ANTICANCER RESEARCH *42*: 555-563 (2022)
doi:10.21873/anticanres.15513

Olaparib-induced Apoptosis Through EBNA1-ATR-p38 MAPK Signaling Pathway in Epstein-Barr Virus-positive Gastric Cancer Cells

SUNG HO MOON[1*], NAM-SOOK PARK[2*], MIN HYE NOH[2], YEONG SEOK KIM[2], SOON HO CHEONG[1] and DAE YOUNG HUR[2]

[1]*Department of Anesthesiology and Pain Medicine, Inje University Busan Paik Hospital, Busan, Republic of Korea;*
[2]*Department of Anatomy and Tumor Immunology, Inje University College of Medicine, Busan, Republic of Korea*

Abstract. *Background: Epstein-Barr virus (EBV)-associated gastric cancer has been identified as a cancer subtype with definitive clinical and molecular characteristics. Although olaparib, a poly ADP ribose polymerase (PARP) inhibitor, is considered a potential effective agent for gastric cancer, the effect and underlying mechanism of olaparib on gastric cancer depending on EBV infection is not fully understood. Materials and Methods: EBV-positive SNU719 and EBV-negative SNU638 gastric cancer cell lines were used to identify the effects of olaparib using the trypan blue exclusion method and annexin V staining assay. To observe the underlying cellular signaling mechanisms of olaparib-induced cell death, Epstein–Barr virus nuclear antigen 1 (EBNA1) and signaling related molecule expression were assessed using transfection, silencing of specific genes using small interfering RNA (siRNA), western blotting and signaling inhibition assay. Results: Olaparib decreased the cell viability of EBV-positive SNU719 gastric cancer cells through caspase-3-dependent apoptosis in a dose dependent manner, whereas EBV-negative SNU638 gastric cancer cells showed drug resistance to olaparib. EBNA1 was expressed in SUN719 gastric cancer cells; however, ataxia telangiectasia and Rad3 related (ATR) and phosphorylated ATR kinase were expressed in SNU638 gastric cancer cells. EBNA1 transfection decreased ATR phosphorylation through p38 mitogen-activated protein kinase (MAPK) phosphorylation in SUN638 gastric cancer cells, and silencing of ATR kinase increased the susceptibility of these cells to olaparib treatment. Moreover, VE-821, an ATR kinase specific inhibitor, also increased the sensitivity of SNU638 cells to olaparib. In contrast, SB203580, a p38 MAPK inhibitor, inhibited this increase in sensitivity to olaparib by EBNA1 transfection. Conclusion: Olaparib treatment led to different cellular responses depending on EBV infection in gastric cancer cell lines. These results provide new insights into the mechanism of olaparib-induced apoptosis in gastric cancer cells and suggest that EBV infection should be considered when developing new potential therapeutic agents for gastric cancer.*

Although gastric cancer incidence has a gradually declining tendency globally, it is still the major cause of cancer-associated deaths and is especially one of the most frequently diagnosed cancers in Eastern Asia (1-3). Treatments for gastric cancer include surgical treatment, chemotherapy, radiotherapy, targeted molecular therapy and gene therapy; all these have increased survival rates of patients with gastric cancer (4-6). Recently targeted molecular therapy and radiation therapy have attracted attention (7, 8). However, the development of a new chemotherapy for gastric cancer treatment is still necessary on account of a low response rate to targeted therapies, drug resistance and recurrence.

Epstein-Barr virus (EBV)-related gastric carcinoma accounts for almost 8-10% of all gastric cancers (9). EBV-associated gastric cancer has been identified as a subtype with definitive clinical and molecular characteristics by recent molecular subtyping (10, 11). The most distinct feature of EBV-associated cancers is EBV-latent gene expression. The carcinogenic impact of EBV-latent genes on other EBV-related cancers including Burkitt's lymphoma, natural killer/T-cell lymphoma and Hodgkin lymphoma have been widely studied and published (12-14). EBV nuclear antigen-1 (EBNA1) is among these genes and is critical for carcinogenesis in EBV-positive gastric carcinomas (15).

*These Authors contributed equally to this study.

Correspondence to: Prof. Dae Young Hur, Department of Anatomy and Tumor Immunology, Inje University College of Medicine, 75 Bokji-ro, Busanjin-gu, Busan 47392, Republic of Korea. Tel: +82 518906796, Fax: +82 518966634, e-mail: dyhur@inje.ac.kr

Key Words: Olaparib, apoptosis, Epstein-Barr virus nuclear antigen-1, EBNA-1, ataxia telangiectasia and Rad3 related kinase, ATR, gastric cancer cell.

Furthermore, EBV-positive gastric carcinoma shows discrete features not only in pathologic and molecular characteristics, but also in clinicopathologic signatures such as frequency of lymph node involvement (16, 17). EBNA1 is the most significantly expressed gene for latent pattern in EBV-positive gastric carcinoma (18). EBNA1 promotes cell survival in Burkitt's lymphoma and genomic instability and metastasis in nasopharyngeal carcinoma through reactive oxygen signaling and mitogen-activated protein kinase activation (19, 20). EBNA1 contributes to the carcinogenesis of EBV-positive gastric carcinoma via the disruption of promyelocytic leukemia nuclear body associated with tumor suppression (21). However, the definite role of EBNA1, which has been comprehensively researched in EBV-positive gastric carcinoma, still remains unclear. Therefore, investigating the definite pathogenesis of EBNA1-induced carcinogenesis in EBV-positive gastric carcinomas could contribute to the improvement of treatment strategies for EBV-associated gastric carcinomas.

Olaparib is a new drug approved as a poly ADP ribose polymerase (PARP) inhibitor, exhibiting anti-cancer effects in triple-negative breast and ovarian cancers (22-24). The main mechanism of action of olaparib is the inhibition of DNA repair (PARP is an enzyme engaged in genomic stability) (25, 26). The PARP inhibitor olaparib has shown better effects in breast cancer susceptibility gene (BRCA)-mutant cancers (27). Comprehensive studies have shown that most cancers display genomic instability associated with accumulating DNA damage (28, 29). Furthermore, the other genes that are necessary to repair irradiation-related DNA damage, such as ATR and Rad 51, also show mutations or copy number alterations (30). Classical treatments of cancer by chemotherapy and radiation therapy also use the DNA damage mechanism (31). These previous studies imply that olaparib might be a possible effective agent for various cancers including gastric cancer. However, we have little knowledge of its effects and mechanisms in gastric carcinoma.

In the present study, we investigated whether olaparib treatment produces different cellular responses in two different gastric cancer cell lines depending on EBV infection. The underlying cell signaling mechanism mediated by EBV-related viral protein expression, specific receptor kinase expression and pro-apoptotic gene activation was assessed in two EBV-positive/-negative gastric cancer cells.

Materials and Methods

Cell culture and reagents. Human gastric cancer cell lines SNU-638 and SNU-719 were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). Cells were maintained in RPMI 1640 culture medium (Mediatech Inc., Mansssas, VA, USA) supplemented with 10% fetal bovine serum (Tissue Culture Biologicals, Tulare, CA, USA), 100 μg/ml streptomycin (HyClone, Logan, UT, USA) and 100 units/ml penicillin (HyClone) at 37°C in a 5% CO_2 incubator. Olaparib (Selleckchem, Houston, TX, USA), VE-821 (Selleckchem), a specific inhibitor of ATR, z-DEVD-FMK (Sigma-Aldrich, St. Louis, MO, USA), a specific inhibitor of caspase-3, and SB203580 (Sigma-Aldrich), a specific inhibitor of p38 MAPK, were purchased for this study. This study was permitted by the Institutional Bioethics Review Board at the Inje University Busan Paik Hospitals (Busan, Republic of Korea).

Cell viability assay and Annexin V staining analysis. Cells were treated with olaparib at different concentrations (0, 1, 2.5, 5 μM) for 24 h and cell viability was measured using a trypan blue exclusion method (Sigma-Aldrich). For the Annexin V staining assay (BD Biosciences, San Jose, CA, USA), cells were treated with olaparib for 24 h and then cells were trypsinized, washed with PBS, treated with annexin V-FITC binding buffer (BD Biosciences) for 15 min and measured by FACSCalibur flow cytometry (BD Bioscience).

Plasmid, DNA and transient transfection. SNU638 cells were seeded at $5x10^5$ in 60-mm dishes and transiently transfected with MSCV or MSCV-EBNA1 (Plasmid #37954, Addgene, Watertown, MA, USA) using lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) at different doses for 48 h according to manufacturer's instructions.

Small interference RNA (siRNA) transfection. Cells were incubated with scramble siRNA (5'-GCC UGU UUCb GGG UAG AGG AUU GAA U-3') and ATR siRNA (5'-CCU CCG UGA UGU UGC UUG AdTdT-3'). In our study, transient siRNA transfections were carried with lipofectamine 2000 transfection reagent (Invitrogen) for 24 h according to the recommended guidelines. All siRNAs were obtained from Genolution Pharmaceuticals Inc. (Seoul, Republic of Korea).

Western blotting. Drug-treated cell lysis was performed with cell lysis buffer (pH7.4, 50 mM HEPES, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 2.5 mM EGTA, 0.1% Triton X-100) supplemented with a phosphatase and protease inhibitor cocktail (Sigma). Total cell lysates (20 μg per well) were taken to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transmitted to an Immobilon-PVDF membrane (Millipore Corporation, Billerica, MA, USA). Membranes were incubated with 5 % non-fat skim milk to block in tris-buffered saline (TBS)-T buffer (pH 7.4, 0.1% Tween 20, 150 mM NaCl, 20 mM Tris-HCl). The primary antibody reaction was performed with the following antibodies: anti-EBNA1 (#sc-81581, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-β-actin (#sc-47778, 1:1,000, Santa Cruz Biotechnology), anti-AT and Rad3-related kinases (ATR, #2790S, 1:1,000, Cell Signaling Technology, Beverly, MA, USA), anti-phospho ATR (#2909S, 1:1,000, Cell Signaling Technology), anti-phospho p38 mitogen activated protein kinases (MAPK, #9211S, 1:1000, Cell Signaling Technology), anti-p38 MAPK (#9212S, 1:1,000, Cell Signaling Technology) and anti-cleaved caspase-3 antibodies (#9661S, 1:1,000, Cell Signaling Technology). The primary antibodies reacted with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies (1:1,000, Cell signaling Technology) and visualized using chemiluminescence (Amersham, Buckinghamshire, UK).

Statistical analysis. All experimental data are showed as mean±SD, and each value represents at least two different experiments. Statistical analysis was performed using SAS software program

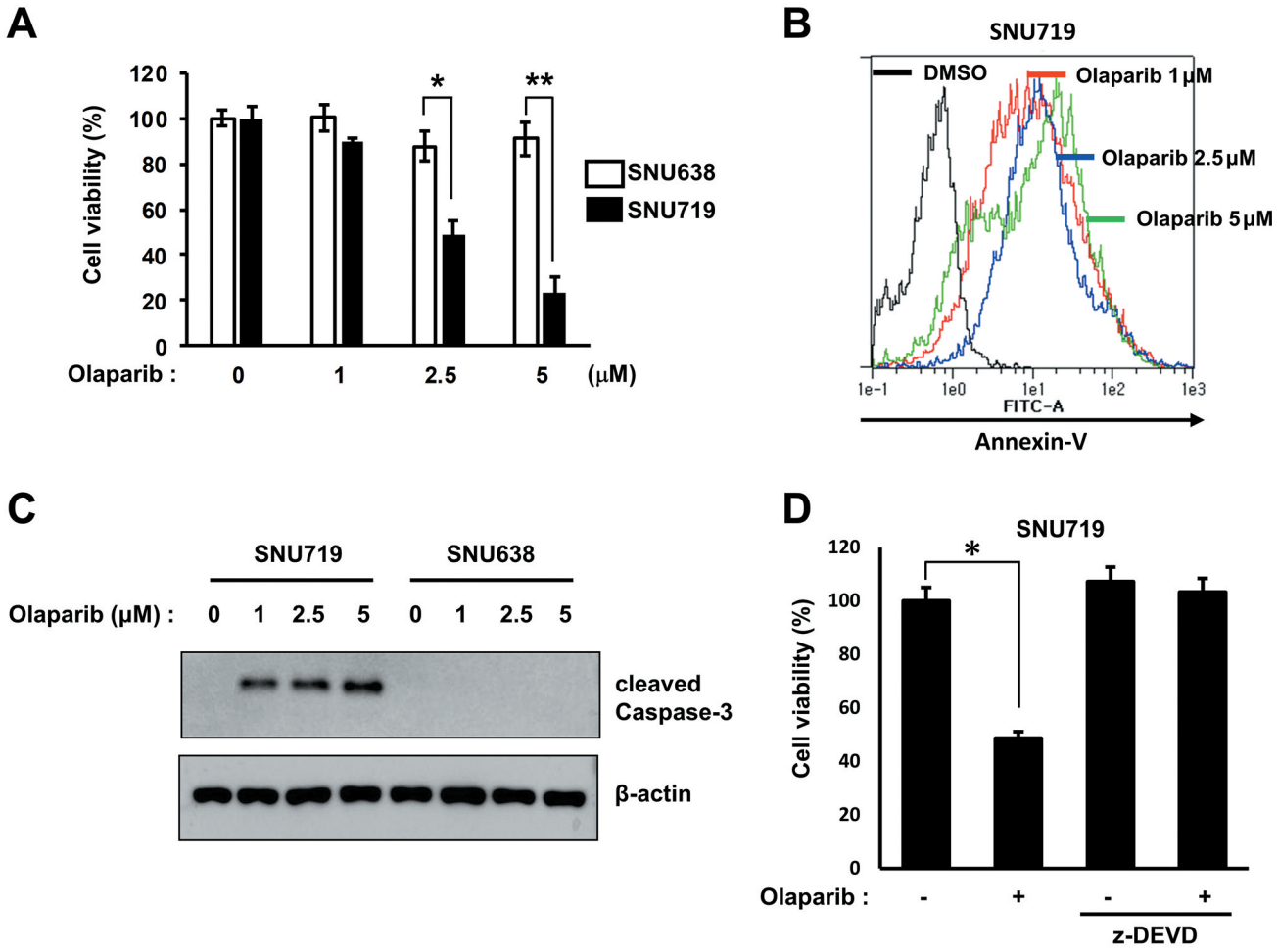

Figure 1. *Olaparib induced apoptotic cell death only in EBV-positive gastric cancer cells via caspase 3 activation. (A) Cell viability was measured with trypan blue exclusion assay after olaparib treatment for 48 h (1, 2.5, and 5 μM concentrations). Values are presented as S.E. of three independent experiments performed in duplicate. (B) Apoptotic cells were identified with annexin V staining and measured by flow cytometry. Cells were harvested and stained with FITC-conjugated annexin V antibodies. Left shifting histograms indicate an increase in apoptotic cell number. Colored histograms indicate respective concentrations as stated in the legend. These histograms represent three independent experiments. (C) Cells were incubated with olaparib under the same conditions as described in the previous experiment. Total cell lysates were collected and immunoblotting was performed to observe caspase 3 cleavage. (D) Cells were pre-treated with z-DEVD (a specific caspase 3 inhibitor, 20 μM, 1 h), and washed and incubated with or without olaparib. Cell viability was then assessed with the trypan blue exclusion method. Values are reported as S.E. of four independent experiments performed in duplicate. Results represent three independent experiments. β-actin is used as a control. z-DEVD; z-DEVD-FMK. Cell viability values were analyzed using one-way ANOVA with Tukey correction. *$p<0.05$ and **$p<0.01$.*

(9.4, Cary, NC, USA). Comparisons between individual data points were assayed by one-way analysis of variance (ANOVA) with Tukey correction. A *p*-Value <0.05 was regarded as statistically significant.

Results

Olaparib induced apoptotic cell death only in EBV-positive gastric cancer cells through the caspase 3 signaling pathway. To observe the effects of olaparib in gastric cancer cells, two different gastric cancer cell lines (SNU638, an EBV-negative gastric cancer cell line, and SNU719, an EBV-positive gastric cancer cell line) were tested with cell viability assay after olaparib treatment (1, 2.5, and 5 μM concentrations). Olaparib decreased the cell viability of only EBV-positive SNU719 gastric cancer cells in a dose dependent manner (Figure 1A). More specifically, olaparib significantly decreased the cell viability of SNU719 gastric cancer cells in both 2.5 and 5 μM concentrations (*$p<0.05$ and **$p<0.01$, Figure 1A). Next, we investigated whether the SNU719 cell death was related to apoptosis using flow

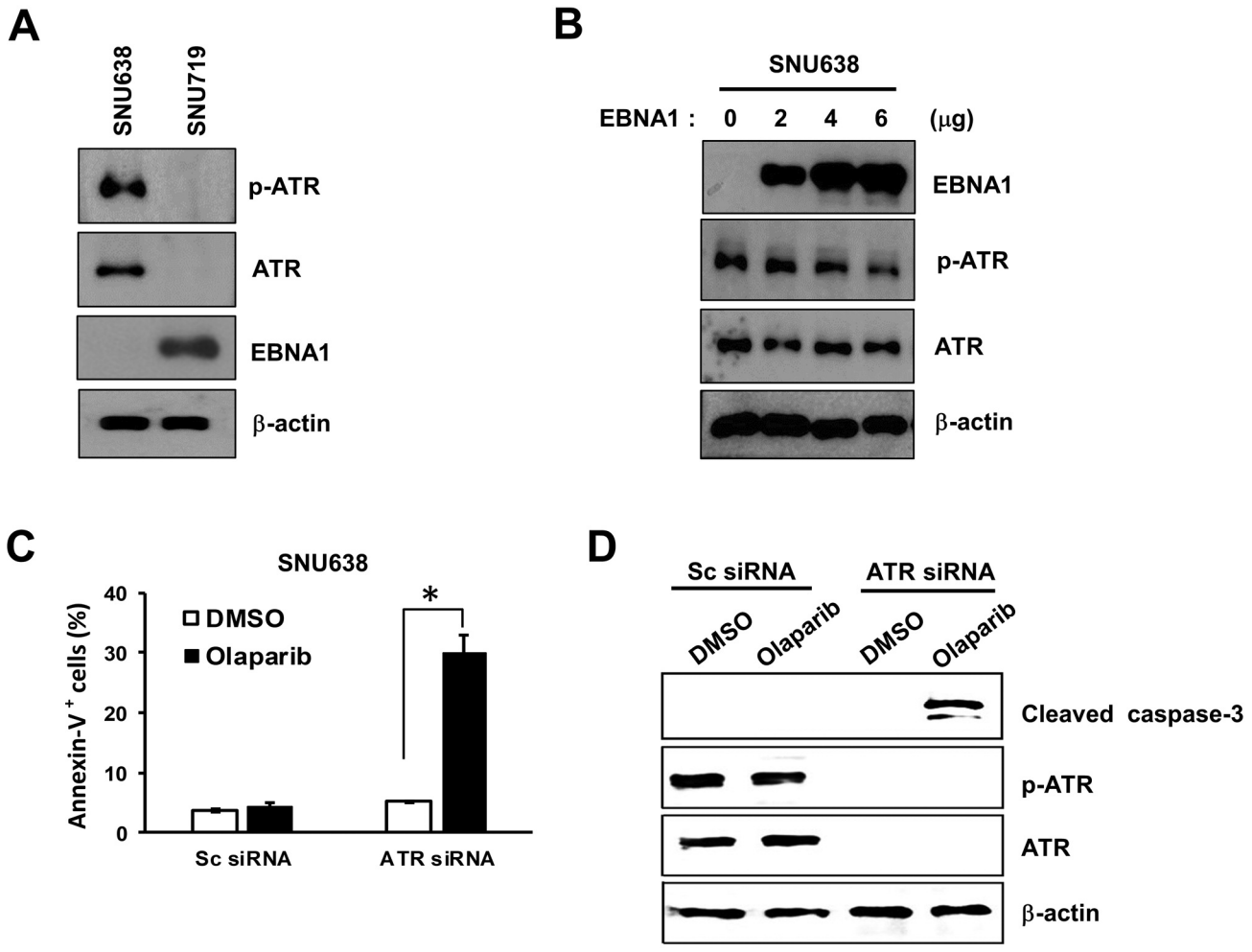

Figure 2. *EBNA1 transfection increased the susceptibility of EBV-negative gastric cancer cells to olaparib through ATP phosphorylation. (A) Total cell lysates of two gastric cancer cell lines were collected. Immunoblot was performed to observe ATR and EBNA1 expression and ATR kinase phosphorylation in two gastric cancer cell lines. (B) To observe the relationship between EBNA1 and ATR kinase, EBNA1 transfection was performed in EBV-negative SNU638 gastric cancer cells. The total cell lysates of two gastric cancer cell lines were then collected and immunoblotting was performed. The results represent three independent experiments. β-actin was used as loading control. (C) Silencing of the ATR gene using siRNA was performed for 24 h; cells were then treated with olaparib at 48 h, apoptotic cells were analyzed with annexin V staining and measured with flow cytometry. Cells were harvested and stained with FITC-conjugated annexin V antibodies. (D) Cell lysates were prepared for evaluation of ATR and phosphorylated ATR expression using Western blotting. Results represent three independent experiments. EBNA 1: EBNA 1 transfection. The cell number values were analyzed using one-way ANOVA with Tukey correction. *p<0.05.*

cytometry. Olaparib induced apoptosis (Annexin-V positive cells) of SNU719 gastric cancer cells in a dose dependent manner (Figure 1B).

To identify the signaling mechanism of olaparib-induced apoptosis in gastric cancer cells, the cleavage of caspase 3 were observed with western blotting. Olaparib induced the cleavage of caspase 3 only in SNU 719 cells in a dose dependent manner (Figure 1C). Moreover, z-DEVD, a specific inhibitor for caspase 3, restored cell viability in the same cells (Figure 1D).

EBNA1 transfection increased the susceptibility of EBV-negative SNU638 gastric cancer cells to olaparib through down-regulation of ATP phosphorylation. Ataxia telangiectasia and Rad3-related kinase (ATR) expression and phosphorylation were investigated in EBV-negative SNU638 and EBV-positive SNU719 gastric cancer cells, to identify the resistance mechanism of SNU638 gastric cancer cells to olaparib. Both ATR and phosphorylated ATR (p-ATR) were expressed only in SNU638 EBV-negative gastric cancer cells (Figure 2A). However, as expected, Epstein–Barr nuclear

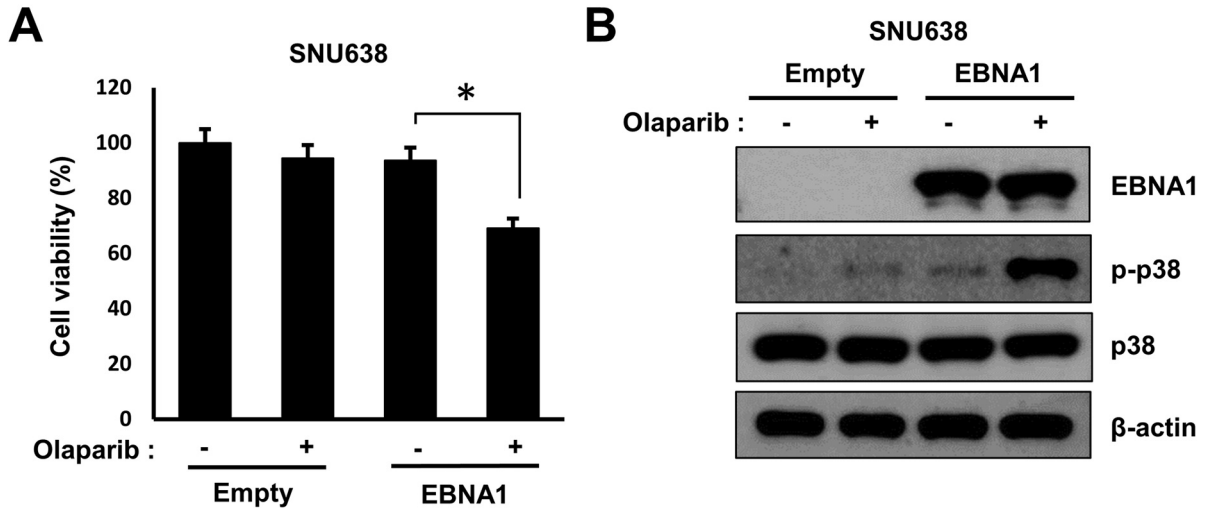

Figure 3. *EBNA1 transfection increased the susceptibility of EBV-negative gastric cancer cells to olaparib through p38 phosphorylation. (A) EBNA1 transfection was performed, and cells were then treated with olaparib (2.5 μM concentration) for 48 h. Cell viability was measured with the trypan blue exclusion assay. Values are presented as S.E. of four independent experiments performed in duplicate. (B) After EBNA1 transfection, cells were collected, and then cell lysates were prepared for evaluation of p38 and phosphorylated p38 expression using Western blotting. Results represent four independent experiments. β-actin was used as a loading control. Cell viability values were analyzed using one-way ANOVA with Tukey correction. *p<0.05.*

antigen 1 (EBNA1), dimeric viral protein associated with EBV, was expressed only in SUN719 EBV-positive gastric cancer cells (Figure 2A). To assess whether the EBNA 1 expression was related to ATR expression, ATP expression and phosphorylation were observed after EBNA1 transfection in SNU638 EBV-negative gastric cancer cells. EBNA1 transfection decreased ATP phophorylation in a dose dependent manner, but total ATP expression was not changed (Figure 2B).

To identify whether the differences in ART expression and phosphorylation are related with the susceptibility of two gastric cancer cells to oraparib, annexin-V staining assay was performed with silencing of ART using siRNA in SNU638. Silencing of ART increased significantly the annexin-V SNU638 positive cellsupon oraparib treatment (*p<0.01, Figure 2C). Moreover, silencing of ART also induced the cleavage of caspase-3 in SUN638 cells upon oraparib treatment (Figure 2D).

EBNA1 transfection increased the susceptibility of EBV-negative SNU638 gastric cancer cells to olaparib via p38 phosphorylation. In previous results, EBNA1 transfection decreased ATP phophorylation, therefore it was evaluated whether EBNA1 transfection itself increases the olaparib-induced cell death of SNU638 cells. EBNA1 transfection induced significant cell death of SUN638 gastric cancer cells with olaparib treatment (*p<0.01, Figure 3A). In addition, EBN1 transfection and olaparib induced the phosphorylation of p38 kinase, a pro-apoptotic kinase, synergistically (Figure 3B).

The VE-821 ATP inhibitor increased the sensitivity of SNU638 cells to olaparib. To verify the participation of the ATP phophorylation in olaparib-induced apoptosis of gastric cancer cells, an inhibition assay was implemented using the ATP inhibitor VE-821 along with EBNA1 transfection. VE-821 effectively inhibited ATP phosphorylation in SNU638 cells in a dose dependent manner (Figure 4A). VE-821 increased the sensitiviy of SNU638 cells to olaparib, and its application together with EBNA transfection increased the sensitivity to olaparib synergistically through inhibition of ATP phosphorylation (*p<0.05 and **p<0.01, Figure 4B and C).

The SB203580 p38 kinase inhibitor inhibited the increase of sensitivity to olaparib by EBNA1 transfection. In the previous results, EBN1 transfection and olaparib induced the phosphorylation of p38 kinase synergistically. Therefore, the effect of the p38 kinase inhibitor, SB203580, was observed in the same experimental condition. SB203580 inhibited the increased sensitivity of olaparib induced by EBNA1 transfection and also inhibited ATR phosphorylation (**p<0.01, Figure 5A and B).

Discussion

EBV-related gastric carcinoma is a discrete subtype of gastric cancer, constituting 8-10% of total gastric cancers (32, 33). EBV-related gastric carcinoma is the malignant proliferation of EBV-infected gastric epithelial cells and all EBV-infected cells exhibit more than one latent protein such

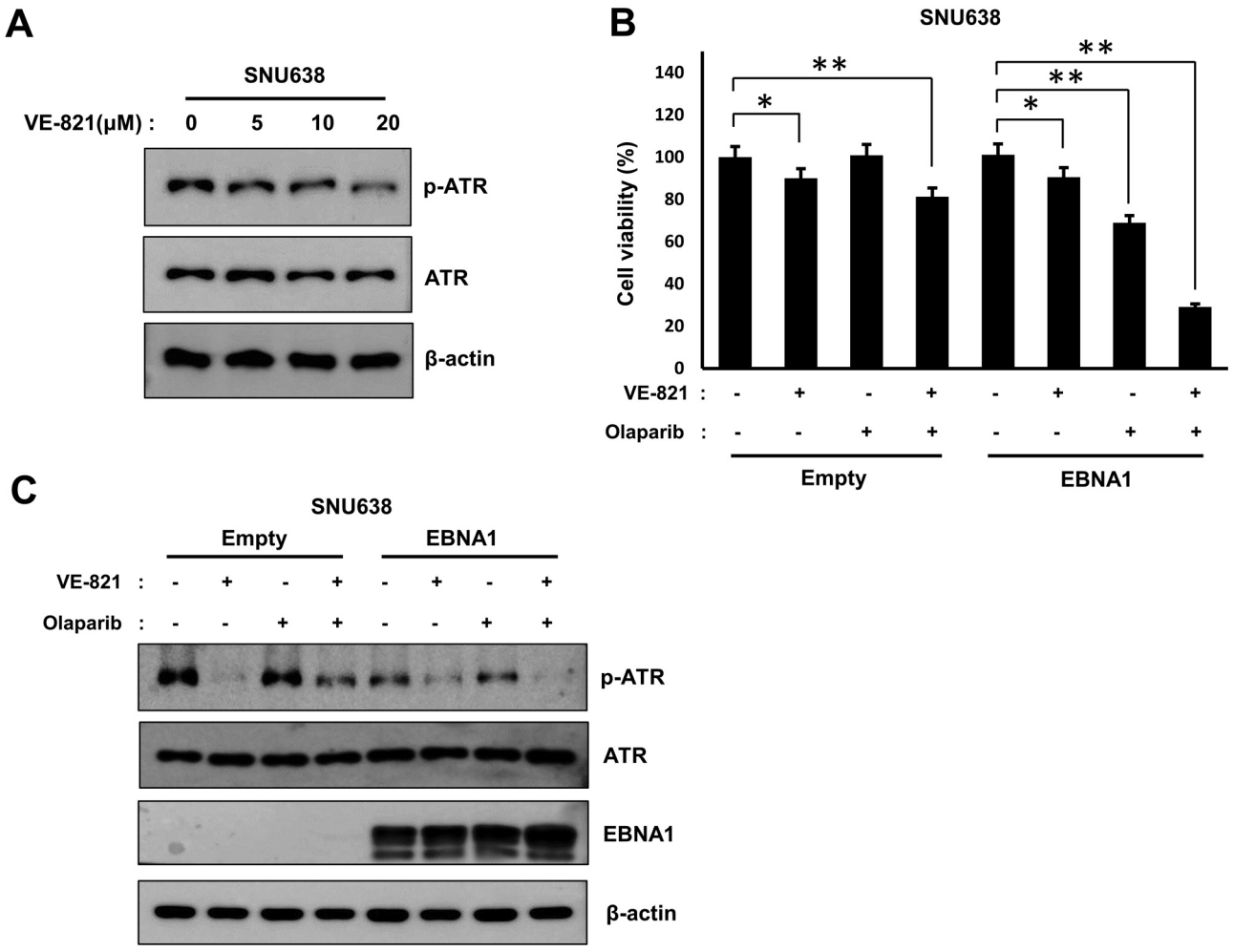

Figure 4. *VE-821 and EBNA1 transfection decreased the olaparib resistance of EBV-negative SNU638 cells through inhibition of ATR phosphorylation. (A) SNU638 gastric cancer cells were incubated with VE-821 in different concentrations. Total cell lysates were collected and immunoblotting was performed to confirm the inhibition effects on ATR phosphorylation. VE-821, an ATR inhibitor, inhibited ATR phosphorylation of SNU638 cells in a dose-dependent manner. (B) SNU638 gastric cancer cells were incubated with VE-821 for 1 h. EBNA1 transfection and olaparib treatment (2.5 μM concentration) were then performed as described previously. Then, cell viability was measured with a trypan blue exclusion assay. Values represent S.E. of four independent experiments investigated in duplicate. (C) After all treatments (using the same experimental conditions as the previous experiment), cells were collected and cell lysates were analyzed for evaluation of EBNA1, ATR and phosphorylated ATR expression using Western blotting. Results represent three independent experiments. β-actin was used as loading control. Cell viability values were analyzed using one-way ANOVA with Tukey correction. *p<0.05 and **p<0.01.*

as EBNA1, EBNA2, latent membrane protein1 (LMP1), and LMP2A in EBV-related gastric cancer (34-36). These distinct characteristics of EBV-related gastric cancer imply a different cellular response to the same therapeutic agent with EBV-negative gastric cancer. Interestingly, olaparib, a potential candidate agent for gastric cancer, induced apoptosis only in EBV-positive SNU719 gastric cancer cells through caspase 3 dependent pathway in this study (Figure 1 and Figure 2). Previous studies and these results support and suggest that the establishment of a newly targeted therapeutic strategy for gastric carcinoma should be considered in accordance with EBV infection.

In this respect, the confirmation of the definite cell signaling mechanism causing these disparate cellular responses based on EBV infection is indispensable to overcome the resistance to potential therapeutic agents. Therefore, the defining different kinase activation with or without EBV infection may be a good scheme to define disparate signaling mechanisms. Ataxia telangiectasia mutated and Rad3-related (ATR) kinase is an essential factor induced by DNA replication and DNA damage

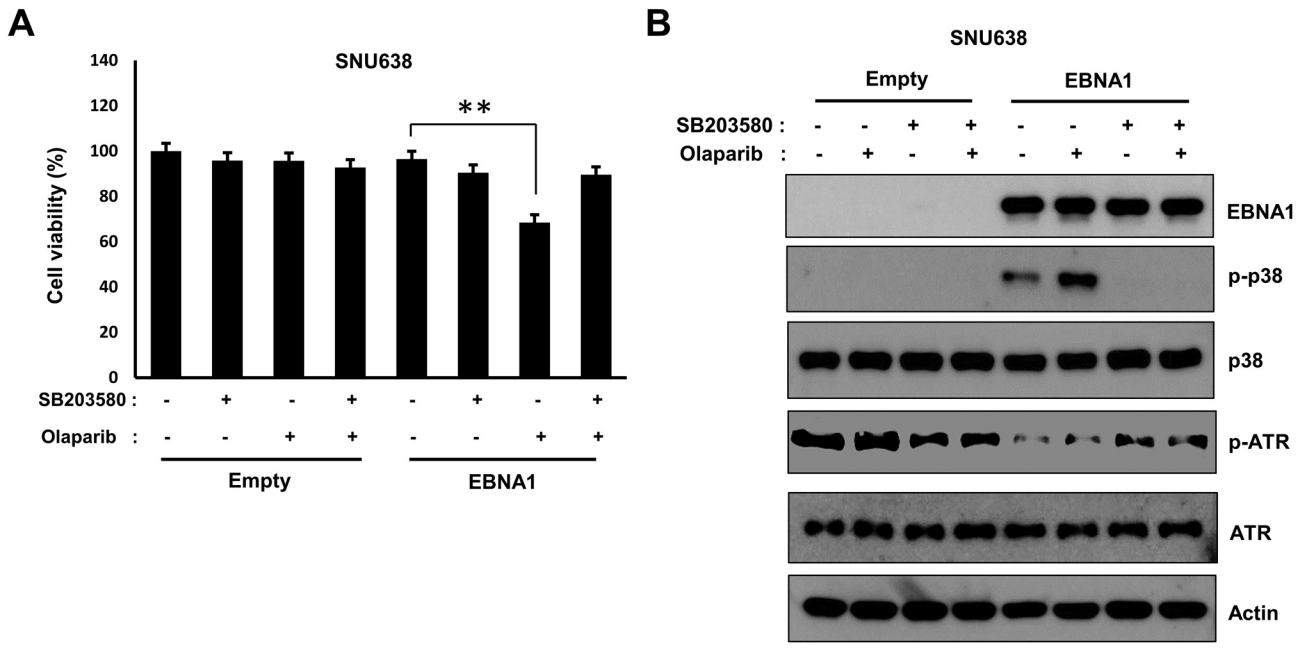

Figure 5. *SB203580, a p38 kinase inhibitor, inhibited the increase of sensitivity to olaparib by EBNA1 transfection in EBV-negative SNU638 cells. (A) SNU638 gastric cancer cells were incubated with SB203580 for 1 h. EBNA1 transfection and olaparib treatment (2.5 μM concentrations) were then performed as described previously. Cell viability was measured with a trypan blue exclusion assay. Values are presented as S.E. of four independent experiments performed in duplicate. (B) After all treatments (using the same experimental conditions as the previous experiment), cells were harvested and cell lysates were prepared for evaluation of EBNA1, p38, phosphorylated p38, ATR and phosphorylated ATR expression using Western blotting. Results represent three independent experiments. β-actin was used as loading control. Cell viability values were analyzed using one-way ANOVA with Tukey correction. **p<0.01.*

in various cancers including pancreatic and prostate cancer (37-39). In the present study, both ATR and phosphorylated ATR (p-ATR) kinases were expressed in only SNU638 EBV-negative gastric cancer cells, and experimental EBNA1, a key EBV-related viral gene, transfection to SNU638 EBV-negative gastric cancer cells decreased ATP kinase phophorylation in a dose dependent manner. Moreover, ATR kinase knock-out using siRNA increased the susceptibility to olaparib in SNU638 EBV-negative gastric cancer cells. These results implied that ATR kinase was a key molecule to determine the susceptibility to olaparib, and that EBNA1 could be the regulator of the ATR kinase phosphorylation in gastric cancer cells.

EBNA1 transfection induced ATR kinase phosphorylation in SNU638 gastric cells. On that account, whether EBNA1 transfection could elicit olaparib-induced apoptosis in SNU638 cells was investigated in sequence. As expected, EBNA1 transfection also increased olaparib-induced cell death in SNU638 cells.

Based on previous studies, p38 mitogen-activated protein kinase (MAPK) is related with apoptosis in EBV-transformed B cells and is activated in the process of EBV reactivation (40, 41). Interestingly, Figure 5 results show that EBNA1 transfection alone slightly induced p38 MAPK phosphorylation, whereas olaparib treatment induced p38 MAPK phosphorylation strongly only after EBNA1 transfection. These results suggest that olaparib induced apoptosis of gastric cancer cells through EBNA1-p38-ATR kinase signaling pathway.

To confirm the signaling cascade of olaparib-induced apoptosis of gastric cancer cells, various inhibition assays were investigated. At first, the inhibitory effect of VE-821, ATR kinase phosphorylation inhibitor, was tested in SNU 638 cells. Then, VE-821 was evaluated in the experimental system established with olaparib treatment and EBNA1 transfection. VE-821 effectively inhibited the phosphorylation of ATR kinase in a dose dependent manner. VE821 induced cell death by itself slightly, enhanced olaparib-induced cell death, and showed synergic effect on EBNA1-transfected cell death in SNU638 cells. These results are consistent with the previous studies (38). On the contrary, SB203580, a p38 MAPK phosphorylation inhibitor, effectively restored the EBNA1-transfected cell death of SNU638 cells. These inhibitor assays support that EBNA1 and ATR kinase are involved in determining the susceptibility to olaparib-induced apoptosis through p38 MAPK kinase phosphorylation in gastric cancer cells.

Collectively, olaparib treatment induced apoptosis through caspase 3 activation in EBV positive SNU719 gastric cancer cells, whereas EBV negative gastric cancer cells showed resistance to olaparib through ATR and phosphorylated ATR expression. EBNA1 transfection increased the susceptibility to olaparib through p38 phosphorylation, ATR inactivation in EBV negative gastric cancer cells.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization, Sung Ho Moon and Dae Young Hur; Data curation, Sung Ho Moon, Nam-Sook Park, and Min Hye Noh; Formal analysis, Sung Ho Moon and Yeong Seok Kim; Funding acquisition, Dae Young Hur; Investigation, Nam-Sook Park; Project administration, Nam-Sook Park and Dae Young Hur; Writing – original draft, Sung Ho Moon; Writing, review and editing, Soon Ho Cheong and Dae Young Hur.

Acknowledgements

This work was supported by the 2019 Inje University research grant.

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Received October 5, 2021
Revised November 17, 2021
Accepted November 19, 2021