Biochemical and Biophysical Research Communications 522 (2020) 342–347

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Efficacy of glutathione inhibitors for the treatment of ARID1A-deficient diffuse-type gastric cancers

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ARTICLE INFO

Article history:
Received 2 November 2019
Accepted 13 November 2019
Available online 22 November 2019

Keywords:
Diffuse-type gastric cancer
Glutathione (GSH)
Reactive oxygen species (ROS)
APR-246
ARID1A

ABSTRACT

ARID1A, a subunit of the SWI/SNF chromatin remodeling complex, increases the intracellular levels of glutathione (GSH) by upregulating solute carrier family 7 member 11 (SLC7A11). Diffuse-type gastric cancer is an aggressive tumor that is frequently associated with ARID1A deficiency. Here, we investigated the efficacy of GSH inhibition for the treatment of diffuse-type gastric cancer with ARID1A deficiency using ARID1A-proficient or -deficient patient-derived cells (PDCs). ARID1A-deficient PDCs were selectively sensitive to the GSH inhibitor APR-246, the GCLC inhibitor buthionine sulfoximine, and the SLC7A11 inhibitor erastin. Expression of SLC7A11, which is required for incorporation of cystine, and the basal level of GSH were lower in ARID1A-deficient than in ARID1A-proficient PDCs. Treatment with APR-246 decreased intracellular GSH levels, leading to the excessive production of reactive oxygen species (ROS), and these phenotypes are suppressed by supply of cystine and GSH compensators. Taken together, vulnerability of ARID1A-deficient gastric cancer cells to GSH inhibition is caused by decreased GSH synthesis due to diminished SLC7A11 expression. The present results suggest that GSH inhibition is a promising strategy for the treatment of diffuse-type gastric cancers with ARID1A deficiency.

1. Introduction

Loss-of-function mutations of genes encoding subunits of the SWI/SNF chromatin remodeling complex are found in approximately 20% of all human cancers [1]. Such mutations promote tumorigenesis by impairing chromatin remodeling for transcription, DNA damage repair, DNA replication, and chromatin segregation, thereby disturbing transcriptional homeostasis. The *ARID1A* gene, which encodes a component of the SWI/SNF chromatin remodeling complex, is frequently mutated in several intractable cancers. *ARID1A* mutations are present in 46% of ovarian clear cell carcinoma (OCCC), 33% of gastric carcinoma, 27% of cholangiocarcinoma, and 15% of pancreatic carcinoma cases [2–5], which all lack effective molecular targeting therapies. *ARID1A* deficiency is associated with poor prognosis in various cancers [6]. Thus, much effort has been devoted to elucidating the effects of *ARID1A* deficiency to develop effective therapeutic modalities against these intractable cancers [7–11].

Antioxidants have been proposed as targets for cancer therapy mediated by the induction of reactive oxygen species (ROS) and DNA damage [12,13]. Cellular ROS levels are determined by the balance between ROS generation and elimination, and are regulated by antioxidant defense mechanisms [12]. Because high levels of ROS cause cell damage and cell death, targeting antioxidant defense systems is an attractive therapeutic strategy. We recently proposed a novel therapeutic strategy for ARID1A-deficient ovarian cancers mediated by targeting the vulnerability of glutathione (GSH) metabolism [14]. This strategy is based on the finding that ARID1A deficiency impairs the transcription of solute carrier family 7 member 11 (*SLC7A11*), which maintains the intracellular cysteine balance for GSH synthesis, thereby decreasing the basal GSH level. A low basal level of GSH in ARID1A-deficient cancers may underlie

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https://doi.org/10.1016/j.bbrc.2019.11.078
0006-291X/

the sensitivity to inhibition of GSH metabolism. ARID1A shows a synthetic lethal relationship with several GSH synthesis-related genes. APR-246, an investigational drug with GSH inhibiting activity, and the glutamate-cysteine ligase catalytic subunit (GCLC) inhibitor buthionine sulfoximine (BSO) are effective for the treatment of ovarian cancers with *ARID1A*-deficiency. These two drugs decrease intracellular GSH levels in *ARID1A*-deficient cancer cells with low basal GSH. This leads to increased ROS production and the perturbation of antioxidant system homeostasis [14]. Therefore, ARID1A-deficient ovarian cancer cells with low GSH levels are vulnerable to GSH metabolism inhibition. However, whether GSH inhibition is effective for the treatment of other types of tumors with ARID1A deficiency remains unknown.

Gastric cancer is a common malignancy with a high prevalence in Asian countries, and it is the second cause of cancer-related death worldwide [15]. Gastric cancer is classified into two histological types, namely, intestinal and diffuse [16]. Diffuse-type gastric cancer is infiltrative and often shows aggressive invasion into the gastric wall, resulting in metastasis and the spread of gastric cancer cells into the peritoneal cavity followed by ascites accumulation [17]. Diffuse-type gastric cancers, which are more intractable and have a worse prognosis than intestinal-type tumors, are frequently associated with ARID1A deficiency [17]. This led us to hypothesize that GSH inhibition may be effective for the treatment of diffuse-type gastric cancers associated with ARID1A deficiency. Here, we investigated the efficacy of GSH inhibitors using diffuse-type gastric cancer cell lines established from patient-derived ascites.

2. Material and methods

2.1. Reagents

APR-246 (Cat# 9000487) and erastin (Cat# 17754) were purchased from Cayman. L-buthionine-sulfoximine (Cat# B2515-500 MG), glutathione monoethyl ester (GSH-MEE) (Cat# G1404-25 MG), and L-cystine dimethyl ester dihydrochloride (CC-DME) (Cat# 857327-5G) were purchased from Sigma-Aldrich.

2.2. Establishment of diffuse-type gastric cancer cell lines

Tumor samples and ascites were obtained from patients with diffuse-type gastric cancer who underwent surgery or cell-free and concentrated ascites reinfusion therapy at the National Cancer Center Hospital or Kanamecho Hospital (Tokyo, Japan) and were cultured *in vitro*. The study protocol was approved by the Institutional Review Board of the National Cancer Center (Tokyo, Japan), and written informed consent was obtained from the patients. Whole ascetic cells were pelleted by centrifugation at 1500 rpm for 5 min at room temperature and then incubated in hemolysis buffer (0.75% NH_4Cl and 17 mM Tris-HCl, pH 7.65) for 10 min. After centrifugation, pellets were washed with PBS and cultured in RPMI 1640 containing 10% FBS for 1 week, after which the culture medium was replaced with DMEM containing 10% FBS to remove lymphocytes. Cells were cultured for an additional week. Adherent cells were cultured in RPMI 1640 containing 10% FBS for several weeks with weekly medium exchanges until the appearance of multiple colonies. When necessary, cultured cells were treated repeatedly with 0.05% trypsin-EDTA for a short duration to remove fibroblasts or other cell types such as mesothelial cells. The culture was passaged when colonies became dense.

2.3. Histological analysis of cell line-derived xenografts

Six-week-old female CAnN.Cg-Foxn1nu/CrlCrlj (BALB/c-nu/nu) mice (Charles River Laboratories Japan were bred at room temperature with a 12 h light/dark daily cycle. The mice were maintained under specific pathogen-free conditions and were provided sterile food, water, and cages. Approximately 5×10^6 cancer cells were suspended with 100 μl phosphate-buffered saline and were injected subcutaneously into mice using a 26.5-gauge needle. All experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain and were approved by the Committee for Ethics in Animal Experimentation of the National Cancer Center. Specimens fixed in formalin and embedded in paraffin were cut into 8 μm sections, which were dewaxed and dehydrated for routine hematoxylin and eosin staining.

2.4. Immunoblot analysis

Immunoblot analysis was performed according to method described in the previous study [14].

2.5. Cell viability assay

Cell viability was examined by measuring cellular ATP levels using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). To measure cell viability after drug treatment, cells were trypsinized, counted, reseeded at the specified density in 96-well plates, and exposed to the indicated concentrations of drugs. Cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay. Luminescence was measured using an Envision Multi-label plate reader (PerkinElmer).

2.6. mRNA quantification

mRNA quantification was performed according to method described in the previous study [14].

2.7. Detection of GSH and ROS

GSH and ROS were detected using the GSH/GSSG-Glo Assay (Promega) and/or the GSH-Glo Assay (Promega) and the ROS-Glo Assay (Promega). To measure the levels of GSH and ROS after drug treatment, cells were trypsinized, counted, reseeded at the specified density in 96-well plates, and exposed to the indicated concentrations of drugs. After 24–48 h, luminescence was measured using an Envision Multi-label plate reader (PerkinElmer). Cell viability was also measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). GSH and ROS levels were normalized to cell viability. The GSH/GSSG ratio was calculated as the GSH-GSSG signal divided by the GSSG/2 signal. Relative signal ratios in treated samples were normalized to those in untreated samples.

2.8. Statistical analysis

Statistical analyses were performed using Microsoft Excel and GraphPad Prism. Data are expressed as the mean ± SD or mean ± SEM, as indicated in the figure legends. The sample size (n) is indicated in the figure legends and represents biological replicates. Statistical significance was evaluated using the two-tailed Student's *t*-test. Statistically significant differences are indicated by asterisks as $^*p < 0.05$.

3. Results

3.1. Selection of ARID1A-deficient and -proficient patient-derived cells established from the ascites of patients with diffuse-type gastric cancer

Of over 100 patient-derived cells (PDCs) obtained from the ascites of 65 patients with diffuse-type gastric cancer, we selected 13 cell types (NSC-4X1a, -7C, −14C, −20C, −22C, −34C, −48CA, −58C, −64C, −65C, −67C, −68C, and −70C) showing adherent cell growth and lower dispersion in the drug-sensitivity test than floating cells. ARID1A protein expression was investigated by immunoblot analysis. Eight PDCs were selected for further analysis based on whole exome data. Of these eight PDCs, four (NSC−7C, −58C, −65C, and −67C) lacked ARID1A protein expression (ARID1A-deficient: ARID1A^{-}) and four (NSC−48CA, −64C, −68C, and −70C) retained ARID1A protein expression (ARID1A-proficient: ARID1A^{+}) (Fig. 1A). SLC7A11 expression was lower in ARID1A-deficient PDCs than ARID1A-proficient PDCs (Fig. 1A), consistent with the pattern observed in ovarian cancer [14]. Consistent with ARID1A protein levels, three (NSC−7C, −58C, and −67C) of four ARID1A-deficient PDCs had homogeneous frame-shift mutations in the *ARID1A* gene, and the remaining PDC (NSC−65C) had a homogeneous stop codon mutation (R1461X). The four ARID1A-proficient PDCs had no mutations. Xenograft tumors derived from established PDCs retained the histological properties of diffuse-type gastric cancers (Fig. 1B). Xenograft tumors derived from established PDCs retained the histological properties of diffuse-type gastric cancers. Representative histological data are shown in Fig. 1B.

3.2. ARID1A-deficient gastric cancer cells are sensitive to GSH inhibitors

We next examined the sensitivity of ARID1A-deficient gastric cancer cells to GSH inhibitors. The IC_{50} values for the GSH inhibitor APR-246 were markedly lower in ARID1A-deficient PDCs than in ARID1A-proficient PDCs (Fig. 2A and B). Treatment with BSO, an inhibitor of the GSH synthesis enzyme GCLC, sensitized ARID1A-deficient PDCs more efficiently than ARID1A-proficient PDCs (Fig. 2C). These results indicate that sensitivity to APR-246 or BSO is associated with ARID1A deficiency in gastric cancer, which is consistent with the results obtained in ovarian cancer [14]. Taken together, these data indicate that GSH inhibition might be a promising strategy for the treatment of diffuse-type gastric cancers with ARID1A-deficiency.

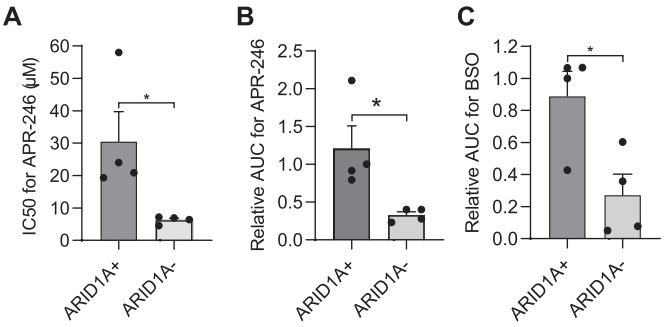

Fig. 2. ARID1A-deficient diffuse-type gastric cancer PDCs are sensitive to GSH inhibitors.
(A) IC_{50} values for APR-246 in DGC PDCs including four ARID1A-WT PDCs and ARID1A-deficient PDCs after treatment for 6 days.
Data are expressed as the mean ± SEM. (n = 4) *p < 0.05; two-tailed *t*-test.
(B) Relative area under the curve (AUC) values for cell viability in PDCs including four ARID1A-WT PDCs and ARID1A-deficient PDCs treated with APR-246 for 6 days.
Data are expressed as the mean ± SEM. (n = 4) *p < 0.05; two-tailed *t*-test.
(C) Relative AUC values for cell viability in PDCs including four ARID1A-WT PDCs and ARID1A-deficient PDCs treated with BSO for 6 days.
Data are expressed as the mean ± SEM. (n = 4) *p < 0.05; two-tailed *t*-test.

3.3. ARID1A-deficient gastric cancer cells are vulnerable to GSH inhibition due to low basal levels of GSH

Next, we investigated whether low expression of the SLC7A11 protein in ARID1A-deficient gastric cancers is associated with decreased *SLC7A11* transcription. *SLC7A11* mRNA levels were lower in ARID1A-deficient than in ARID1A-proficient PDCs (Fig. 3A, Fig. S1A). Since SLC7A11 is required for GSH synthesis by supplying intracellular cysteine, we examined whether SLC7A11 downregulation leads to decreased GSH synthesis. The basal levels of GSH were considerably lower in ARID1A-deficient than in ARID1A-proficient PDCs (Fig. 3B, Fig S1B). These results indicate that ARID1A-deficiency downregulates SLC7A11 expression and decreases the basal levels of GSH in diffuse-type gastric cancer cells, consistent with the findings in ovarian cancer [14].

APR-246 inhibits GSH activity by reacting with thiol groups [18]. Therefore, we next examined whether APR-246 preferentially inhibits GSH in ARID1A-deficient cancer cells. APR-246 treatment markedly decreased GSH levels in ARID1A-deficient PDCs and not in ARID1A-proficient PDCs (Fig. 3C, Fig S1C). Consistent with the antioxidant activity of GSH, ROS levels were increased more markedly in ARID1A-deficient than in ARID1A-proficient PDCs (Fig. 3D, Fig S1D). These results indicate that the excessive increase of oxidative stress induced by GSH inhibitors in ARID1A-deficient cells decreased cell viability.

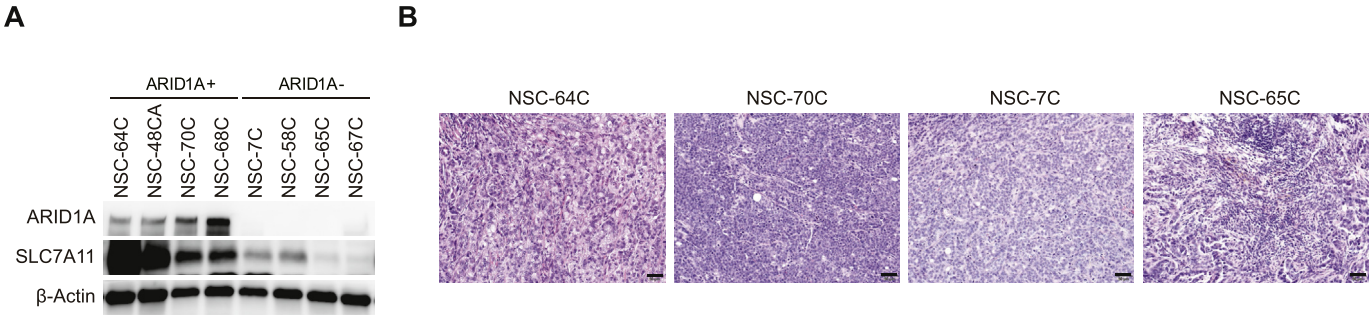

Fig. 1. ARID1A protein expression in diffuse-type gastric cancer patient-derived cell (PDC) lines and histology of xenograft tumors.
(A) Immunoblotting for ARID1A, SLC7A11, and β-actin in whole-cell extracts of diffuse-type gastric cancer PDCs. (B) Hematoxylin and eosin staining of xenograft tumors derived from PDCs. NSC-64C, NSC-70C, and NSC-7C tumors showed poorly differentiated histology. NSC-65C tumors showed poorly or moderately differentiated histology. Scale bar, 50 μm.

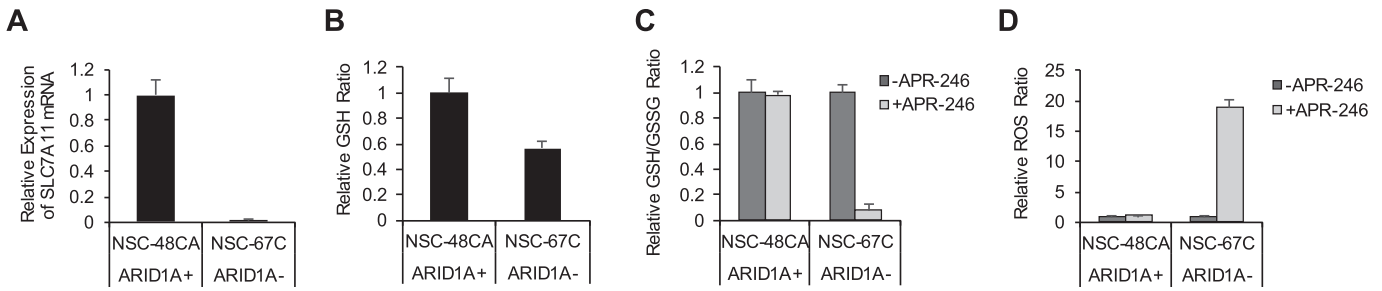

Fig. 3. ARID1A-deficient gastric cancer cells are vulnerable to GSH inhibition due to low basal levels of GSH.
(A) Relative expression of SLC7A11 mRNA in PDCs: ARID1A-WT NSC-48CA cells and ARID1A-deficient NSC-67C cells. Data are expressed as the mean ± SD.
(B) Basal GSH levels in PDCs: ARID1A-WT NSC-48CA cells and ARID1A-deficient NSC-67C cells. Data are expressed as the mean ± SD.
(C) Relative levels of GSH/GSSG, which indicates the ratio of reduced GSH to the oxidized form GSH disulfide (GSSG), in ARID1A-WT NSC-48CA cells and ARID1A-deficient NSC-67C cells treated with 40 μM APR-246 for 24 h. Data are expressed as the mean ± SD
(D) Relative ROS levels in ARID1A-WT NSC-48CA cells and ARID1A-deficient NSC-67C cells treated with 40 μM APR-246 for 48 h. Data are expressed as the mean ± SD.

3.4. Vulnerability of ARID1A-deficient gastric cancer cells to GSH inhibition is caused by decreased GSH synthesis due to diminished SLC7A11 expression

We next examined whether the vulnerability of ARID1A-deficient cancer cells is related to cysteine shortage and consequent GSH shortage. The APR-246-induced GSH decrease, ROS increase, and cell death in ARID1A-deficient cancer cells were markedly suppressed by co-treatment with the cystine compensator cystine dimethyl ester (CC-DME) or the GSH compensator glutathione monoethyl ester (GSH-MEE), cell-permeable versions of cystine and GSH, respectively, suggesting that these cell-permeable metabolites were able to compensate for impairment of cystine uptake due to diminished SLC7A11 expression (Fig. 4A–C). GSH is synthesized from cysteine, glutamate, and glycine. SLC7A11 contributes to GSH synthesis by transporting cysteine into the cell. Therefore, we next examined the sensitivity to erastin, an SLC7A11 inhibitor [19], in ARID1A-deficient PDCs. The IC_{50} values for erastin were markedly lower in ARID1A-deficient PDCs than in ARID1A-proficient PDCs (Fig. S2A). Erastin treatment markedly decreased GSH levels in ARID1A-deficient PDCs and not in ARID1A-proficient PDCs (Fig. S2B). These data indicate that a cysteine shortage and consequent GSH shortage secondary to diminished SLC7A11 expression in ARID1A-deficient cancer cells are the cause of their sensitivity to GSH inhibition.

4. Discussion

In previous work from our group, we demonstrated the potential of GSH inhibitory therapy for the treatment of OCCC, a malignant type of ovarian cancer prevalent in Asian countries [14]. We then expanded our research to other cancer types that may respond to the same strategy. In this study, we focused on diffuse-type gastric cancer and demonstrated that this malignancy is also sensitive to metabolic pathway inhibitors, such as APR-246, BSO and erastin, associated with ARID1A deficiency. The molecular basis of the sensitivity was similar to that observed in ovarian cancer [14]: ARID1A deficiency downregulated the expression of SLC7A11, which is required for the supply of cysteine for GSH synthesis. Decreased SLC7A11 expression leads to a decrease in the basal level of GSH, which increases the sensitivity of cells to GSH inhibitor-mediated perturbation of the homeostatic balance between GSH and ROS, and the death of ARID1A-deficient cancer cells. We recently identified that ARID1A-deficient OCCC cells are selectively sensitive to gemcitabine [20]. Inhibition of SLC7A11 by erastin potentiated gemcitabine sensitivity [19]. These observations suggest that gemcitabine sensitivity in ARID1A-deficient cancer cells is associated with diminished SLC7A11 expression. The prognosis of patients with advanced diffuse-type gastric cancer has remained poor over the past decade because of a higher rate of peritoneal dissemination in diffuse-type (78%) than in intestinal-type (45%) tumors [17]. In particular, the prognosis of scirrhous gastric cancer (Borrmann's type IV carcinoma), which accounts for 40% of diffuse-type gastric cancers, remains extremely poor. The 5 year overall survival rate is approximately 10% and ranges from 18% to 29% even after curative surgery [20]. However, effective molecular-targeted therapeutic drugs are not available. Because approximately 30% of patients with gastric cancer have ARID1A deficiency [21], GSH inhibitory therapy may improve the prognosis of this intractable disease.

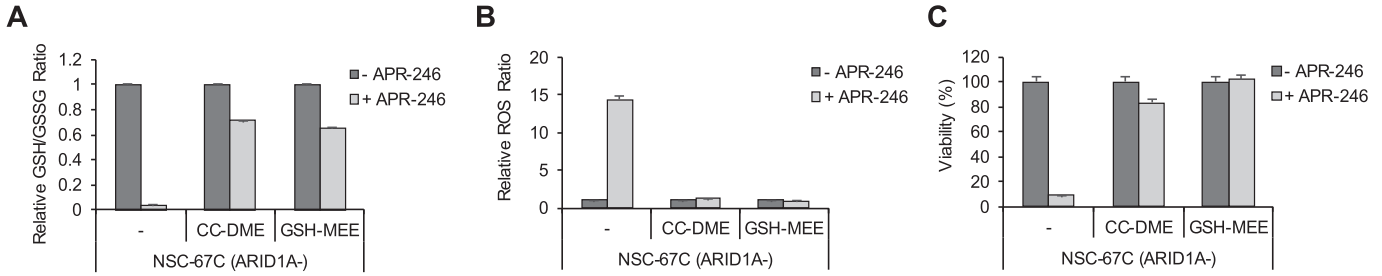

Fig. 4. Vulnerability of ARID1A-deficient gastric cancer cells to GSH inhibition is caused by decreased GSH synthesis due to diminished *SLC7A11* expression.
(A–C) Relative GSH levels (A), relative ROS levels (B), and cell viabilities (C) in ARID1A-deficient NSC-67C cells after treatment with 20 μM APR-246 for 24 h with or without 100 μM CC-DME or 2.5 mM GSH-MEE co-treatment.

Because APR-246 inhibits multiple proteins containing thiol groups [22], toxicity associated with off-target effects is an issue of concern. However, a recent phase I clinical trial of APR-246 for hematologic malignancies did not report any serious side effects [23]. APR-246 was originally developed as a drug targeting p53 mutants [24], and the therapeutic effects of APR-246 were examined in clinical trials of cancers with frequent *TP53* mutations, such as high-grade serous ovarian cancer and hematological malignancies [22,25,26]. Gastric cancers frequently show mutations in both *ARID1A* (about 30%) and *TP53* (about 50%) genes [21]. Because *ARID1A* and *TP53* mutations tend to be mutually exclusive [1], APR-246 may be effective in a large proportion (about 80%) of gastric cancer patients. We recently showed that the proposed GSH inhibitory strategy can be applied to cholangiocarcinoma, another aggressive cancer prevalent in Asian countries [14]. In addition, deficiency of other SWI/SNF chromatin remodeling proteins in addition to ARID1A may improve the response to GSH inhibitory therapy. These data suggest that the applications of this therapeutic strategy may be further expanded.

Funding

This study was supported in part by the Practical Research for Innovative Cancer Control from the Japan Agency for Medical Research and Development, Japan (JP19ck0106374), Ono Pharmaceutical Corporation, Japan (C2018-130) and by the National Cancer Center Research and Development Fund, Japan (29-A-2).

Author contributions

Conceptualization: M.S., H.O.; methodology: M.S., F.C., T. Kuroda, H.S., and H.O.; formal analysis and investigation: M.S. and T. Kuroda; resources: F.C., M.K., K.M., and H.S.; writing – original draft preparation: M.S., H.O.; writing – review and editing: M.S., T. Kohno, H.S., and H.O.; supervision: T. Kohno and H.O.; project administration: H.O.; funding acquisition: H.S. and H.O.

Declaration of competing interest

H.O. receives research funding from Ono Pharmaceutical Corporation, Japan. The other authors declare that they have no competing interests.

Acknowledgments

We acknowledge and appreciate our colleagues for their valuable efforts and comments on this paper.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.11.078.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.11.078.

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