SGLT2 inhibitor ipragliflozin attenuates breast cancer cell proliferation

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Abstract. Cancer is currently one of the major causes of death in patients with type 2 diabetes mellitus. We previously reported the beneficial effects of the glucagon-like peptide-1 receptor agonist exendin-4 against prostate and breast cancer. In the present study, we examined the anti-cancer effect of the sodium-glucose cotransporter 2 (SGLT2) inhibitor ipragliflozin using a breast cancer model. In human breast cancer MCF-7 cells, SGLT2 expression was detected using both RT-PCR and immunohistochemistry. Ipragliflozin at 1–50 μM significantly and dose-dependently suppressed the growth of MCF-7 cells. BrdU assay also revealed that ipragliflozin attenuated the proliferation of MCF-7 cells in a dose-dependent manner. Because the effect of ipragliflozin against breast cancer cells was completely canceled by knocking down SGLT2, ipragliflozin could act via inhibiting SGLT2. We next measured membrane potential and whole-cell current using the patch clamp technique. When we treated MCF-7 cells with ipragliflozin or glucose-free medium, membrane hyperpolarization was observed. In addition, glucose-free medium and knockdown of SGLT2 by siRNA suppressed the glucose-induced whole-cell current of MCF-7 cells, suggesting that ipragliflozin inhibits sodium and glucose cotransport through SGLT2. Furthermore, JC-1 green fluorescence was significantly increased by ipragliflozin, suggesting the change of mitochondrial membrane potential. These findings suggest that the SGLT2 inhibitor ipragliflozin attenuates breast cancer cell proliferation via membrane hyperpolarization and mitochondrial membrane instability.

Key words: SGLT2 inhibitor, Breast cancer, Membrane potential, Mitochondria

RECENTLY, cancer has emerged as a major cause of death in patients with diabetes mellitus [1]. This is especially true in Japan, where cancer is the leading cause of death in patients with type 2 diabetes mellitus (T2DM). Accordingly, the Japan Diabetes Society and Japan Cancer Association have issued a warning regarding the increased cancer risk in patients with diabetes mellitus [2]. Notably, cases with T2DM and metabolic syndrome caused by obesity have been suggested to be associated with a higher risk of many cancers [3]. This evidence suggests the need for a therapeutic strategy for T2DM that can decrease not only glucose levels but also cancer risk and progression. In our previous work, we studied the anti-cancer effect of a glucagon-like peptide-1 (GLP-1) receptor agonist using prostate cancer [4] and breast cancer models [5]. In addition, we observed a further reduction of prostate cancer growth upon combined therapy with metformin and GLP-1 receptor agonist [6]. Sodium-glucose cotransporter 2 (SGLT2) inhibitors are anti-diabetic agents currently approved for clinical application. Because of their unique glucose-lowering mechanism and cardiovascular protective effect, they have received substantial attention. The SGLT2 inhibitor ipragliflozin is made in Japan [7] and was the first medication of its type to be used clinically in the country. We previously reported that ipragliflozin increased adiponectin and HDL-cholesterol and decreased HbA1c, body mass index, serum C-peptide level, and blood pressure in Japanese patients with T2DM [8]. In the present study, we examined the anti-cancer effect of the SGLT2 inhibitor ipragliflozin using breast cancer cells.
Materials and Methods

Cell culture and cell proliferation assays
The MCF-7 and MDA-MB-231 human breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The KPL-1 human breast cancer cell line was kindly provided by Dr. Junichi Kurebayashi, Kawasaki Medical School [9]. All cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cell proliferation assays were performed as described previously [4-6] with minor modifications. Briefly, cells were seeded in 12-well tissue culture plates and maintained in complete medium with 0–50 μM ipragliflozin (kindly provided by Astellas Pharma Inc., Tokyo, Japan). Cell proliferation was analyzed 0–4 days later by cell counting using a hemocytometer.

Immunohistochemistry
Paraffin sections were incubated with anti-SGLT2 (ab37296; Abcam, Cambridge, UK) and subsequently incubated with Alexa Fluor 488 goat anti-rabbit IgG (A-11008; Thermo Fisher Scientific, Rockford, IL, USA). Sections were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) and visualized by confocal microscopy.

Reverse transcription and quantitative real-time polymerase chain reaction (PCR)
Reverse transcription and quantitative real-time PCR were performed as described previously [4, 6]. Total mRNA from breast cancer cells was isolated using RNEasy Mini Kits (Qiagen, Venlo, the Netherlands) and reverse-transcribed into cDNA. PCR reactions were performed using a Light Cycler 2.0 (Roche, Basel, Switzerland) and SYBR Premix Ex Taq™ II (Takara, Otsu, Japan). Sections were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) and visualized by confocal microscopy.

Bromodeoxyuridine (BrdU) assays
The BrdU incorporation assay was performed using Cell Proliferation ELISA kits (1647229; Roche Applied Science, Mannheim, Germany). Briefly, MCF-7 cells were plated at 5,000 cells/well in 96-well culture plates in complete media. After attaining 60%–70% confluence, cells were treated with media containing 10% FBS with 0–100 μM ipragliflozin for 24 h. BrdU solution (10 μM) was added during the last 2 h of stimulation. The cells were dried and fixed, and cellular DNA was denatured with FixDenat solution (Roche Applied Science) for 30 min at room temperature. A peroxidase-conjugated mouse anti-BrdU monoclonal antibody (Roche Applied Science) was added to the culture plates and cells were incubated for 90 min at room temperature. Tetramethylbenzidine substrate was added and the plates were incubated for 15 min at room temperature. The absorbance of samples was measured using a microplate reader at 450–620 nm. Mean data are expressed as the ratio relative to the proliferation of control (untreated) cells.

Small interfering (si)RNA knockdown of SGLT2 and cell proliferation assay
To knock down SGLT2, we used SGLT-2 siRNA (sc-106547; Santa Cruz Biotechnology, CA, USA), which was designed to target human SGLT2; control siRNA (sc-37007; Santa Cruz Biotechnology) was used as a negative control. MCF-7 cells were plated at 2 × 10^4 cells/well in six-well plates and transfected with 10 nmol/L SGLT-2 siRNA or negative control siRNA using MISSION siRNA Transfection Reagent (Sigma-Aldrich). Seventy-two hours after transfection, cells were subject to cell proliferation assays. Briefly, cells were detached and re-plated in 24-well tissue culture plates in complete media with or without 10 μM ipragliflozin. At 0–4 days after treatment, cells were collected and counted using a hemocytometer.

Patch clamp measurements
Whole-cell patch recording for current and voltage clamps was performed using the nystatin-performed patch technique in MCF-7 cells at room temperature (22–25°C) with an Axopatch 200B (Molecular Devices, Sunnyvale, CA, USA) patch clamp amplifier, as described previously [10]. For whole-cell recording, we used Na+-based bath solutions, which contained (in mM) 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 D-glucose (pH adjusted to 7.4 with NaOH and osmolality adjusted to 320 mosmol/kg-H2O with D-mannitol). The pipette solution contained (in mM) 55 K2SO4, 20 KCl, 5 MgCl2, 0.2 EGTA, and 5 HEPES (pH adjusted to 7.4 with KOH and osmolality adjusted to 300 mosmol/kg-H2O with D-mannitol).

Mitochondrial permeability potential
Mitochondrial membrane potential (ΔΨm) was examined using a JC-1 mitochondrial membrane potential detection kit (#10009172; Cayman Chemicals, Ann
Arbor, MI, USA), according to the company’s instructions. MCF-7 cells treated with or without 10 μM ipragliflozin were stained with the cationic dye JC-1, which exhibits potential-dependent accumulation in mitochondria. At low membrane potential, JC-1 exists as a monomer and produces green fluorescence (emission at 527 nm). At high membrane potential and polarization, JC-1 forms J aggregates and produces red fluorescence (emission at 590 nm).

**Statistical analysis**

Unpaired $t$-tests and two-way ANOVA for repeated measures were performed for statistical analysis as appropriate. $P$-values less than 0.05 were considered statistically significant. Results are expressed as mean ± SEM.

**Results**

*SGLT2 is expressed in human breast cancer cells*

To detect SGLT2 expression on human breast cancer cells, we performed immunohistochemical analysis. As depicted in Fig. 1A, SGLT2 was expressed on the membrane of breast cancer cells. *SGLT2* gene expression was determined by quantitative RT-PCR using a set of primers targeting a 94-bp coding region of *SGLT2*. *TBP* expression was used for normalization. Unpaired $t$-tests were performed to calculate statistical significance. ($^*p < 0.05$ vs. KPL-1 cells) ($n = 3$).

![Fig. 1 SGLT2 is expressed in human breast cancer cells](image-url)

(A) Immunohistochemistry was performed to examine SGLT2 expression in breast cancer cell lines. All samples were counterstained with DAPI (magnification, 400×). (B) Quantitative RT-PCR was performed using a set of primers targeting a 94-bp coding region of *SGLT2*. *TBP* expression was used for normalization. Unpaired $t$-tests were performed to calculate statistical significance. ($^*p < 0.05$ vs. KPL-1 cells) ($n = 3$).
examined by quantitative PCR in three different breast cancer cell lines, highly estrogen-sensitive MCF-7 cells, estrogen-independent MDA-MB-231 cells, and low-estrogen-sensitive KPL-1 cells (Fig. 1B). Compared with KPL-1 cells, MCF-7 cells showed significantly higher SGLT2 gene expression. Thus, in subsequent experiments, we used MCF-7 cells, which are among the most widely used human breast cancer cell lines.

SGLT2 inhibitor attenuates breast cancer cell proliferation

We next treated MCF-7 cells with 0–50 μM ipragliflozin and created a growth curve. Ipragliflozin decreased the number of MCF-7 cells in a dose-dependent manner (Fig. 2A). Upon knocking down SGLT2 expression using siRNA, the attenuation of cell proliferation induced by ipragliflozin was completely canceled (Fig. 2B), suggesting that ipragliflozin attenuated breast cancer cell proliferation through SGLT2 inhibition. Furthermore, BrdU assay revealed that ipragliflozin at a high dose significantly inhibited DNA synthesis of MCF-7 cells (Fig. 2C). The efficacy of knockdown by siRNA was confirmed by RT-PCR (data not shown).

SGLT2 inhibitor ipragliflozin induces hyperpolarization of MCF-7 cell membrane

Because SGLT2 takes up not only glucose but also sodium into the cytoplasm, we next examined membrane potential using the patch clamp technique. As shown in Fig. 3A, 10 μM ipragliflozin induced hyperpolarization of the MCF-7 cell membrane similar to that upon treatment with glucose-free medium. Measurement of ΔV revealed the significant reduction of membrane potential by glucose-free medium and ipragliflozin (Fig. 3A), suggesting that the inhibition of glucose uptake through SGLT2 induced hyperpolarization of MCF-7 cell membrane potential. To confirm the pivotal role of SGLT2 in ipragliflozin-induced membrane hyperpolarization, we knocked down SGLT2 using siRNA and treated MCF-7 cells with medium with or without glucose. The change in membrane potential induced by the deletion of glucose in culture medium was abolished by siSGLT2 (Fig. 3B). Both glucose sensitive (Fig. 3C) and ipragliflozin sensitive (Fig. 3D) current density were significantly decreased by knocking down SGLT2.

SGLT2 inhibitor ipragliflozin induces mitochondrial membrane instability

We next examined the effect of ipragliflozin on ΔΨm because mitochondria are critical intracellular organelles that determine cell death and proliferation. JC-1 dye, which measures mitochondrial potential, is an indicator of cell viability. Red fluorescence indicates healthy and
Fig. 3  SGLT2 inhibitor ipragliflozin induced hyperpolarization of MCF-7 cell membrane

(A) Left panel: representative time course of changes in the membrane potential. Effects of membrane potential on glucose removal or addition of 10 μM ipragliflozin. Right panel: changes in membrane potential with glucose removal (Glucose–) and addition of 10 μM ipragliflozin (Ipragliflozin). Unpaired $t$-tests were performed to calculate statistical significance. (*$p < 0.05$ vs. Control) ($n = 6–7$). (B) Representative traces of the $I–V$ curve for membrane currents with or without glucose in control siRNA or SGLT2 siRNA treated MCF-7 cells. The differences in membrane currents between glucose-containing and -free conditions are shown as red curves. Ramp pulses ($–80$ mV to $+60$ mV, 0.28 V/s) were applied every 10 s from a holding potential of $+40$ mV. (C) Columns represent the means ± S.E.M. of difference currents (normalized to cell capacitance) in the presence and absence of external glucose. Unpaired $t$-tests were performed to calculate statistical significance. (*$p < 0.05$ vs. Control) ($n = 6–7$). (D) Columns represent the means ± S.E.M. of difference currents (normalized to cell capacitance) in the presence and absence of external ipragliflozin. Unpaired $t$-tests were performed to calculate statistical significance. (*$p < 0.05$ vs. Control) ($n = 6–7$).
intact mitochondria, while green fluorescence indicates unhealthy mitochondria and presages cell death via necrosis or apoptosis. As depicted in Fig. 4A, substantially increased green fluorescence was observed in MCF-7 cells treated with ipragliflozin. Furthermore, plotting the amounts of JC-1 fluorescence revealed that mitochondrial membrane instability was induced at the early phase of ipragliflozin treatment (Fig. 4B).

**Discussion**

In the present study, we investigated the effect of the SGLT2 inhibitor ipragliflozin against breast cancer through cell membrane hyperpolarization and mitochondrial membrane instability. SGLT2 inhibitor is a newly identified anti-diabetic agent that has received substantial attention for its glucose-lowering effects, without producing body weight gain and hypoglycemia, as well as its cardiovascular protective effects. Notably, recent reports from basic experimental studies also suggested that SGLT2 inhibitor also exhibits effects against cancers such as pancreatic, prostate [11], liver [12], and colon cancers [13]. However, to the best of our knowledge, no studies have examined the effect of SGLT2 inhibitor on breast cancer. Breast cancer is one of the most critical cancers associated with T2DM and obesity. Recently published data based on the National Health Interview Survey in the USA reported a 65.3% decline of mortality in breast cancer patients with diabetes compared with that in patients without diabetes [14]. Accordingly, gly-
cemic control while also inhibiting breast cancer progression is important for female patients with DM.

In the present study, we investigated SGLT2 expression in human breast cancer cells, which is not expressed in human normal mammary gland. Our findings revealed that the SGLT2 inhibitor ipragliflozin attenuated breast cancer cell proliferation and DNA synthesis (Fig. 1). The dose of ipragliflozin that attenuated breast cancer cell proliferation, 1–10 μM, was similar to its pharmacological concentration in serum [14], suggesting that our data generally mirror clinical conditions. Furthermore, orally administrated ipragliflozin distributes into glandular tissues in similar or higher concentrations compared with serum (unpublished data by Astellas Pharma). Although growth was also suppressed at a lower dose of ipragliflozin (Fig. 2A), ipragliflozin at a high dose, 50–100 μM, reduced DNA synthesis in the BrdU assay (Fig. 2C). These findings suggest that ipragliflozin attenuated breast cancer cell proliferation through not only inhibiting DNA synthesis but also other mechanisms, such as cell death including apoptosis. We examined apoptosis by TUNEL assay, however, reproducible apoptosis was not observed. Further examination using other methods is required. We focused on the sodium transport by SGLT2 because sodium uptake is emerging as a mechanism of cancer biology including breast cancer [15]. Ipragliflozin shut down sodium uptake through SGLT2 and induced membrane hyperpolarization of MCF-7 cells. We also found that ipragliflozin induced instability of ΔΨm, which may lead to apoptosis and necrosis of host cells. The mechanism by which SGLT2 induces mitochondrial membrane instability may involve either inhibition of glucose or inhibition of sodium transport. Intracellular sodium could alter ΔΨm via sodium-calcium and sodium-hydrogen exchangers [16, 17]. However, low glucose might not regulate ΔΨm; glucose transporter 1 is also expressed in breast cancer cells and is an important energy regulator and therapeutic target [18]. Further experiments may reveal other effects of SGLT2 inhibitors on cancer cells and explore combination treatment with SGLT2 inhibitor, metformin and GLP-1. A meta-analysis and case report suggested anticancer effects of SGLT2 inhibitors [19, 20].

In conclusion, in this study, we showed that the SGLT2 inhibitor ipragliflozin attenuates breast cancer cell proliferation via membrane hyperpolarization and mitochondrial membrane instability.

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Author Contributions

S.K. and T.K. performed experiments and data analysis. T.No. wrote the manuscript and conceived the research hypothesis and design. T.Nu. performed the patch clamp measurements and wrote the manuscript. Y.H., C.I., T.H., Y.F.T., N.H., R.M., T.I., and D.K. reviewed the manuscript. T.Y. conceived the research design and reviewed the manuscript.

Disclosure

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References

1. Emerging Risk Factors Collaboration, Seshasai SR, Kaptoge S, Thompson A, Di Angelantonio E, Gao P, et al. (2011) Diabetes mellitus, fasting glucose, and risk of cause-specific death. N Engl J Med 364: 829–841.
2. Kasuga M, Ueki K, Tajima N, Noda M, Ohashi K, et al. (2013) Report of the JDS/JCA joint committee on diabetes and cancer. Diabetol Int 4: 81–96.
3. Esposito K, Chiodini P, Colao A, Lenzi A, Giugliano D (2012) Metabolic syndrome and risk of cancer: a systemic review and meta-analysis. Diabetes Care 35: 2402–2411.
4. Nomiyama T, Kawanami T, Irie S, Hamaguchi Y, Terawaki Y, et al. (2014) Exendin-4, a glicagon-like peptide-1 receptor agonist, attenuates prostate cancer growth. Diabetes 63: 3891–3905.
5. Iwaya C, Nomiyama T, Komatsu S, Kawanami T, Tsutsumi Y, et al. (2017) Exendin-4, a glucagonlike
peptide-1 receptor agonist, attenuates breast cancer growth by inhibiting NF-κB activation. *Endocrinology* 158: 4218–4232.

6. Tsutsumi Y, Nomiyama T, Kawanami T, Hamagichi Y, Terawaki Y, *et al.* (2015) Combined treatment with Exendin-4 and metformin attenuates prostate cancer growth. *PLoS One* 10: e0139709

7. Tahara A, Kurosaki E, Yokono M, Yamajuku D, Kihara R, *et al.* (2012) Pharmacological profile of ipragliflozin (ASP1941), a novel selective SGLT2 inhibitor, in vitro and in vivo. *Naunyn Schmiedebergs Arch Pharmacol* 385: 423–436.

8. Nomiyama T, Shimono D, Horikawa T, Fujimura Y, Ohsako T, *et al.* (2018) Efficacy and safety of sodium-glucose cotransporter 2 inhibitor ipragliflozin on glycemic control and cardiovascular parameters in Japanese patients with type 2 diabetes mellitus; Fukuoka Study of Ipragliflozin (FUSION). *Endocr J* 65: 859–867.

9. Kurebayashi J, Kurosuni M, Sonoo H (1995) A new human breast cancer cell line, KPL-1 secretes tumor-associated antigens and grows rapidly in female athymic nude mice. *Br J Cancer* 71: 845–853.

10. Numata T, Murakami T, Kawashima F, Moroue N, Heuser JF, *et al.* (2012) Utilization of photoinduced charge-separated state of donor-acceptor-linked molecules for regulation of cell membrane potential and ion transport. *J Am Chem Soc* 134: 6092–6095.

11. Scafoglio C, Hirayama BA, Kepe V, Liu J, Ghezzi C, *et al.* (2015) Functional expression of sodium-glucose transporters in cancer. *Proc Natl Acad Sci USA* 112: E4111–E4119.

12. Shiba K, Tsuchiya K, Komiya C, Miyachi Y, Mori K, *et al.* (2018) Canagliflozin, an SGLT2 inhibitor, attenuates the development of hepatocellular carcinoma in a mouse model of human NASH. *Sci Rep* 8: 2362.

13. Saito T, Okada S, Yamada E, Shimoda Y, Osaki A, *et al.* (2015) Effect of dapagliflozin on colon cancer cell. *Endocr J* 62: 1133–1137.

14. Kadokura T, Saito M, Utsuno A, Kazuta K, Yoshida S, *et al.* (2011) Ipragliflozin (ASP1941), a selective sodium-dependent glucose cotransporter 2 inhibitor, safely stimulates urinary glucose excretion without inducing hypoglycemia in healthy Japanese Subjects. *Diabetol Int* 2: 172–182.

15. Mao W, Zhang J, Komer H, Jiang Y, Ying S (2019) The emerging role of voltage-gated sodium channels in tumor biology. *Front Oncol* 9: 124.

16. Baysal K, Jung DW, Gunter KK, Gunter TE, Brierley GP (1994) Na(+)-dependent Ca2+ efflux mechanism of heart mitochondria is not a positive Ca2+/2Na+ exchanger. *Am J Physiol* 266: C800–C808.

17. Numata M, Petrecca K, Lake N, Orlowski J (1998) Identification of a mitochondrial Na+/H+ exchanger. *J Biol Chem* 273: 6951–6959.

18. Martel F, Guedes M, Keating E (2016) Effect of polyphenols on glucose and lactate transport by breast cancer cell. *Breast Cancer Res Treat* 157: 1–11.

19. Tang H, Dai Q, Shi W, Zhai S, Song Y, *et al.* (2017) SGLT2 inhibitors and risk of cancer in type 2 diabetes: a systematic review and meta-analysis of randomised control trials. *Diabetologia* 60: 1862–1872.

20. Okada J, Matsumoto S, Kaira K, Saito T, Saito T, *et al.* (2018) Sodium glucose cotransporter 2 inhibition combined with cetuximab significantly reduced tumor size and carcinoembryonic antigen level in colon cancer metastatic to liver. *Clin Colorectal Cancer* 17: e45–e48.