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Histone methyltransferase SMYD2 selective inhibitor LLY-507 in combination with poly ADP ribose polymerase inhibitor has therapeutic potential against high-grade serous ovarian carcinomas

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ABSTRACT

Dysfunction of histone methylation is known to be related to cancer progression. The histone methyltransferase SMYD2 methylates histone protein H3 and non-histone proteins, including poly ADP ribose polymerase 1 (PARP1). There have been reports of SMYD2 overexpression in several types of cancers. However, there are no reports regarding its role in high-grade serous ovarian carcinomas (HGSOCs). Therefore, we investigated the expression profile and conducted functional analysis on SMYD2 in HGSOC cells. In addition, we verified whether SMYD2 inhibition increases the susceptibility of HGSOC cells to PARP inhibitors. We analyzed the expression of histone methyltransferase SMYD2 by quantitative real-time polymerase chain reaction and immunohistochemistry using HGSOC clinical tissues (n = 35). We performed functional analyses, including cell proliferation assay, cell cycle analysis, and immunoblotting, after treatment with SMYD2 siRNAs and SMYD2 selective inhibitor LLY-507 in HGSOC cells. We also performed colony-formation assay after combination treatment with LLY-507 and PARP inhibitor olaparib in HGSOC cells. The expression profiles of SMYD2 showed significant overexpression of SMYD2 in HGSOC clinical tissues. The knockdown or inhibition of SMYD2 by siRNAs or LLY-507, respectively, suppressed cell growth by increasing the proportion of apoptotic cells. LLY-507 showed additive effect with olaparib in the colony-formation assay. These findings suggest that LLY-507 can be used alone or in combination with a PARP inhibitor for the treatment of patients with HGSOC.

1. Introduction

Ovarian cancer patients have the highest mortality rate amongst patients with gynecologic cancers. The 10-year survival rate of ovarian cancer patients is <30%. High-grade serous ovarian carcinoma (HGSOC) accounts for 70–80% of ovarian cancer-related deaths. Most patients with HGSOC are diagnosed at advanced stages of the disease [1]. Genomic analysis of HGSOC has revealed genetic mutations in some genes, such as *TP53* and *BRCA1/2*. Among these genes, the rate of somatic *TP53* mutations observed in HGSOC was over 95%. Some HGSOC patients have germline mutations in *BRCA1/2*, which is related to hereditary breast and ovarian cancer (HBOC) syndrome. As a new molecular targeted therapy for HGSOC, the PARP inhibitor, olaparib, was developed for *BRCA1/2* mutated HGSOC. The PARP inhibitor is a pharmacological inhibitor of the enzyme PARP, which is involved in DNA repair. PARP inhibitor can

Abbreviations: DMEM/F12, Dulbecco's modified Eagle medium/nutrient mixture F-12; DMSO, dimethyl sulfoxide; ERα, estrogen receptor α; FBS, fetal bovine serum; HGSOC, high-grade serous ovarian carcinoma; HMT, histone methyltransferase; IHC, immunochemistry; IS, intensity score; PARP, poly ADP ribose polymerase; PBS, phosphate-buffered saline; PS, proportion score; RPMI, Roswell Park Memorial Institute; siRNA, small interfering RNA; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; PI, Propidium iodide.

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selectively induce synthetic lethality in cancer cells with *BRCA1/2* mutation [2].

Histone methylation is an important epigenetic modification, along with histone acetylation, ubiquitination, sumoylation, poly ADP-ribosylation, and phosphorylation, and is generally associated with relevant gene expression.

Several studies have indicated the role of dysregulation of histone methylation in carcinogenesis and cancer progression [3,4]. Several histone methyltransferases (HMTs) and demethylases are frequently upregulated in many types of cancers [5,6]. Our previous study showed that the HMT, SUV39H2, conferred therapeutic resistance to lung cancer cells [7]. In addition, we have reported that the overexpression of the HMT, EZH2, is observed in endometrial cancer cells, and that the knockdown of EZH2 expression and treatment with an EZH2-selective inhibitor suppress cancer growth and induce apoptosis [8]. Thus, there is substantial evidence suggesting that the inhibition of HMTs is a promising and novel strategy for cancer therapy.

Another HMT, SMYD2, has been identified as a member of the SMYD family and is known to function as an oncogene. It is a SET and MYND domain-containing histone (lysine) methyltransferase that methylates histone proteins H3K4 and H3K36 associated with active transcription [9]. SMYD2 also methylates various types of non-histone proteins, including p53, Rb, HSP90, ERα, EML4-ALK, β-catenin, and poly ADP ribose polymerase (PARP)1, thereby regulating various cellular function [10–15].

Although it has been reported that SMYD2 is upregulated in several types of cancers, there are no reports on its role in HGSOC. Therefore, we examined the expression and conducted functional analysis of SMYD2 in HGSOC. The goal of our study was to elucidate the involvement of SMYD2 in HGSOC and its therapeutic potential.

2. Materials and methods

2.1. Fresh frozen clinical samples

Thirty-five primary HGSOC and six normal ovarian tissue samples were obtained from the University of Tokyo Hospital from 2010 to 2016. This study was approved by the Human Genome, Gene Analysis Research Ethics Committee of the University of Tokyo.

2.2. Cell lines and cell culture conditions

High-grade serous ovarian carcinoma cell lines, JHOS2, JHOS3, JHOS4, and HTOA, were purchased from the RIKEN Cell Bank (Ibaraki, Japan). The KURAMOCHI, OVSAHO, OVKATE, and TYK-nu cell lines were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). The OVCAR3 cell line was purchased from the American Type Culture Collection (ATCC; Manassas). All cells were maintained at 37 °C in humidified air with 5% CO_2.

2.3. siRNA transfection

siRNA transfection (100 nM) was performed using Lipofectamine-RNAi MAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Small interfering RNAs (siRNAs) specific to SMYD2 (siSMYD2 #1: sense: 5′-GAAUGACCGGUUAAGAGA-3′ and antisense: 5′- UCUCUUAACCGGUCAUUUC-3′; siSMYD2 #2: sense: 5′- GAUUUGAUUCAGAGUGACA-3′ and antisense: 5′- UGUCACUCUGAAUCAAAUC-3′) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Negative control siRNA (siNC; MISSION siRNA Universal Negative Control; Sigma-Aldrich) was used as the control.

2.4. RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR was performed using the One-Step SYBR Prime Script RT-PCR Kit (TaKaRa Bio, Tokyo, Japan) in a Light Cycler instrument (Roche Diagnostics, IN, USA). The mRNA expression levels were normalized using β-actin (housekeeping gene) mRNA expression level. The sequences of primers (final concentration, 10 pmol/μl) used for qRT-PCR are as follows: SMYD2 forward: 5′-ATCTCCTGTACCCAACGGAAG-3′ and reverse: 5′-CACCTTGGCCTTATCCTTGTCC-3′; β-actin forward: 5′-CACACTGTGCCCATCTACGA-3′ and reverse: 5′-CTCCTTAATGTCACGCACGA-3′.

2.5. Cell viability assay

Cell Count Kit-8 solution (Dojindo, Kumamoto, Japan) was added to each well, and the cells were incubated for 2 h. The absorbance of the solution at 450 nm was measured on a microplate reader (BioTek, Winooski, VT, USA). Cell viability was normalized by 0.02% dimethyl sulfoxide (DMSO; Sigma-Aldrich) or siNC-treated cells [16].

2.6. Antibodies and inhibitors

The following antibodies were obtained from the indicated suppliers: rabbit anti-SMYD2 (#9734; Cell Signaling, Danvers, MA, USA, 1:200 for immunohistochemistry (IHC) and 1:1000 for western blotting), rabbit anti-cleaved PARP (#5125; Cell Signaling, 1:1000 for western blotting), mouse anti-β-actin (A2228; Sigma-Aldrich, Frankfurt, Darmstadt, Germany, 1:5000 for western blotting), anti-rabbit IgG, HRP-linked antibody (#7074S; Cell Signaling, 1:3000 for western blotting), and anti-mouse IgG, HRP-linked antibody (#7077S; Cell Signaling, 1:3000 for western blotting). The SMYD2 selective inhibitor LLY-507 was purchased from Selleck (No. S7575), and the PARP inhibitor olaparib was purchased from Cayman Chemical (No. 10621).

2.7. Cell cycle analysis

The pelleted cells were washed with phosphate-buffered saline (PBS) and centrifuged twice at 1500 rpm for 5 min. The cells were then fixed with 70% ethanol, followed by incubation at 4 °C overnight. The cells were washed twice with PBS to remove the ethanol, and then centrifuged twice at 1500 rpm for 5 min. RNase A stock solution (final concentration 0.5 μg/ml) was added to the cells, and the cells were then incubated at 37 °C for 20 min. The cells were stained with propidium iodide (PI, 50 μg/ml; Sigma-Aldrich) at 4 °C for 15 min in the dark and analyzed by fluorescence-activated cell sorting (FACS) with an Epics XL instrument (Beckman Coulter, Brea, CA, USA) using Cell Quest pro software v3.1. (BD Bioscience, Franklin Lakes, NJ, USA) (16). Data are shown as mean ± SD of three independent experiments.

2.8. Immunohistochemical staining

The tissue sections (6-μm thick) were deparaffinized using xylene and dehydrated using Target Retrieval Solution with a pH of 9 (Agilent, Santa Clara, CA, USA). The activity of endogenous peroxidase was blocked using Dako REAL™ Peroxidase-Blocking Solution and incubated at 4 °C overnight with rabbit monoclonal anti-SMYD2 antibodies (#9734; Cell Signaling, 1:200 for IHC). The slides were stained using Dako REAL™ EnVision™ horseradish peroxidase-conjugated rabbit/mouse secondary antibodies for 8 min. The stained tissue sections were visualized using Dako REAL™ DAB + CHROMOGEN. The nuclei were stained blue by

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hematoxylin counterstaining. The IHC score was calculated by the sum of proportion score (PS) of positive cells (score 0: 0%, score 1: <1%, score 2: 1%–10%, score 3: 10%–33%, score 4: 33%–67%, score 5: >67%) and intensity score (IS) of staining of positive cells (score 0: background, score 1: weak staining, score 2: moderate staining, score 3: strong staining). The IHC score was graded as follows: 0–4: negative, and 5–8: positive according to a previous report [17,18].

2.9. Protein extraction and western blotting

Protein was extracted with RIPA lysis buffer (Wako, Osaka, Japan, 188–02453) supplemented with protease inhibitor cocktail (Roche, 11836153001). The samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (BIO-RAD Mini-PROTEAN® TGX™ Gels) and transferred to nitrocellulose membranes. Protein bands were detected using Amersham ECL Select (GE Healthcare Life Sciences, Piscataway, NJ, USA) and ImageQuant LAS 4000 mini (GE Healthcare Life Sciences, USA).

2.10. Colony-formation assay

The cells (1.2×10^3 cells/well) were seeded in 6-well plates. After 24 h of incubation, the cells were treated with LLY-507 (0.5 and 1.0 μM), olaparib (0.1 or 1.0 μM), or a combination of both drugs. The colonies were visualized by fixing with 100% ethanol for 2 h. After staining with 0.5% Giemsa (Wako) for 60 min, the number of colonies (consisting of over 50 cells) was counted and normalized by the number of 0.08% DMSO-treated colonies [8].

2.11. Statistical analyses

Statistical significance was determined by Student's *t*-test and Pearson's chi-square test using Excel and JMP Pro. v.12 (SAS, Cary, NC, USA). $^{**}P < 0.05$ and $^{*}P < 0.01$ indicate different degrees of statistical significance as noted in the figures.

3. Results

3.1. SMYD2 is highly expressed in HGSOC tissues

To analyze the involvement of HMTs in the development of HGSOC, the expression level of several HMT genes in HGSOC tissues was examined by qRT-PCR (data not shown). SMYD2 was significantly overexpressed in the 35 HGSOC specimens compared with its expression in the six normal ovaries (Fig. 1A and B). Next, we performed immunohistochemistry to analyze the protein levels of SMYD2. The IHC score was calculated by the sum of proportion score (PS) and intensity score (IS) of SMYD2 positive cells. SMYD2 expression was positive in 0 of the 6 (0%) normal ovary tissues and in 26 of the 30 (86.7%) HGSOC tissues ($p < 0.0001$). (Fig. 1C, Tables 1A and 1B). The IHC data indicated that the expression of SMYD2 was significantly upregulated in HGSOC. Our data suggested that SMYD2 expression is upregulated in HGSOC cells.

3.2. SMYD2 regulates the growth of HGSOC cells

To examine whether SMYD2 overexpression has an important role in HGSOC cell proliferation, we performed knockdown experiments using two independent siRNAs targeting SMYD2 and siNC in HGSOC cell lines. We confirmed that the expression of SMYD2 was suppressed after treatment with siRNA targeting SMYD2 (Fig. 2A and B).

Next, we performed cell-proliferation assay. Our data showed that knockdown of SMYD2 with siRNA also suppressed the growth of HGSOC cells compared to that of the negative control (Fig. 2C). To elucidate the mechanism of the anti-tumor effect induced by SMYD2 knockdown, we examined the FACS analysis which confirmed that the proportion of HGSOC cells in the sub G1 phase — apoptotic cells — was significantly increased (Fig. 2D). Moreover, knockdown of SMYD2 induced the cleavage of PARP, a hallmark of apoptosis (Fig. 2E). These data suggest that the knockdown of SMYD2 induces apoptosis.

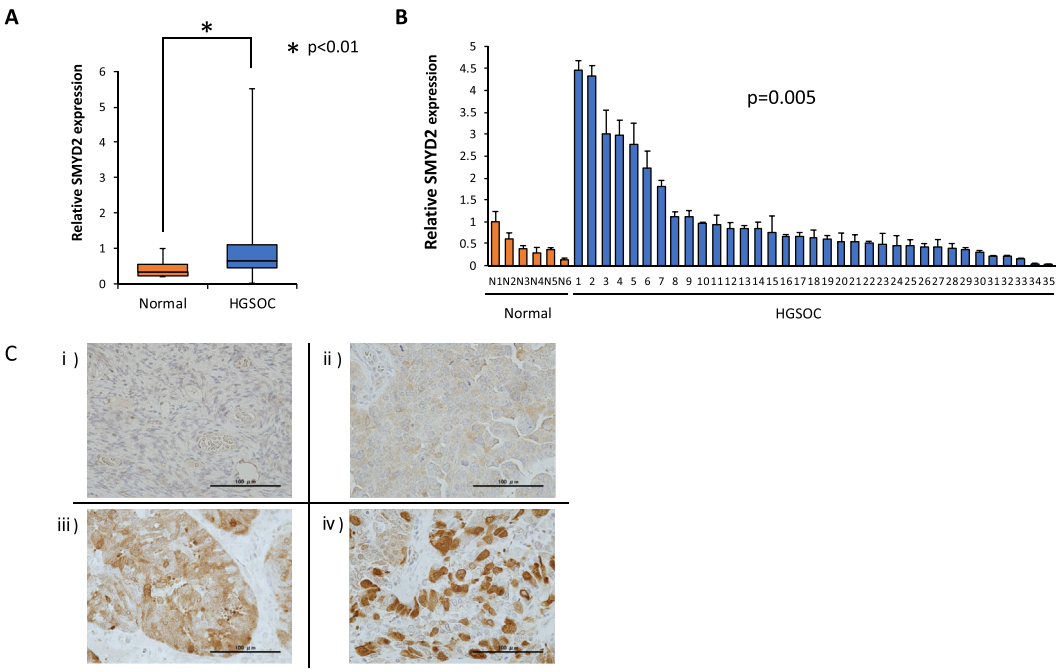

Fig. 1. SMYD2 was overexpressed in HGSOC tissues. (A, B) Quantitative real time PCR of SMYD2 in HGSOC and normal ovarian tissues. SMYD2 mRNA was significantly overexpressed in HGSOC tissues (n = 35) compared with that in normal ovarian tissues (n = 6). (C) Immunohistochemical staining of SMYD2 in HGSOC (n = 30) and normal ovary (n = 6) tissues. Intensity score, IS, i) Normal ovary, score 0: background. ii) HGSOC, score 1: weak staining, iii) HGSOC, score 2: moderate staining, iv) score 3: strong staining.

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Table 1A
IHC scores of SMYD2 positive cells in HGSOC tissues.

IHC score	PS	IS	n (%)
3	2	1	1 (3.3)
4	3	1	3 (10.0)
	2	2	1 (3.3)
5	4	1	1 (3.3)
	3	2	3 (10.0)
	4	1	2 (6.7)
6	5	1	3 (10.0)
	4	2	5 (16.7)
	3	3	1 (3.3)
7	4	3	4 (13.3)
	5	2	3 (10.0)
8	5	3	3 (10.0)

IHC score: PS + IS.
PS: proportion score IS: intensity score.

Table 1B
SMYD2 expression in normal ovarian tissues and HGSOC tissues.

Tissue sample	SMYD2 expression (%)		p-value
	Negative (n = 11)	Positive (n = 25)	
Normal ovarian tissues (n = 6)	6 (100.0)	0	<0.0001
HGSOC tissues (n = 30)	5 (16.7)	25 (83.3)	

IHC score: (0–4) Negative, (5–8) Positive.
HGSOC: high-grade serous ovarian carcinoma.
SMYD2: SET and MYND domain containing 2.

3.3. SMYD2-selective inhibitor suppresses cell growth and induces apoptosis in HGSOC cells

We examined the anti-tumor effect of an SMYD2 selective inhibitor. After treatment with the SMYD2 inhibitor, LLY-507, we performed the MTT assay. The blockage of SMYD2 by LLY-507 suppressed the proliferation of HGSOC cells in a dose-dependent manner. The IC_{50} of LLY-507 in HGSOC cell lines ranged from 1.77 to 2.90 μM (Fig. 3A). In addition, we confirmed the induction of apoptosis by SMYD2 inhibitor. We also found that SMYD2 inhibitor induced the cleavage of PARP, similar to that observed after SMYD2 knockdown (Fig. 3B). The cell cycle analysis suggested that SMYD2 inhibitor increased the population of sub G1 cells (Fig. 3C). To determine the long-term effect of SMYD2, the colony-formation assay was performed. We found that LLY-507 also decreased the number of colonies of HGSOC cell lines in a dose-dependent manner (Fig. 4A). Moreover, we evaluated the combination effect of the SMYD2 inhibitor and the PARP inhibitor olaparib, which is a molecular target drug for ovarian cancer. We found that the combination treatment of LLY-507 and the PARP inhibitor additively suppressed the growth of HGSOC cells (Fig. 4B).

4. Discussion

In the present study, we found that SMYD2 was overexpressed in HGSOC tissue samples compared to its levels in the normal ovary tissue samples. Our *in vitro* study results further suggested that the higher expression of SMYD2 enhanced the proliferation of HGSOC cells. In addition, suppression of SMYD2 increased the proportion of apoptotic cells. Overall, selective inhibition of SMYD2 demonstrated that SMYD2 is a potent therapeutic target for HGSOC treatment.

Previously, we showed that some HMTs play vital roles in carcinogenesis in humans [7,8]. Other studies have also suggested the involvement of HMTs in malignant alterations of cells in humans [5,6]. Studies have shown that SMYD2 expression is upregulated in several carcinomas, such as gastric, breast, and bladder cancers [11,19,20].

The qRT-PCR and IHC data of the present study indicated that SMYD2 was overexpressed in HGSOC clinical samples. This is the first report demonstrating the elevated expression of SMYD2 in ovarian cancer.

Some studies have suggested that knockdown of SMYD2 suppresses the proliferation of various types of cancer cells [11]. Consistent with the previous results, we found that knockdown of SMYD2 suppressed cell proliferation in HGSOC cells. We also found that knockdown of SMYD2 induced apoptotic cells that increased in the sub G1 phase in the cell cycle analysis. In addition, knockdown

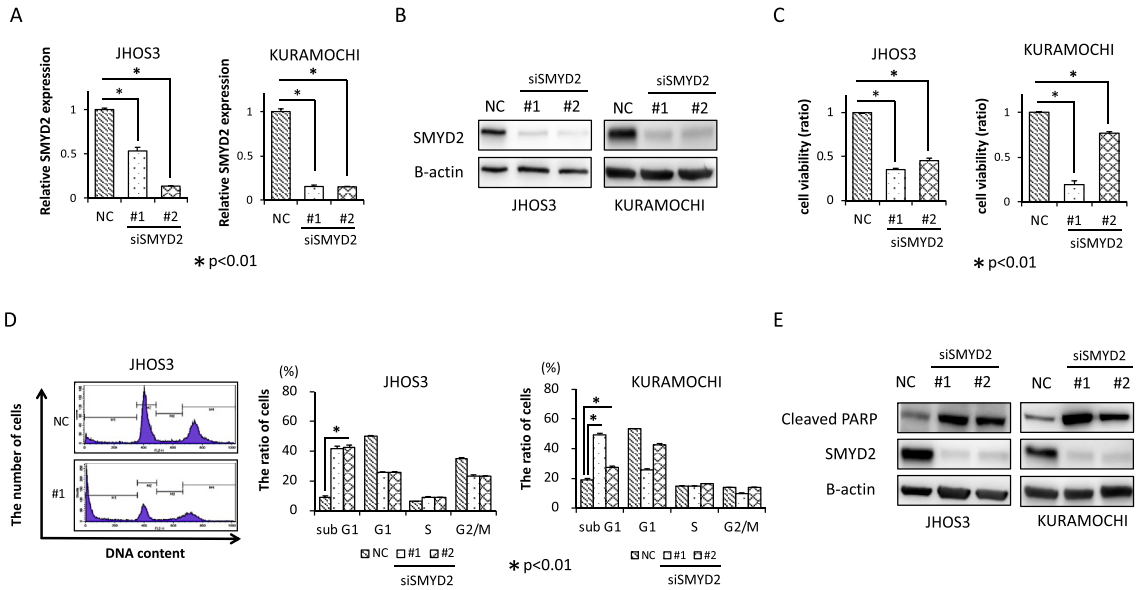

Fig. 2. Knockdown of SMYD2 suppressed proliferation of HGSOC cells by **increasing apoptotic cells.** (A, B) Knockdown of SMYD2 by siRNAs for 72 h suppressed the expression of SMYD2 in JHOS3 and KURAMOCHI cells (*P < 0.01). C) Cell viability assay after treatment with SMYD2 siRNAs for 96 h. Knockdown of SMYD2 suppressed the proliferation of JHOS3 and KURAMOCHI cells (*P < 0.01). D) Knockdown of SMYD2 by siRNAs for 72 h increased the population of sub-G1 phase JHOS3 and KURAMOCHI cells (*P < 0.01). We performed flow cytometry and PI staining to analyze the cell cycle phase. E) Knockdown of SMYD2 by siRNAs for 72 h increased the level of cleaved PARP in JHOS3 and KURAMOCHI cells.

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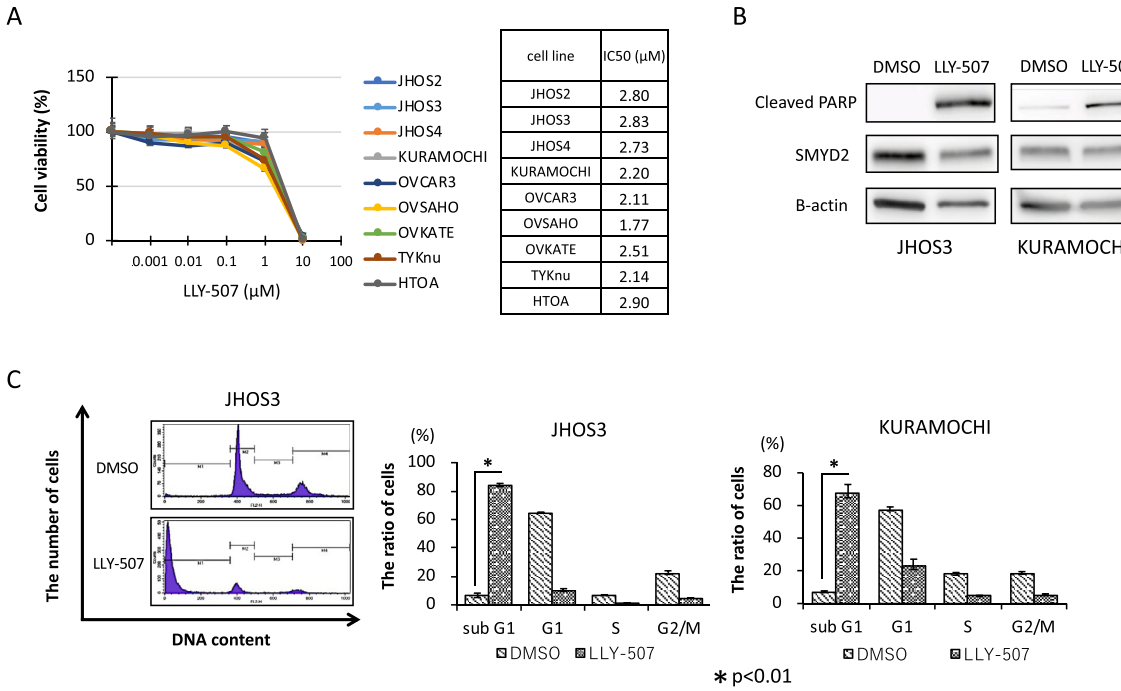

cell line	IC50 (μM)
JHOS2	2.80
JHOS3	2.83
JHOS4	2.73
KURAMOCHI	2.20
OVCAR3	2.11
OVSAHO	1.77
OVKATE	2.51
TYKnu	2.14
HTOA	2.90

Fig. 3. LLY-507 suppressed cell growth and caused apoptosis in HGSOC cells. (A) Various concentrations (0.001–10 μM) of LLY-507 were added to HGSOC cells for 11 days. The IC_{50} values of LLY-507 in HGSOC cell lines ranged from 1.77 μM to 2.90 μM and were normalized to those in 0.02% dimethyl sulfoxide (DMSO)-treated cells. (B) Inhibition of SMYD2 increased the level of cleaved PARP in JHOS3 and KURAMOCHI cells treated with 8 μM LLY-507 or 0.08% DMSO for 72 h. (C) LLY-507 increased the population of sub-G1 phase JHOS3 and KURAMOCHI cells treated with 8 μM LLY-507 or 0.08% DMSO for 72 h (*P < 0.01). We performed flow cytometry and PI staining to analyze the cell cycle phase.

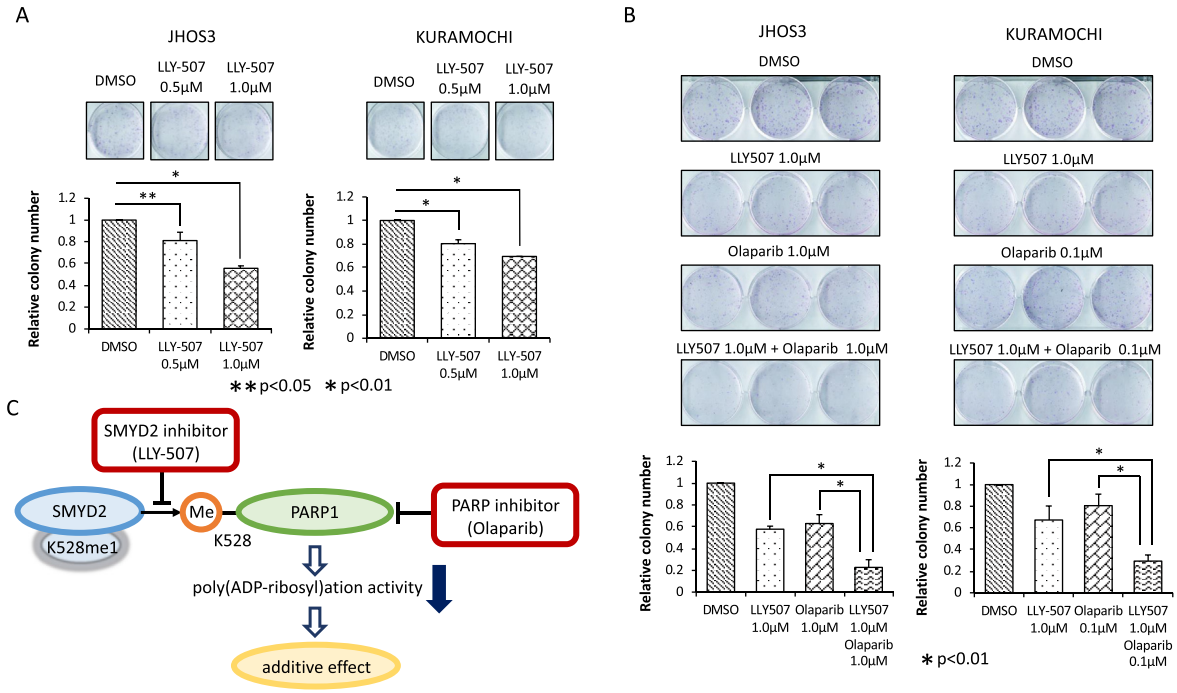

Fig. 4. LLY-507 has an additive effect with PARP inhibitor in HGSOC cells. (A) Colony-formation assay in JHOS3 and KURAMOCHI cells treated with LLY-507 (0.5 μM and 1.0 μM) for 11 days. Inhibition of SMYD2 significantly suppressed the growth of colonies in a dose dependent manner (*P < 0.01, **P < 0.05). (B) Colony-formation assay in JHOS3 and KURAMOCHI cells treated with LLY-507 (1 μM), PARP inhibitor olaparib (0.1 or 1.0 μM), or combination of both drugs for 11 days. LLY-507 in combination with olaparib showed additive effect in JHOS3 and KURAMOCHI cells. *P < 0.01. (C) Schematic presentation of the effect of LLY-507 in combination with olaparib. SMYD2 monomethylates K528 of poly(ADP-ribose) polymerase 1 (PARP1) and enhances its activity. Inhibition of SMYD2 results in the reduction of PARP1 enzymatic activity. Me: Methylation.

of SMYD2 increased the cleavage of PARP, which is a marker of apoptosis. Studies have reported that the mechanism of anti-tumor effect involves SMYD2 suppression, leading to the induction of cell cycle arrest in the G1/G0 phase in various types of cancers [19,20]. It has also been reported that knockdown of SMYD2 with siRNA enhances p53-mediated apoptosis via reducing p53 methylation

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[10,20]. However, there might be other mechanisms of apoptosis induction in HGSOC because HGSOC is characterized by *TP53* mutation, which involves loss of function [2]. Further, SMYD2 methylates several non-histone proteins, such as PTEN. *PTEN* is known to be a tumor suppressor gene and is known to induce apoptosis [21]. Therefore, another possible mechanism of apoptosis is that the knockdown of SMYD2 decreases PTEN methylation and increases PTEN activity [22].

In the present study, LLY-507 suppressed the proliferation of HGSOC cells via induction of apoptosis. Moreover, the colony-formation assay showed the long-term effects of SMYD2 inhibitors on HGSOC. It has been reported that LLY-507 induces an anti-tumor effect in several types of cancers *in vitro*, including esophageal, liver, and breast cancer cell lines, in a dose-dependent manner [23]. The results of the present study suggest that LLY-507 has a therapeutic potential for the treatment of HGSOC. This is the first study to report the anti-tumor effect of LLY-507 against HGSOC cells.

PARP inhibitors have attracted considerable attention as one of the major molecular-targeted therapeutics for inhibiting DNA damage response. Currently, there are several completed phase III trials of olaparib, such as the SOLO1 and SOLO2 studies. The SOLO1 study was a randomized, double-blind, phase III trial to examine the efficacy of olaparib as a maintenance therapy for advanced ovarian cancer, primary peritoneal cancer, or fallopian-tube cancer patients with BRCA1, BRCA2, or BRCA1/2 mutations. The results showed that the risk of disease progression or death was 70% lower with olaparib than with placebo [24]. The SOLO2 trial was performed to examine the anti-tumor effect of olaparib as a maintenance therapy for patients with platinum-sensitive, recurrence, and germline BRCA-mutated ovarian cancer. The results revealed that olaparib significantly improved progression-free survival compared with placebo in patients with a germline BRCA1 or BRCA2 (BRCA1/2) mutation and platinum-sensitive, relapsed ovarian cancer [25].

Olaparib has been clinically applied for treating patients with certain recurrent ovarian cancers associated with *BRCA1/2* mutations in the United States. In Japan, it has been used to treat patients with platinum-sensitive relapsed ovarian cancer [25]. Our results suggest that combination therapy with small-molecule SMYD2 inhibitors, such as LLY-507 and olaparib can be a plausible strategy for effective treatment of HGSOC. It has been reported that SMYD2 methylates PARP1 and promotes its activity in cancer cells [12]. The combination effect of SMYD2 inhibitor and PARP inhibitor may be due to the decrease in the activity of methylated PARP and inhibition of PARP activity (Fig. 4C).

There are some limitations in this study. First, biomarkers for SMYD2 inhibitors need to be identified. Second, *ex vivo* experiments might be needed to further examine the anti-tumor effect of LLY-507 in HGSOC. Third, we need to clarify the involvement of histone and non-histone methylation by SMYD2 in HGSOC.

Based on these results, we conclude that the role of SMYD2 overexpression in HGSOC is similar to its role in other cancers, suggesting that it could be a potential therapeutic target for various types of cancers. Monotherapy with a selective SMYD2 inhibitor, such as LLY-507, or combination molecular targeted therapy with selective SMYD2 and PARP inhibitors might be a promising strategy to effectively treat high-risk HGSOC patients.

Conflicts of interest

K. Sone has a research grant from Daiichi-Sankyo Co., Ltd. K. Oda has a research grant from Daiichi-Sankyo Co., Ltd. and Astrazeneca plc and lecture fee from Chugai Pharmaceutical Co., Ltd. and Astrazeneca plc. The other authors have no competing interests to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.03.155.

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