular (austere) tumor microenvironment. Agents that retard this tolerance to nutrient starvation represent a novel antiausterity strategy in anticancer drug discovery. (+)-Grandifloracin (GF), isolated from *Uvaria dac*, has shown preferential toxicity to PANC-1 human pancreatic cancer cells under nutrient starvation, with a $PC_{50}$ value of 14.5 μM. However, the underlying mechanism is not clear. In this study, GF was found to preferentially induce PANC-1 cell death in a nutrient-deprived medium via hyperactivation of autophagy, as evidenced by a dramatic upregulation of microtubule-associated protein 1 light chain 3. No change was observed in expression of the caspase-3 and Bcl-2 apoptosis marker proteins. GF was also found to strongly inhibit the activation of Akt, a key regulator of cancer cell survival and proliferation. Because pancreatic tumors are highly resistant to current therapies that induce apoptosis, the alternative cell death mechanism exhibited by GF provides a novel therapeutic insight into antiausterity drug candidates.

**Keywords:** (+)-grandifloracin, antiausterity strategy, PANC-1, nutrient starvation

## Introduction

Human pancreatic cancer is the most fatal form of cancer worldwide, with a 5-year survival rate of less than 5%.[1] Each year, approximately 29,000 people are diagnosed with pancreatic cancer in Japan.[2] The annual mortality rate from this malignancy closely approximates the annual incidence rate.[3,4] Once diagnosed, the average life expectancy is 6 months. It is the fifth leading cause of cancer-related mortality in Japan and other industrialized countries.[4] Until now, no effective treatment has been available.[5,6] Human pancreatic cancer shows resistance to most conventional chemotherapeutic drugs in clinical use, such as paclitaxel, doxorubicin, and cisplatin.[7] At present, gemcitabine and S-1 (tegafur + gimeracil + oteracil potassium) are the only standard regimens for advanced pancreatic cancer.[8–11] Therefore, effective chemotherapeutic agents against this disease are urgently needed. Human pancreatic tumors are hypovascular in nature,[12] causing a limited supply of nutrients and oxygen to reach the aggressively proliferating tumor cells.[13] As tumor cells proliferate, the demand for essential nutrients and oxygen exceeds the supply. Consequently, large areas of tumor survive under the hostile environment characterized by nutrient and oxygen starvation. Yet, human pancreatic tumor cells show the extraordinary ability to tolerate such extreme states through the modulation of energy metabolism.[14] While normal human cells die within 24 hours under nutrient starvation, some human pancreatic cancer cell lines can survive up to 72 hours in the complete absence of nutrients such as glucose, amino acids, and serum.[14]

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This remarkable tolerance to nutrient starvation is one of the key factors for survival and progression of pancreatic tumors. Therefore, agents that retard the tolerance of cancer cells to nutrient starvation represent a novel approach in anticancer drug discovery.[15] Using this hypothesis, we established a novel antiausterity strategy for the discovery of anticancer agents that preferentially target tolerance to nutrient starvation by cancer cells. Previous work on this strategy has led to the discovery of a number of potent anticancer agents, such as arctigenin,[15] angelmarin,[16] kayeassamins A–I,[17,18] and panduratins,[19,20] from the medicinal plants used in Japanese Kampo medicine and Southeast Asian countries.[21] Interestingly, these compounds also strongly suppressed tumor growth in a xenograft model using pancreatic cancer cells.[15] In our continued work, we recently found that a dichloromethane extract of the stem of *Uvaria dac* preferentially inhibited PANC-1 human pancreatic cancer cell survival under nutrient deprivation.[22] Work-up of this bioactive extract led to the discovery of (+)-grandifloracin (GF) as a potent antiausterity agent that showed preferential toxicity to PANC-1 cells with a $PC_{50}$ value of 14.5 μM. In this study, we explored the underlying mechanism of GF-induced modulation of key regulatory proteins involved in tolerance to nutrient starvation in PANC-1 cells.

# Materials and methods

## Reagents

GF (Figure 1) was isolated from the stems of *U. dac* as described previously.[22] GF purity was determined to be 95% by high-performance liquid chromatography. Conventional anticancer agents, ie, gemcitabine, 5-fluorouracil, 2-deoxyglucose, paclitaxel, podophyllotoxin, and camptothecin, were purchased from Sigma-Aldrich (St Louis, MO, USA). Each reagent was dissolved in dimethyl sulfoxide as a 10 mM stock solution and stored at –30°C until use. Dilution to give the desired concentration was performed prior to treatment. Dulbecco's phosphate-buffered saline was purchased from Nissui Pharmaceutical (Tokyo, Japan). Dulbecco's Modified Eagle's Medium. (DMEM) was purchased from Wako Pure Chemical (Osaka, Japan). Sodium bicarbonate, potassium chloride, magnesium sulfate, sodium dihydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, and phenol red were purchased from Wako Pure Chemical. HEPES was purchased from Dojindo Laboratories (Kumamoto, Japan). Fetal bovine serum was purchased from Nichirei Biosciences Inc. (Tokyo, Japan). Antibiotic/antimycotic solution was purchased from Sigma-Aldrich. The WST-8 cell counting kit was purchased from Dojindo Laboratories. Cell culture flasks and 96-well plates were obtained from Falcon Becton Dickinson Labware (BD Biosciences, San Jose, CA, USA). Nutrient-deprived medium was prepared according to a previously described protocol.[14] Rabbit polyclonal antibodies to Akt, phosphoryl Akt (Ser473), mammalian target of rapamycin (mTOR), phosphoryl mTOR (Ser2448), Bcl-2, caspase 3, and LC3A/B were purchased from Cell Signaling Technology (Danvers, MA, USA). A goat polyclonal antibody to actin was purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). Horseradish peroxidase-conjugated goat polyclonal anti-rabbit and rabbit polyclonal anti-goat immunoglobulins were purchased from DakoCytomation (Glostrup, Denmark).

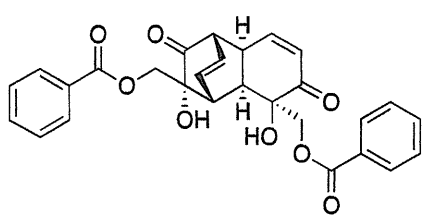


**Figure 1** Chemical structure of (+)-grandifloracin.

## Cell line

The PANC-1 (RBRC-RCB2095) cell line was purchased from the Riken BRC Cell Bank (Ibaraki, Japan) and maintained in standard DMEM with 10% fetal bovine serum supplement, 100 U/mL of penicillin G, 0.1 mg/mL of streptomycin, and 0.25 μg/mL of amphotericin B.

## Preferential cytotoxic activity

The in vitro preferential cytotoxicity of GF was determined using a previously described procedure with a slight modification.[22] In brief, human pancreatic cancer cells were seeded in 96-well plates ($1.5 \times 10^4$/well) and incubated in fresh DMEM at 37°C under humidified 5% $CO_2$ and 95% air for 24 hours. After the cells were washed with Dulbecco's phosphate-buffered saline, the medium was changed to serially diluted test samples in DMEM or nutrient-deprived medium, with the control and blank in each plate. After 24 hours of incubation, 100 μL of DMEM containing 10% WST-8 cell counting kit solution was directly added to each well. After 3 hours of incubation, absorbance at 450 nm was measured (EnSpire® Multilabel Reader, PerkinElmer, Waltham, MA, USA). Cell viability was calculated from the mean values for three wells using the following equation:

$$\text{Cell viability (\%)} = [(\text{Abs}_{(\text{test sample})} - \text{Abs}_{(\text{blank})})/(\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{blank})})] \times 100$$

## Morphologic assessment

Cells were seeded in 60 mm dishes ($1 \times 10^6$ cells) and incubated in a humidified $CO_2$ incubator for 24 hours to allow cell attachment. The cells were then washed twice with Dulbecco's phosphate-buffered saline and treated with 25 μM GF in DMEM, nutrient-deprived medium, and the control. After 12 and 24 hours of incubation, the cells were treated with fluorescein-labeled annexin V and propidium iodide, and cell morphology was observed using an inverted Nikon Eclipse TS 100 microscope (40× objective) with phase-contrast and fluorescence modes. Microscopic images were taken using a Nikon DS-L-2 camera directly attached to the microscope.

## Annexin V/dead cell assay

The annexin V/dead cell assay was performed in a Muse™ cell analyzer (Merck Millipore, Billerica, MA, USA) utilizing a Muse annexin V and dead cell kit. The assay utilizes phycoerythrin-labeled annexin V to detect phosphatidylserine on the external membrane of apoptotic cells. The kit contains the DNA dye, 7-aminoactinomycin D (7-AAD) for the exclusion of nonviable cells. Four populations of cells can be distinguished in this assay: nonapoptotic cells, annexin V (–) and 7-AAD (–); early apoptotic cells, annexin V (+) and 7-AAD (–); late-stage apoptotic and dead cells, annexin V (+) and 7-AAD (+); and necrotic nuclear debris, annexin V (–) and 7-AAD (+). The assay was performed according to the manufacturer's protocol. In brief, the cells were seeded in 60 mm dishes ($1 \times 10^6$ cells) and incubated in a humidified $CO_2$ incubator for 24 hours to allow cell attachment. The cells were then washed twice with Dulbecco's phosphate-buffered saline and treated with 12.5 μM GF, 25 μM GF, or the control of nutrient-deprived medium for the indicated time periods. The cells were then harvested from the dish with trypsin to give single cell suspensions. Finally, 100 μL of annexin V/dead reagent and 100 μL of a single cell suspension were mixed in a microtube and incubated for 20 minutes at room temperature in the dark. The cells were then analyzed using the Muse cell analyzer, and 5,000 cell events were collected for each sample. The images were acquired as the screenshots of the processed data and the text size was edited for clarity.

## Western blot analysis

Proteins were separated by gel electrophoresis on a polyacrylamide gel containing 0.1% sodium dodecyl sulfate and transferred to polyvinylidene fluoride membranes. The membranes were blocked with Block Ace® (DS Pharma Medical, Suita, Japan), washed with Dulbecco's phosphate-buffered saline containing 0.1% polyoxyethylene (20) sorbitan monolaurate (Wako Pure Chemical), and incubated overnight with primary antibodies diluted in Can Get Signal® (Toyobo, Osaka, Japan). After washing, the membranes were incubated for 45 minutes at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-goat immunoglobulins as the secondary antibody. The bands were detected with an enhanced chemiluminescence solution (PerkinElmer). The images were analyzed using Image Studio software version 3.1.4.

## Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test. A $P$-value$<0.05$ was considered to be statistically significant.

# Results

## GF showed preferential cytotoxicity in a concentration-dependent manner

The PANC-1 cell line is highly resistant to nutrient deprivation and shows an extraordinary ability to survive for >48 hours even under complete nutrient starvation. GF remarkably diminished tolerance to nutrient starvation in a concentration-dependent manner (Figure 2A). Cells exposed to GF at 25 μM showed 100% cell death within 24 hours in nutrient-deprived medium, with a $PC_{50}$ value of 14.5 μM; however, no toxicity was observed in nutrient-rich DMEM.

## GF sensitized PANC-1 cell death under glucose/serum-deprived conditions

To determine the conditions under which GF induces sensitivity to nutrient starvation resulting in cell death, the PANC-1 cells were treated with 25 μM GF under various nutrient conditions of glucose, amino acids, and serum. Cell viability was measured 24 hours after treatment. As shown in Figure 2B, GF was found to be toxic during glucose or serum deprivation, irrespective of the presence or absence of amino acids. In the presence of glucose and serum, cell viability was 100%. However, removal of serum led to a decrease in cell viability to 73% and 69% in the presence or absence of amino acids, respectively. Similarly, removal of glucose also led to a significant decrease in cell viability to 66%. Removal of both glucose and serum decreased cell viability to 2%.

## Conventional anticancer agents are ineffective against PANC-1 cells in nutrient-deprived medium

The preferential cytotoxicity of GF was compared with that of several conventional anticancer agents, including

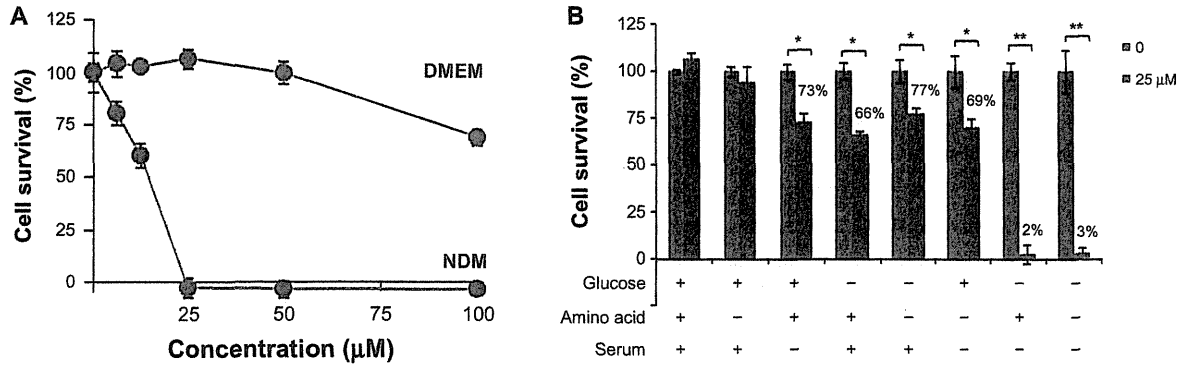


**Figure 2** Effect of (+)-grandifloracin on PANC-1 cell survival after 24 hours in NDM and normal medium (DMEM). **(A)** Effect of (+)-grandifloracin concentration on cell survival in NDM and DMEM. **(B)** Effects of medium components, ie, glucose, amino acids, and serum. Data are expressed as the mean ± standard deviation, n=3. *$P<0.05$; **$P<0.01$ indicate significant difference from the control.
**Abbreviations:** NDM, nutrient-deprived medium; DMEM, Dulbecco's Modified Eagle's Medium.

gemcitabine, 5-fluorouracil, 2-deoxyglucose, paclitaxel, camptothecin, and podophyllotoxin, using PANC-1 cells grown in nutrient-deprived medium versus DMEM (Figure 3). All tested agents were virtually inactive in nutrient-deprived medium; however, paclitaxel and camptothecin showed weak activity in nutrient-rich DMEM at the maximum tested dose of 100 μM after 24 hours. Because some of the conventional anticancer agents showed weak activity in DMEM, their effects during prolonged treatment were also evaluated by monitoring their cytotoxicity after 24, 48, and 72 hours. As shown in Figure 4, gemcitabine and 5-fluorouracil weakly decreased cell viability 72 hours after treatment. However, these compounds did not show a clear concentration-dependent effect. 2-Deoxyglucose was completely inactive. Paclitaxel and podophyllotoxin were found to reduce cell viability after 72 hours, but the effect was not concentration-dependent. On the other hand, camptothecin exhibited strong activity with cell viability of <25% at 10 μM 48 hours after treatment.

## Assessment of GF-induced apoptosis

To investigate whether GF-induced cell death in nutrient-deprived medium involves apoptosis, the cell morphology was examined. As shown in Figure 5, at 25 μM, GF induced a marked change in PANC-1 cell morphology within 8 hours. However, the cells lacked the classical signs of apoptosis, such as shrinkage or fragmentation into membrane-bound apoptotic bodies. Instead, swelling and rupture of cell membranes and disruption of cellular organelles appeared to be closer to a necrotic-type cell death. Staining with annexin V/propidium iodide reagent showed an increased population of cells containing Annexin V (green fluorescence) and propidium iodide (red fluorescence). Annexin V is a $Ca^{2+}$-dependent phospholipid-binding protein with

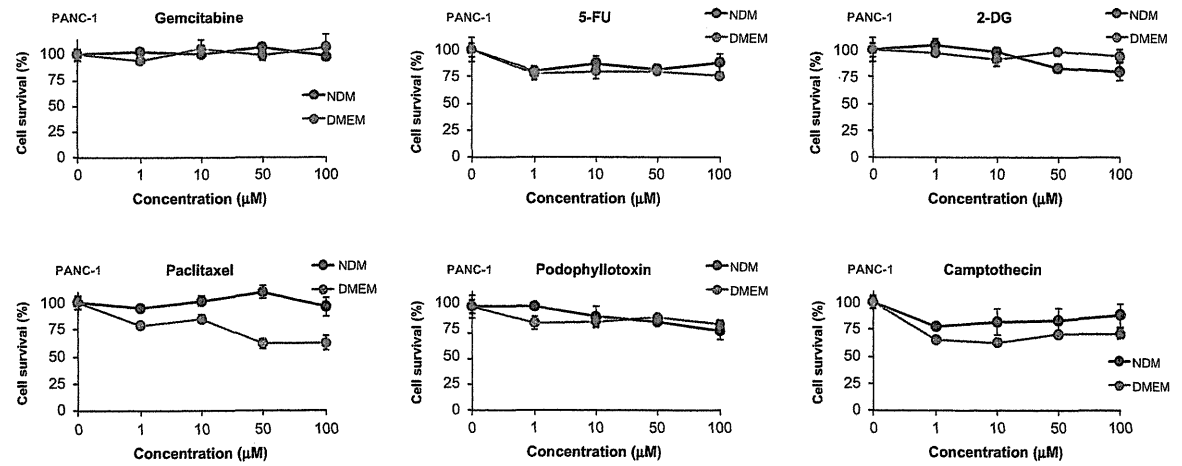


**Figure 3** Effect of conventional anticancer agents against PANC-1 cells after 24 hours in NDM and DMEM. Data are expressed as the mean ± standard deviation, n=3.
**Abbreviations:** NDM, nutrient-deprived medium; DMEM, Dulbecco's Modified Eagle's Medium; 5-FU, 5-fluorouracil; 2-DG, 2-deoxyglucose.

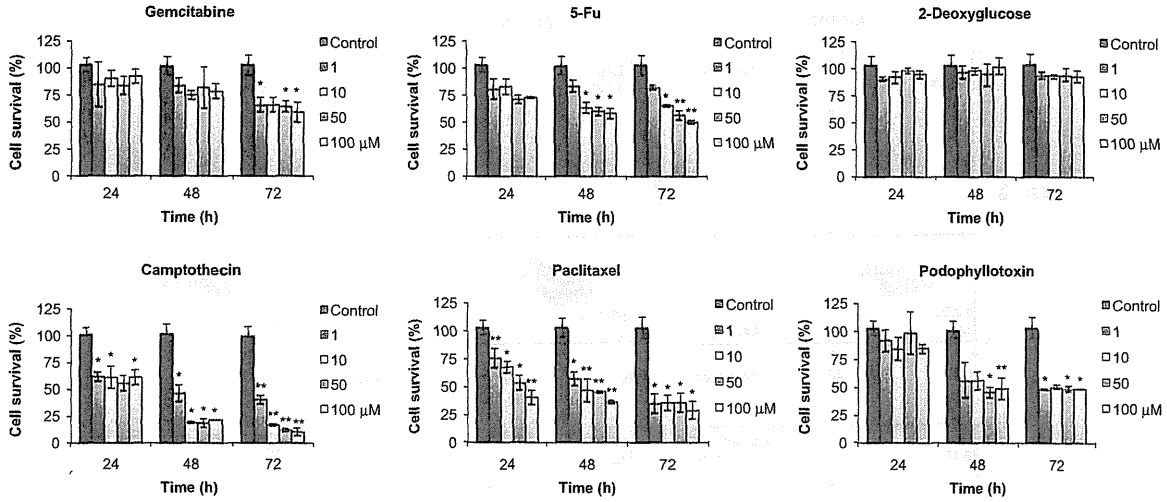


**Figure 4** Assessment of cytotoxicity of conventional anticancer agents against PANC-1 cells in Dulbecco's Modified Eagle's Medium. Data are expressed as the mean ± standard deviation, n=3. *$P<0.05$; **$P<0.01$ indicates a significant difference from the control.
**Abbreviation:** 5-FU, 5-fluorouracil.

high affinity for phosphatidylserine. Translocation of phosphatidylserine to the external cell surface occurs both in apoptosis and necrosis. We further performed flow cytometric analysis of cells treated with GF utilizing the Muse Annexin V and dead cell kit, which contains 7-AAD as a dye for exclusion of nonviable cells. 7-AAD is impermeable to viable cells and does not stain viable or early apoptotic cells. In late apoptotic and necrotic cells, the integrity of the cell membrane decreases, which allows 7-AAD to pass through the membranes, intercalate into nucleic acids and DNA, and display red fluorescence. As shown in Figure 6, the cells are predominantly stained with both Annexin V and 7-AAD within 12 hours in a concentration-dependent manner. In the control of nutrient-deprived medium, more than 90% of the cells survived. After treatment with GF, this cell population decreased markedly to 72% (12.5 μM) and 29% (25 μM), with an increase in the late apoptotic/necrotic cell population from 1% (control) to 15% (12.5 μM) and 61% (25 μM), respectively (Figure 6). We further performed Western blot analysis to examine GF-induced apoptosis. Treatment with GF neither led to cleavage of caspase-3 nor showed Bcl-2 inhibition (data not shown).

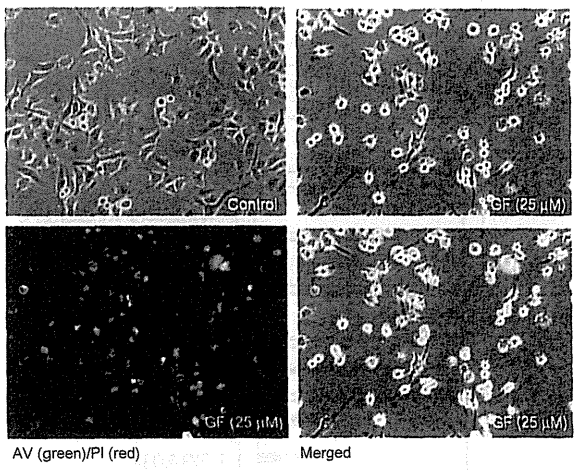


**Figure 5** Effect of GF (25 μM) on PANC-1 cell morphology after 8 hours in NDM. Phase-contrast (upper left), fluorescent (lower left), and merged (lower right) images of PANC-1 cells.
**Abbreviations:** AV, Annexin V; PI, propidium iodide; NDM, nutrient-deprived medium; GF, (+)-grandifloracin.

## GF inhibits Akt/mTOR activation

Akt is a prosurvival factor that is activated in a majority of tumors and regulates cellular functions such as cell cycle progression, cell migration, invasion, and angiogenesis. High Akt activation has been associated with tolerance to nutrient starvation and survival in an austerity environment.[14] Therefore, the effect of GF on Akt activation was investigated by Western blot analysis. As shown in Figure 6, Akt phosphorylation at Ser473 was completely inhibited by GF in a concentration-dependent as well as time-dependent manner in nutrient-deprived medium. GF also strongly suppressed total Akt. mTOR is a downstream effector of Akt and is frequently activated in various cancer types, where it is involved in tumor progression and metastasis.[23] Therefore, we tested whether GF has any modulatory activity against mTOR activation. As shown in Figure 7, addition of 25 μM GF completely inhibited mTOR phosphorylation at Ser2448 6 hours after treatment.

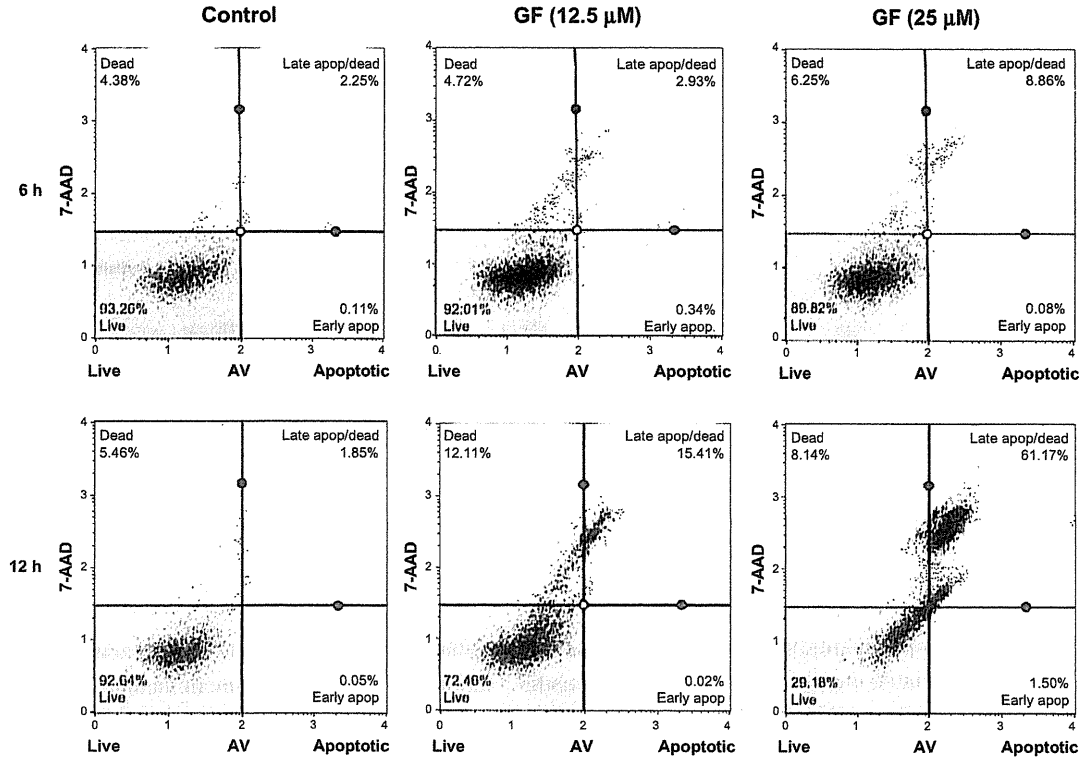


**Figure 6** Assessment of apoptosis by GF. PANC-1 cells were treated with vehicle or GF (12.5 μM and 25 μM) in nutrient-deprived medium. After treatment (6 hours and 12 hours), the cells were treated with Annexin V/7-AAD reagent and cytometric analysis was performed.
**Abbreviations:** Apop, apoptotic; AV, Annexin V; GF, (+)-grandifloracin; 7-AAD; 7-aminoactinomycin D.

## GF-induced autophagy in PANC-1 cells

Because no apoptotic cell death was observed in cells treated with GF, we speculated that GF might have induced autophagy. Therefore, expression of the autophagic marker microtubule-associated protein-light chain 3 (LC3), the cytoplasmic form of LC3-I (16 kDa), and the preautophagosomal and autophagosomal membrane-bound form of LC3-II (14 kDa) was examined by Western blot. The PANC-1 cells were cultured for varying time periods at different GF concentrations. As shown in Figure 7, no apparent differences were observed in LC3-I

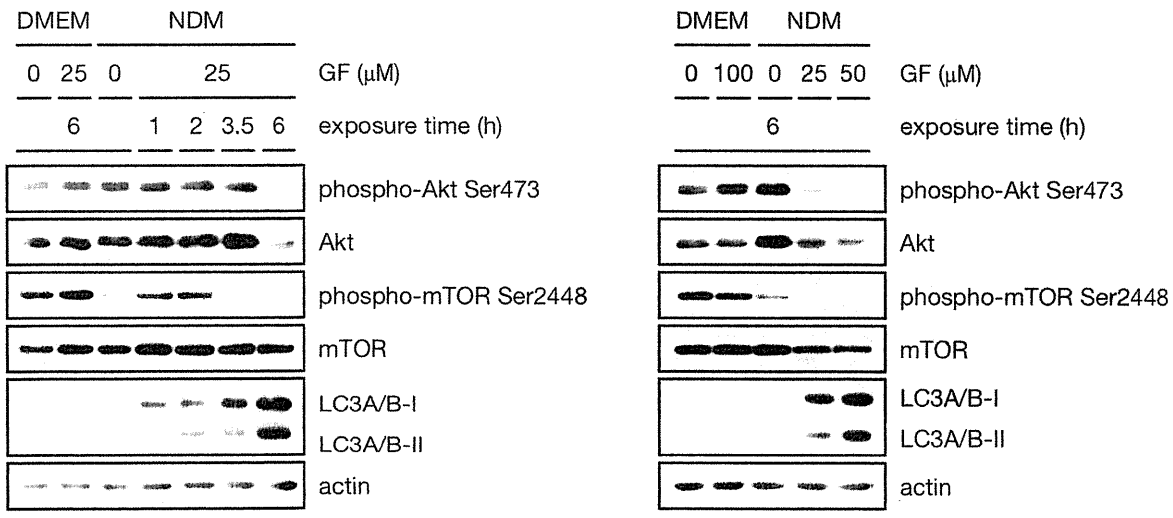


**Figure 7** Effect of GF against Akt, mTOR, LC3A/B I, and LC3A/B II.
**Abbreviations:** GF, (+)-grandifloracin; NDM, nutrient-deprived medium; DMEM, Dulbecco's Modified Eagle's Medium.

and LC3-II expression in the controls of both DMEM and nutrient-deprived medium. However, treatment with GF led to an enhancement in the expression of both LC3-I and LC3-II in a concentration-dependent as well as time-dependent manner. In nutrient-deprived medium, treatment with 25 μM GF led to incremental increases of eight-fold, 13-fold, and 22-fold in LC3-I expression with respect to the control after 2, 3.5, and 6 hours, respectively. Similarly, increases of 141-fold, 146-fold, and 659-fold in LC3-II expression were observed with respect to the control after 2, 3.5, and 6 hours, respectively.

## Discussion

Pancreatic cancer is associated with the lowest 5-year survival rate of any known cancer and is largely resistant to conventional chemotherapeutic agents. Although the median survival rate of the disease is only 6 months, some recent progress has been reported with FOLFIRINOX (folinic acid + 5-fluorouracil + irinotecan + oxaliplatin) and erlotinib.[24,25] However, new alternatives are urgently needed to improve the clinical outcome for patients diagnosed with pancreatic cancer. Pancreatic tumors are hypovascular and supply only a limited amount of essential nutrients and oxygen to aggressively proliferating cells. Consequently, these cells live in a hostile microenvironment under chronic metabolic stress conditions. For survival, these cells activate adaptive mechanisms such as autophagy.[26,27]

Autophagy is a homeostatic and evolutionarily conserved cellular pathway whereby cellular proteins and organelles are engulfed by autophagosomes, digested in lysosomes, and recycled in order to sustain cellular metabolism.[28] The process is activated in response to nutrient and energy starvation and acts as a survival mechanism to cope with diverse stresses in the tumor microenvironment.[28] Autophagy has been reported to be activated in colorectal cancer cells and to contribute to the tolerance to nutrient deprivation.[29] However, in the clinical setting, autophagy has been reported to serve as an alternative mechanism of programmed cell death that leads to tumor suppression.[30] One of the notable examples of a proautophagic cytotoxic drug that has demonstrated therapeutic benefits in several apoptosis-resistant cancer types in a clinical trial is temozolomide.[30] Several mechanisms have been suggested to explain the role of autophagy in suppression of tumorigenesis. Maintenance of genomic stability by clearance of damaged mitochondria and protein aggregates is considered one of the major mechanisms of tumor suppression by autophagy.[31] Further, excessive metabolic stresses in the tumor microenvironment often lead to necrotic cell death. Activation of autophagy under such circumstances prevents necrotic cell death and suppresses inflammation, which is known to increase tumor growth. Because the therapeutic goal of cancer treatment has been to trigger tumor-selective cell death, accelerating autophagy in apoptosis-resistant cancer cells would be an attractive alternative strategy in cancer therapy.

In the present study, GF does not appear to induce apoptosis but rather to operate by an alternative mechanism of programmed cell death, ie, autophagy. A marked activation of the autophagy marker LC3-II was observed after treatment with GF in a concentration-dependent and time-dependent manner. This was observed not only under nutrient-deprived conditions but also under nutrient-rich conditions, suggesting that GF is indeed an activator of autophagy. However, the effect of GF in nutrient-deprived medium was found to be highly significant compared with that in the control of nutrient-deprived medium at concentrations of 25 μM and 50 μM within 6 hours. Although a basal level of LC3-II protein is observed in the control of nutrient-deprived medium, it is activated within one hour after treatment with GF, which was found to be hyperactivated with respect to time as shown in Figure 5. This suggests that GF-induced autophagy mediates the death of PANC-1 cells preferentially during nutrient starvation.

The serine/threonine kinase Akt/mTOR pathway is constitutively activated in a majority of human pancreatic cancer cell lines. Activation of this pathway has been attributed to the survival of cancer cells in the heterogeneous tumor microenvironment, which confers resistance to chemotherapy and radiotherapy.[14] Akt has been found to be overexpressed in pancreatic cancer cells during extreme nutrient deprivation. Increased Akt expression is one of the austerity markers that enables tumor cells to survive and proliferate in the hostile hypovascular tumor microenvironment.[14] Therefore, inhibition of the Akt pathway might have therapeutic value in cancer patients. A number of antiausterity agents such as arctigenin, kigamicin D, and pyrvinium pamoate have been found to strongly suppress Akt activation, which suggests that inhibition of Akt phosphorylation by these compounds is partially responsible for the preferential cytotoxicity observed under nutrient deprivation.[15,32,33] However, the manner in which Akt inhibition affects downstream signaling under austerity conditions remains largely unknown. In the present study, GF suppressed both total Akt and phospho(Ser473) Akt in a time-dependent as well as concentration-dependent manner. It has been reported that mTOR is frequently inappropriately activated in many cancer types, and development of drugs that inhibit mTOR is an alluring therapeutic target in cancer therapy. mTOR is a downstream effector of the PI3K/AKT pathway and is composed of two distinct complexes,

ie, mTORC1 and mTORC2. In the present study, although the effects of GF on each multiprotein complex were not elucidated, complete inhibition of mTOR phosphorylation at Ser2448 was observed. mTOR inhibitors, such as temsirolimus and everolimus, have been approved by the US Food and Drug Administration for the treatment of renal cell carcinoma, primitive neuroectodermal tumor, and giant cell astrocytoma.[34] In this regard, GF is a dual inhibitor of the principal survival factors, Akt and mTOR, in tumors. Because pancreatic tumors are highly resistant to current chemotherapeutic agents that induce apoptosis, induction of an alternative cell death mechanism exhibited by GF represents a novel attractive candidate for preclinical evaluation.

## Acknowledgment

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## Disclosure

The authors report no conflict of interest in this work.

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# Identification of a lung adenocarcinoma cell line with CCDC6-RET fusion gene and the effect of RET inhibitors *in vitro* and *in vivo*

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**Rearrangements of the proto-oncogene *RET* are newly identified potential driver mutations in lung adenocarcinoma (LAD). However, the absence of cell lines harboring *RET* fusion genes has hampered the investigation of the biological relevance of RET and the development of RET-targeted therapy. Thus, we aimed to identify a *RET* fusion positive LAD cell line. Eleven LAD cell lines were screened for *RET* fusion transcripts by reverse transcription-polymerase chain reaction. The biological relevance of the *CCDC6-RET* gene products was assessed by cell growth, survival and phosphorylation of ERK1/2 and AKT with or without the suppression of RET expression using RNA interference. The efficacy of RET inhibitors was evaluated *in vitro* using a culture system and in an *in vivo* xenograft model. Expression of the *CCDC6-RET* fusion gene in LC-2/ad cells was demonstrated by the mRNA and protein levels, and the genomic break-point was confirmed by genomic DNA sequencing. Mutations in *KRAS* and *EGFR* were not observed in the LC-2/ad cells. CCDC6-RET was constitutively active, and the introduction of a siRNA targeting the *RET 3'* region decreased cell proliferation by downregulating RET and ERK1/2 phosphorylation. Moreover, treatment with RET-inhibitors, including vandetanib, reduced cell viability, which was accompanied by the downregulation of the AKT and ERK1/2 signaling pathways. Vandetanib exhibited anti-tumor effects in the xenograft model. Endogenously expressing CCDC6-RET contributed to cell growth. The inhibition of kinase activity could be an effective treatment strategy for LAD. LC-2/ad is a useful model for developing fusion RET-targeted therapy. (*Cancer Sci* 2013; 104: 896–903)**

Lung cancer is the most common cause of cancer death worldwide.[1] The identification of oncogenic driver genes is to select the increasing number of small molecule inhibitors targeting these gene products.[2,3] In particular, in lung adenocarcinoma (LAD), the most dominant histological subtype of lung cancer, the application of kinase inhibitors for cases with specific gene alterations has been successful, that is, gefitinib and erlotinib for *EGFR* mutation-positive cases and crizotinib for *ALK* fusion-positive cases.[4–7] Furthermore, accumulating evidence has demonstrated somatic mutations and rearrangements of potential oncogenes, including *BRAF, ERBB2* and *ROS1*, in LAD.[8–10]

*RET* is one of the newest LAD driver genes.[11–15] *RET* gene is located on chromosome 10 and encodes a receptor tyrosine kinase,[16,17] and the oncogenic potential of this gene product has been suggested in several tumors, including thyroid cancer.[18–20] Recently, five independent groups identified aberrant fusion genes, *KIF5B-RET* and *CCDC6-RET* in clinical samples of LAD.[11–15] Ectopically expressed RET fusion products afforded NIH3T3 cells with anchorage-independent growth and tumorigenicity in nude mice.[11,14] Furthermore, KIF5B-RET-expressing H1299 cells exhibited growth factor-independent growth.[11] These findings strongly suggest the oncogenic activity of RET fusion products and also suggest the potential therapeutic efficacy of multi-kinase inhibitor targeting of RET using the abovementioned cells. However, LAD-derived cell lines harboring *RET* fusion genes had not been identified. Recently, Matsubara *et al.*[21] screened LAD cell lines that were sensitive to a RET inhibitor vandetanib and found a *CCDC6-RET* fusion gene-harboring cell line, LC-2/ad.

We have independently screened cell lines established from Japanese LAD samples by RT-PCR and found that LC-2/ad cells expressed the *CCDC6-RET* fusion gene product. We further examined whether LC-2/ad cells depend on RET fusion-mediated signaling. In addition, the antitumor effect of RET inhibitors in LC-2/ad cells was evaluated *in vitro* and *in vivo*.

## Materials and Methods

Complete materials and methods were described in the supplementary information (Data S1. Materials and Methods).

**Purchased materials.** Cell lines were purchased from RIKEN Bio Resource Center, the Immuno-Biological Laboratories (Fujioka, Japan) and American Type Culture Collection. Procedures for western blotting was previously described.[22] Primary antibodies specific for RET and phospho-RET Tyr-905 were purchased from Epitomics (Burlingame, CA, USA) and Cell Signaling Technologies (Danvers, MA, USA), respectively. RET-targeting siRNA was purchased from Life Technologies (Carlsbad, CA, USA). Gefitinib, sunitinib malate and sorafenib were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), Sigma-Aldrich (St. Louis, MO, USA) and Toronto Research Chemicals (Toronto, ON, Canada),

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respectively. Vandetanib, AZD6244 and BEZ235 were purchased from Selleck (Houston, TX, USA).

**Multiplex RT-PCR.** Reported *KIF5B/CCDC6-RET* fusion variants were detected by multiplex RT-PCR according to the procedures described elsewhere.[11,14]

**Genomic DNA sequencing.** LC-2/ad DNA was captured with custom hybridization probes targeting *CCDC6* intron 1 and *RET* whole gene (Agilent) followed by parallel sequencing on the MiSeq system (Illumina).

**Real-time RT-PCR.** Procedures for real-time RT-PCR was previously described.[22] The PCR primers used in the present study are shown in Table S1.

***In vivo* studies.** LC2/ad cells at $5.0 \times 10^6$ were subcutaneously inoculated to 8-week-old athymic nude mice (Clea Japan).[23] Vandetanib was administered once daily as a homogeneous suspension by oral gavage at a dosage of 50 mg/kg body weight.[24] The tumor volume was calculated as the product of a scaling factor ($\pi/6$) and the tumor length, width and height.[22] The study was approved by the Institutional Ethics Review Committee for animal experiments at the National Cancer Center.

**Immunohistochemical analysis.** The procedure for hematoxylin eosin staining and immunohistochemical (IHC) was previously described.[22,25]

**Microarray analysis.** Background information of clinical samples was described in a previous report.[26] The study was approved by the Institutional Review Boards of the National Cancer Center. Total RNA was analyzed using Affymetrix (Santa Clara, CA, USA) U133Plus2.0 arrays. The data were processed by the MAS5 algorithm, and the mean expression level of a total of 54 675 probes was adjusted to 1000 for each sample.

## Results

**Identification of the *CCDC6-RET* fusion gene in a Japanese LAD cell line.** To identify *RET* fusion-derived mRNA expression in human LAD cell lines, all reported *KIF5B-RET* and *CCDC6-RET* gene products were screened by multiplex RT-PCR in 11 cell lines derived from Japanese patients. LC-2/ad cells were found to express *CCDC6-RET* mRNA at significantly higher levels, whereas the other cell lines did not exhibit any fusion gene products (Fig. 1a). The expressed fusion *RET* product was sequenced, and an in-frame fusion of *CCDC6* exon 1 and *RET* exon 12, which was identical to the previously reported *CCDC6-RET* fusion products, was identified (Fig. 1b).[14] We then identified a breakpoint of chromosome 10 by retrieving genomic DNA fragments, including the entire *RET* gene and intron 1 of *CCDC6*, by target capture system followed by parallel sequencing. The identified break-point between *CCDC6* intron 1 and *RET* exon 11 was confirmed by Sanger sequencing (Fig. 1b). Quantitative RT-PCR revealed that the expression of 3′ end of *RET* was increased comparable to that of *CCDC6*, whereas the transcript level of the 5′ end of *RET* was significantly lower (Fig. 1c). Consistent with the amount of transcript, western blotting using an antibody recognizing the C-terminus of RET isoform 2 detected a 60-kDa specific band equivalent to

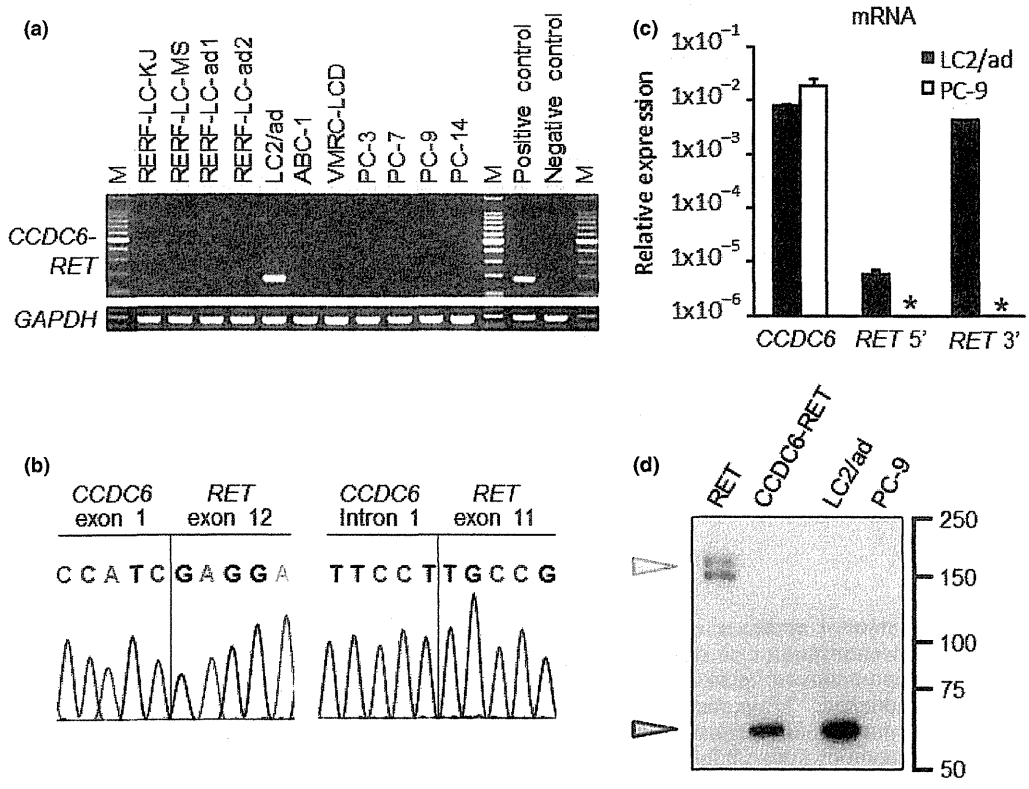


**Fig. 1.** Identification of the *CCDC6-RET* fusion gene. (a) Detection of *RET* fusion transcripts in lung adenocarcinoma (LAD) cell lines by multiplex reverse transcription-polymerase chain reaction (RT-PCR). (b) Sanger sequencing around the fusion point of the cDNA (left) and the breakpoint of the genomic DNA (right) of *CCDC6-RET* in LC-2/ad cells. (c) 3′ region-specific expression of *RET* mRNA in LC-2/ad cells. The 5′ or 3′ region of *RET* and *CCDC6* cDNA level was normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression. The data are shown as the mean ± standard deviation (SD) ($n = 3$). Asterisks indicate that mRNA expression were below the level of detection. (d) Specific expression of the CCDC6-RET fusion protein. Whole-cell lysates of LC2/ad and PC-9 cells and HEK293 cells transfected with wild-type RET (RET) or CCDC6-RET expression plasmids were subjected to western blot analysis to detect RET protein isoform 2. The LC-2/ad cells showed an approximately 60-kDa (red arrowhead) but not 170-kDa (blue arrowhead) band.

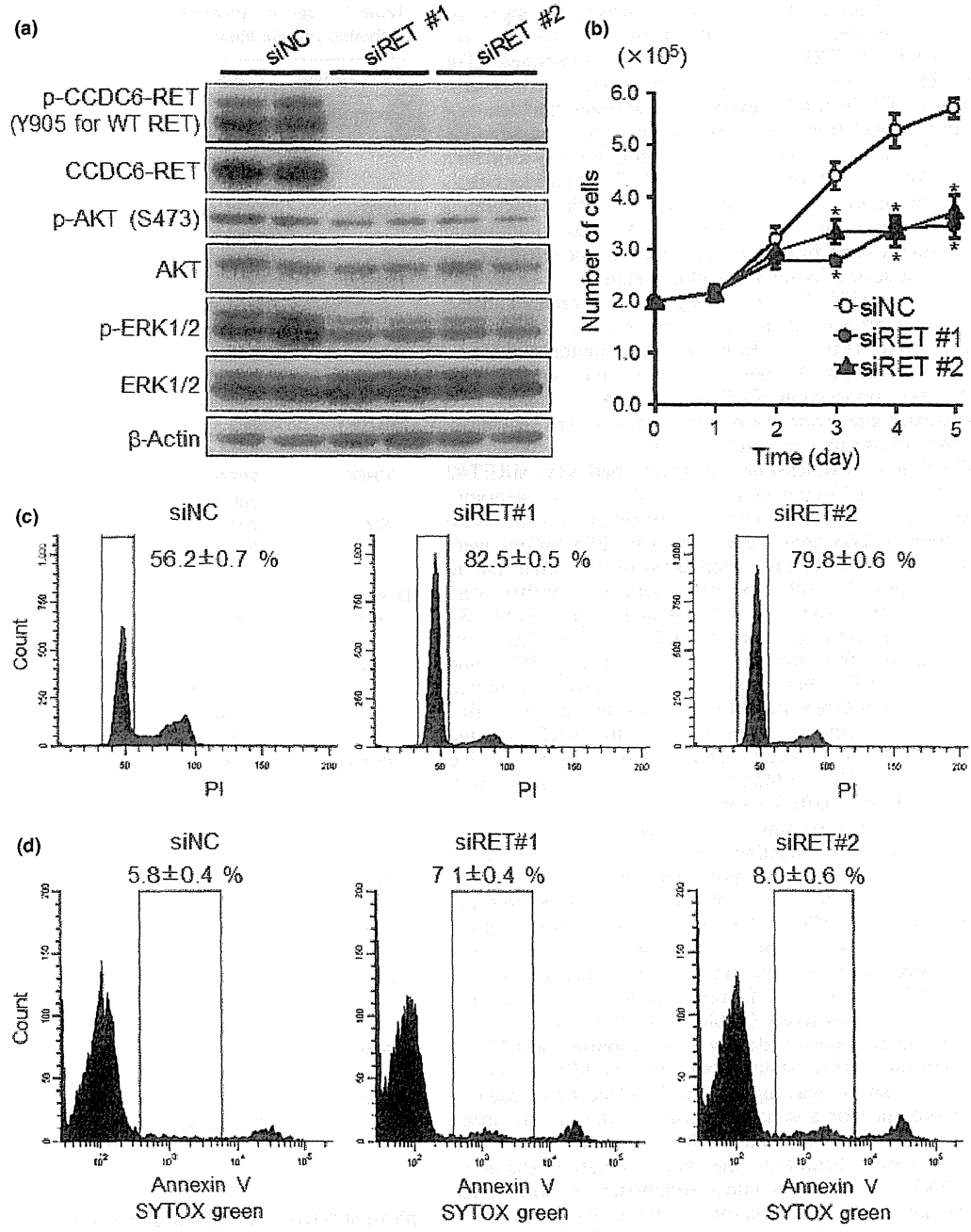


**Fig. 2.** Suppression of CCDC-RET expression by siRNA in LC-2/ad cells. (a) Western blot analysis of siRET-treated LC-2/ad cells. The siRNA transfected cell lysates were applied to the western blotting. (b) Involvement of RET suppression in cell growth inhibition. LC-2/ad cells transfected with siRNAs were incubated for the indicated times. The data are shown as the mean ± standard deviation (SD) ($n$ = 4). *$P$ < 0.01 (Student's $t$-test). (c,d) The DNA ploidy (c) and Annexin V-positive population (d) of siRET-transfected LC-2/ad cells. After 72 h of siRNA transfection, the cells were subjected to DNA ploidy analysis and Annexin V staining. The data are shown as the mean ± SD ($n$ = 4).

the estimated size of the fusion protein composed of 503 amino acids (GeneBank BAM36435), whereas no significant signal was detected that approximated the size of wild-type RET, 170-kDa (Fig. 1d).[11] Taken together, we concluded that LC-2/ad cells express *CCDC6-RET* fusion gene products. *KRAS* exon 2 and *EGFR* exon 19 and 21 were examined by Sanger sequencing, but no obvious mutation was confirmed (Fig. S1).

**CCDC6-RET-dependent ERK1/2 phosphorylation and the proliferation of LC-2/ad cells.** We suppressed *RET* expression by RNAi to characterize the function of CCDC6-RET in LC-2/ad cells. For avoiding off-target siRNA effects, two different sequences of siRNA directed against the 3′ region of *RET* (siRET#1 and #2) and a nontargeting siRNA (siNC) were used. When compared to siNC, a significant reduction in mRNA expression was observed by quantitative RT-PCR detecting the 3′ end of the *RET* mRNA: 66.5% for siRET#1 and 94.2% for siRET#2 (Fig. S2). Western blot analyses also revealed significant decreases in the expression of CCDC6-RET protein (60-kDa) upon the introduction of siRET#1 and #2 compared to the control siNC in the LC-2/ad cells


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