Suppression of TopBP1 function increases the efficacy of chemotherapeutic treatments by enhancing the induction of apoptosis

Yuko Obayashi[1, 2], Ryosuke Fujikane[1, 3], Shou Morita[1, 4], Yuka Uechi[1, 5], Akimitsu Hiraki[2], and Masumi Hidaka[1, 3]

[1] Department of Physiological Science and Molecular Biology, Fukuoka Dental College, Fukuoka, 819-0193, Japan

[2] Department of Oral and Maxillofacial Surgery, Fukuoka Dental College, Fukuoka, 819-0193, Japan

[3] Oral Medicine Research Center, Fukuoka Dental College, Fukuoka, 819-0193, Japan

[4] Department of Oral Rehabilitation, Fukuoka Dental College, Fukuoka, 819-0193, Japan

[5] Department of Oral Growth and Development, Fukuoka Dental College, Fukuoka, 819-0193, Japan

A short running title : Role of TopBP1 in chemotherapeutic treatment

Correspondence author; Masumi Hidaka, PhD

Department of Physiological Science and Molecular Biology, Fukuoka Dental College, 2-15-1, Tamura, Sawara-ku, Fukuoka, 819-0193, Japan

E-mail address: hidaka@college.fdcnet.ac.jp

Abstract

The DNA damage response (DDR) is an important mechanism to maintain genome integrity by arresting cell cycle progression and inducing DNA repair and/or apoptosis. Therefore, the activity of DDR is closely related to the drug sensitivity of cancer cells. Inhibitors of ATR, a key member of protein kinases functioning in DDR, are attractive candidates as sensitizers in chemotherapy. In this study, we explore another candidate of chemosensitizers and report DNA topoisomerase II binding protein 1 (TopBP1), a regulator of ATR-mediated signaling, as a potential target to increase the efficacy of chemotherapeutic treatments. Suppression of *TopBP1* using siRNA increased cancer cell sensitivity to cisplatin and an alkylating agent *N*-methyl-*N*-nitrosourea (MNU), concomitant with a percentage increase of the of sub-G_1 population and caspase-9 activation. The immunoblotting analysis revealed that the phosphorylation of CHK1 was significantly reduced in *TopBP1*-knockdown cells. Consequently, treatment with an ATR inhibitor dramatically increased the production of the sub-G_1 population compared to an ATM inhibitor. Phosphorylation of RPA2 increased after drug treatment in *TopBP1*-knockdown cells. These results suggest that TopBP1 is involved in DDR protecting stalled forks from collapse and preventing apoptosis through the activation of an ATR/CHK1 signaling pathway.

KEYWORDS

apoptosis, cisplatin, DNA damage response, DNA topoisomerase II binding protein 1 (TopBP1), oral oncology

1. INTRODUCTION

DNA in cells is inevitably damaged endogenously by reactive oxygen species produced through cellular metabolism and exogenously by exposure to genotoxic agents or radiation.[1] To maintain the integrity of the genome, cells have equipped sophisticated DNA damage signaling pathways ensuring the resolution of DNA damage and replication stress. The DNA damage response (DDR) involves the recognition of DNA damage, activation of DNA damage-responsive protein kinases and the effector proteins that trigger various cellular processes, such as cell cycle arrest, followed by the induction of DNA repair and/or apoptosis.[2] In this context, genes involved in DNA repair and apoptosis have been considered as tumor suppressors because their loss can lead to cancer-promoting mutations and genomic rearrangement. Numerous hereditary cancer predispositions result from mutations in DNA repair genes.[3, 4] For example, defects in mismatch repair (MMR) genes, such as *MSH2* and *MLH1* genes, are found in a variety of sporadic and hereditary non-polyposis colorectal cancers.[5-7] MMR protein defects render cells hypermutable and promote microsatellite instability. Mutations in *BRCA1* and *BRCA2* genes, which encode proteins involved in repairing DNA double-strand breaks (DSB) by homologous recombination, are also present in familial cases of breast and ovarian cancer.[8, 9]

DDR mechanisms are also relevant to the efficacy of cancer treatment using chemotherapeutic agents.[10] Although these treatments rely on the production of DNA damage that especially exhibits cytotoxicity for highly proliferating cancer cells, the effectiveness of a chemotherapeutic treatment depends on the genetic background of the cells since most of the cancer cells harbor mutations and/or rearrangements in DDR-related genes. Unfortunately, some cancer cells are unable to properly respond to DNA damage and develop resistance to chemotherapy, allowing tumor recurrence after the administration of therapeutic drugs.[11] In this way, the deregulation of DDR causes further mutations and induces constitutive activation of oncogenes and suppression of tumor suppressor genes.[12]

On the other hand, a widespread DDR activation in human cancers has been reported. The investigations indicated that the origin of the genomic instability in cancer cells could be linked to

problems that arise during DNA replication.[13, 14] The expression of oncogenes likely induces replication stress, and cancer cells rely on the constitutive DDR activation to resist and survive replication stress.[15] In this concept, DDR regulators become attractive targets for cancer therapy. The phosphoinositide 3-kinase-related protein kinases, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) are key regulators of DDR.[16, 17] ATM is generally activated by DNA double-strand breaks (DSB) and phosphorylates its downstream target checkpoint kinase 2 (CHK2), while ATR responds to replication stress, base adducts, and DNA crosslinks and activates checkpoint kinase 1 (CHK1) by phosphorylation, with overlapping activities.[18, 19] Increasing evidence indicates that ATM or ATR inhibition can sensitize cancer cells to genotoxic treatments. In recent years, highly selective ATM and ATR inhibitors have been developed and are currently being evaluated in clinical trials.[20-22] In addition, it is highly possible that some other DDR-related components can be targeted to exploit a specific vulnerability in cancer cells.

In this study, we explored the potentials of topoisomerase II binding protein 1 (TopBP1) to regulate cancer cell sensitivity following genotoxic treatments. TopBP1 is a key activator of ATR and interacts with ATR and ATR-interacting protein (ATRIP) through its ATR-activating domain (AAD).[23] We demonstrate evidence to support that the suppression of TopBP1 function sensitizes cancer cells by enhancing the induction of apoptosis, following the treatment with cisplatin that forms DNA crosslink leading to replication fork blockage or a simple alkylating agent *N*-methyl-*N*-nitrosourea (MNU) that produces cytotoxic O^6-methylguanine (O^6-meG) in DNA.[24, 25]

2. MATERIALS AND METHODS

2.1. Cell lines and culture

A human oral squamous cell carcinoma cell line, SAS, was provided by Dr. Hatta (Fukuoka Dental College).[26] The HeLa MR cell line, defective in O^6-methylguanine DNA methyltransferase (MGMT) function, was obtained from our laboratory.[27] Both cell lines were cultivated in Dulbecco's Modified

Eagle Medium (D-MEM; FUJIFILM Wako Pure Chemicals, Osaka, Japan), supplemented with 10% fetal bovine serum and penicillin/streptomycin in 5% CO_2 at 37°C.

2.2. Gene knockdown with siRNA

Silencer Select siRNAs targeting the *TopBP1* gene (#1; 5'-GGAUAUAUCUUUGCGGUUUTT-3' and #2; 5'-GCUCUGUAAUAGUCGACUATT-3') were purchased from Thermo Fisher Scientific. SAS or HeLa MR cells were transfected with siRNAs using Lipofectamine RNAiMax (Thermo Fisher Scientific) according to the manufacturer's instruction manual. The transfected cells were cultured for 2 days and then were used for the assays.

2.3. Chemicals and chemical treatment

Cisplatin and MNU were purchased from Nippon Kayaku and Toronto Research Chemicals, respectively. ATR inhibitor VE-821 and ATM inhibitor KU-60019 were obtained from Selleck Chemicals, Houston, USA.

2.4. Survival assay

Eight hundred cells were plated in 100-mm dishes one day before drug treatment of various Cisplatin or MNU concentrations for 1 h in serum-free D-MEM or in serum-free D-MEM with 20 mM Hepes-KOH (pH6.0), respectively, followed by cultivation in serum-supplemented D-MEM for 10 days. Surviving colonies were fixed with 10% formaldehyde and stained with 0.1% crystal violet. The number of colonies was counted, and survival fractions were calculated.

2.5. Immunoblotting

Protein samples prepared from cells were subjected to SDS-PAGE and were electrotransferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories). The PVDF membrane was

incubated with the primary antibody, and after washing with Tris-buffered saline containing 0.1% Tween 20 (TBS-T), was blotted with HRP-conjugated secondary antibody (GE Healthcare) for 1 h, and finally visualized with a chemiluminescent agent (ImmunoStar LD; FUJIFILM-Wako Pure Chemicals) by an LAS-4010 (GE Healthcare Life Sciences, Amersham, UK).

2.6. Antibodies

Anti-TopBP1 (#A300-111A), anti-β-actin (#A5316), anti-RPA2 (#NA19L), γH2AX-S139 (#05-636), and anti-phospho-ATR-T1989 (#GTX128145) were purchased from Bethyl laboratories, Sigma-Aldrich, Calbiochem, Merck Millipore and GeneTex, respectively. Anti-ATM (#2873), anti-Caspase-9 (#9502), anti-CHK2 (#6334), anti-phospho-ATM-S1981 (#13050), anti-phospho-CHK1-S317 (#12302), anti-phospho-CHK2-T68 (#2661), and anti-phospho-RPA2-S8 (#54762) were obtained from Cell Signaling Tech., MA, USA. Anti-ATR (#sc1887), anti-CHK1 (#sc8408), and anti-PARP1 (#sc8007) were purchased from Santa Cruz Biotech, TX, USA.

2.7. Flow cytometry

SAS and HeLa MR cells transfected with siCont or siTopBP1#1 were treated with drugs and were incubated in a complete medium. SAS cells were also treated with or without cisplatin in combination with inhibitors. The cells were harvested and were suspended with PBS containing 0.1% Triton X-100, 10 μg/ml of RNase A, and 0.25% propidium iodide. Samples were analyzed using a FACS Calibur flow cytometer (BD Biosciences) until the gated events reached 10,000.

3. RESULTS

3.1. Increased sensitivities of *TopBP1*-knockdown cells to cisplatin and MNU treatments

To examine the relevance of TopBP1 function to the efficacy of chemotherapeutic agents in cancer treatment, *TopBP1*-knockdown (*TopBP1*-KD) cells were prepared by introducing two independent small

interference RNAs (siRNAs), targeting to different TopBP1 sequences to a human oral squamous cell carcinoma cell line, SAS. The obvious suppression of TopBP1 protein levels in these knockdown cells was shown by immunoblotting using an anti-TopBP1 antibody (Figure 1A). Using these *TopBP1*-KD cells, we performed a survival assay with various concentrations of the chemotherapeutic drug cisplatin, which is widely used clinically in treating different cancer types.[28] SAS cells transfected with either type of siRNA for *TopBP1* (siTopBP1) showed higher sensitivities than the control cells (siCont) (Figure 1B). This was also the case for HeLa MR, another human-derived cervix cancer cell line (Figure 1C). *TopBP1*-KD cells readily underwent cell death after exposure to cisplatin. Moreover, *TopBP1*-KD also further increased the sensitivity to MNU of HeLa MR cells, which are originally sensitive to the drug,[27] since the cell line is defective in the *MGMT* gene, encoding a specific repair enzyme for O^6-methylguanine (Figure 1D). It should be noted that temozolomide, which induces the same modified base as MNU, is used for malignant glioma treatment.[29] These results clearly indicate that the suppression of *TopBP1* sensitizes cancer cells to the treatment with two types of chemotherapeutic drugs.

3.2. Effects of *TopBP1*-KD on the induction of apoptosis triggered by drug treatments

To examine whether the increased sensitivity of *TopBP1*-KD cells to these drugs was resulted from the enhancement of apoptosis induction, the activation of several apoptotic markers in siTopBP1#1-transfected cells was analyzed. The flow cytometry showed that the sub-G_1 population increased in both SAS and HeLa MR cells transfected with either type of siRNA following cisplatin treatment (Figure 2A). However, the degree of the increase in the SAS-derived *TopBP1*-KD cells (14.2%) was significantly higher than the control cells (3.95%), while 48 h following exposure to cisplatin resulted in 25.8% in HeLa MR-derived *TopBP1*-KD cells compared to 10.9% in control cells. We further analyzed the effect of *TopBP1*-KD on the activation of caspase-9 and cleavage of PARP1 (Figure 2B). On immunoblotting, the cleavage of both caspase-9 and PARP1 was barely seen at 48 h and was detected 72 h after drug treatments in siCont-transfected cells. In contrast, in *TopBP1*-KD cells, the signals for

cleaved-caspase-9 and cleaved-PARP1 were clearly observed even at 48 h in both cell lines and further increased at 72 h in HeLa MR cells. Similar results with slower kinetics, showing that the increased production of sub-G_1 population and cleavage of caspase-9 and PARP1, were also obtained when HeLa MR-derived *TopBP1*-KD cells were treated with MNU (Figure S1). These results indicate that the suppression of TopBP1 enhances the induction of apoptosis triggered by these cytotoxic drugs.

3.3. Impairment of the DNA damage response in *TopBP1*-KD cells

Since TopBP1 is known to activate an ATR/CHK1 signaling pathway, we examined the effect of *TopBP1*-KD on the activation of the DNA damage response after cisplatin treatment. The immunoblotting analysis expectedly revealed that phosphorylation of the DDR-related kinases (ATR, CHK1, ATM and CHK2) was clearly observed in the siCont-transfected SAS and HeLa MR cells 24 and 48 h after treatment with the drug (Figure 3). In contrast, the phosphorylation levels of CHK1, but not ATR, ATM and CHK2, were dramatically decreased in *TopBP1*-KD cells at both 24 and 48 h after drug treatment. Similar results were obtained from the analysis of HeLa MR-derived *TopBP1*-KD cells after treatment with MNU (Figure S2). These results indicate that TopBP1 is involved in the activation of CHK1 on an ATR/CHK1 signaling pathway in response to cisplatin and MNU.

In agreement with this observation, the suppression of the ATR/CHK1 axis by an ATR inhibitor (VE-821) dramatically enhanced the productions of the sub-G_1 population in response to cisplatin treatment than when cells were exposed to an ATM inhibitor (KU-60019) (Figure 4A). Under the conditions, as shown in Figure 4B, an ATR inhibitor expectedly suppressed the phosphorylation levels of ATR and CHK1 whereas an ATM inhibitor reduced the phosphorylation levels of CHK2, respectively. These results indicate that the suppression of an ATR/CHK1 signaling pathway by knockdown of *TopBP1* contributes to sensitize cancer cells to cisplatin treatment.

3.4. Increased phosphorylation levels of RPA2 in *TopBP1*-KD cells after drug treatments

To investigate the effect of *TopBP1*-KD on replication forks interfered by the treatment with cytotoxic drugs, phosphorylation levels of replication protein A (RPA) and H2AX, indicative of fork collapse and DSB formation respectively, were analyzed by immunoblotting. Phosphorylation of RPA2 at S8 was hardly seen at 12 h and detected 24 h after cisplatin treatment in SAS and HeLa MR cells transfected with siCont (Figure 5A). In contrast, the signals for RPA2 phosphorylated at S8 were clearly observed in *TopBP1*-KD cells even at 12 h and further increased at 24 h. With the similar kinetics, slower migrated multiple bands of RPA2 corresponding to RPA2 phosphorylated at multiple residues were observed in both knockdown cells. On the other hand, the appearance of phosphorylated form of H2AX (γH2AX) was comparable in between siCont- and siTopBP1-transfected cells after cisplatin treatment (Figure 5B). Similar results were obtained when siRNA-transfected HeLa MR cells were treated with MNU (Figure S3). *TopBP1*-KD increased the phosphorylation levels of RPA2, with no effect on γH2AX appearance. These results indicate that TopBP1 is involved in protecting replication forks from collapse in response to cytotoxic DNA damage.

4. DISCUSSION

Human TopBP1, originally discovered in two-hybrid studies as a topoisomerase II binding protein,[30] has been implicated in DNA replication and DNA damage response.[31] In this study, we demonstrate that the suppression of the TopBP1 function impairs the activation of an ATR/CHK1 pathway following exposure to two types of anti-cancer drugs and sensitizes cancer cells by enhancing apoptotic induction. We also demonstrate that *TopBP1*-KD dramatically reduces DNA damage-induced CHK1 phosphorylation at S317 but does not affect the autophosphorylation of ATR at T1989. This result is consistent with the previous report that phosphorylation of CHK1 at S345, but not ATR at T1989, is severely suppressed in HCT116-derived *TopBP1*-KD cells after irradiation to ultraviolet (UV).[32] The same group reported that TopBP1 possesses eight BRCA1 carboxy-terminal (BRCT) domains, among which BRCT domains 7 and 8 are important for the binding of TopBP1 to ATR peptide phosphorylated

at T1989, but not the unphosphorylated peptide, indicating the function of TopBP1 downstream of phosphorylated ATR in an ATR/CHK1 axis. Taken together, it is suggested that TopBP1 is involved in the recognition of substrates, instead of the activation of ATR *per se*, among various downstream targets of ATR kinase.

During the process of the induction of apoptosis, accelerated phosphorylation of RPA2 at S8 were observed in *TopBP1*-KD cells after treatment with cisplatin or MNU (Figures 5 and S3); the former produces DNA crosslink, which arrests the movement of DNA replication fork and induces apoptosis unless repaired properly, the latter induces O^6-meG in DNA which can pair with thymine and cytosine during DNA replication, and the resulting O^6-meG/T mismatch leads to MMR-dependent apoptosis.[27] Although O^6-meG does not inhibit DNA replication, MMR-processing at O^6-meG/T mismatch also compromises DNA replication and creates replication stress, evidenced by the report that delayed progression of the S phase was observed in alkylating agent-treated cells in an MMR-dependent manner.[33] Therefore, in response to these replication stresses, TopBP1 possibly activates an ATR/CHK1 signaling pathway and induces cell cycle arrest at G_2/M boundary, crucial for protecting stalled replication forks from collapse and preventing apoptosis. In contrast, ATR is phosphorylated but unable to activate both CHK1 and cell cycle checkpoint in TopBP1-defective cells, and as a consequence, RPA2 binding to ssDNA, generated through uncoupling of the helicase and DNA polymerase activities of the replisome, is hyperphosphorylated and can lead to the induction of apoptosis. This proposed scenario is supported by the recent finding that CHK1 inhibition by a chemical inhibitor induces extensive RPA2 hyperphosphorylation in response to replication stress and promotes apoptosis, concomitant with the reduction of chromatin-binding RAD51, required for recombinational repair of DSB.[34]

In preclinical studies, ATR inhibitors have shown promising potential in therapy to sensitize cancer cells in which oncogene-induced replication stress is induced.[35] Nevertheless, other DDR-related factors we still need to be identified as potential targets that can exploit specific vulnerabilities in cancer cells. In this study, it is strongly suggested that TopBP1 is one of attractive targets for this purpose.

Moreover, the functional status of TopBP1 in cancer cells may be an important determinant of their relative sensitivity to chemotherapeutic drugs since the expression levels of TopBP1 vary considerably among cancer cell lines, and higher expression of TopBP1 is reported to contribute to chemoresistance in a non-small cell lung cancer (NSCLC)-derived cell line.[36] This may be of clinical importance since the levels of TopBP1 expression is relevant to how the protein activates ATR/CHK1 signaling to resist replication stress induced in cancer cells with different genetic backgrounds.

The molecular mechanism of the involvement of TopBP1 in the activation of ATR/CHK1 signaling is still unknown. AAD in TopBP1 is essential for the induction of ATR/CHK1 signaling. It should be noted that Ewing's tumor-associated antigen 1 (ETAA1), recently identified as another activator of ATR in vertebrates, also possesses AAD and functions in parallel with, but independent of, the TopBP1 pathway in stimulating ATR-mediated response.[37-39] Further studies to clarify the roles of AAD in the activation of the ATR signaling pathway remain in progress.

5. CONCLUSION

In this study, using two types of cell lines treated with cisplatin or an alkylating agent, we demonstrated that TopBP1, a regulator of ATR-mediated signaling, is a novel potential target to increase the efficacy of chemotherapeutic treatments. We also showed that suppression of *TopBP1* impairs the DNA damage response and enhances the induction of apoptosis triggered by chemotherapeutic agents. Thus, we propose that targeting TopBP1 can be effective for sensitizing cancer cells in chemotherapy.

ACKNOWLEDGMENTS

The authors would like to thank Dr. M. Hatta at Fukuoka Dental College for providing the SAS cell line. This work was supported by The Private University Research Branding Project and JSPS KAKENHI Grant-in-Aid for Scientific research (C) Grant Number 20K09915 (to M.H.).

CONFLICT OF INTEREST

Authors declare no Conflict of Interests for this article.

ORCID

Yuko Obayashi https://orcid.org/0000-0002-7489-3150

Ryosuke Fujikane https://orcid.org/0000-0002-6621-3848

Akimitsu Hiraki https://orcid.org/0000-0001-8654-1294

Masumi Hidaka https://orcid.org/0000-0002-1901-2955

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FIGURE LEGENDS

FIGURE 1 The sensitivities of *TopBP1*-knockdown cells to the treatment with cisplatin or MNU. Two independent siRNAs targeting the *TopBP1* gene were transfected into SAS or HeLa MR cells. (A) The TopBP1 protein levels at 2 and 3 days after siRNA-transfection were analyzed by immunoblotting. β-actin was the loading control. (B) The survival fraction of siRNA-transfected SAS cells after cisplatin treatment. (C, D) The survival fraction of siRNA-transfected HeLa MR cells after cisplatin (C) or MNU (D) treatment. The cells were treated with various drug concentrations, and the number of colonies formed 10 days after the treatment was counted. The mean values of survival fraction obtained from three independent experiments is shown with the S.E. *$p < 0.05$ (Student's *t*-test).

FIGURE 2 Enhancement of apoptotic cell death in *TopBP1*-knockdown cells treated with cisplatin. siRNA-transfected SAS and HeLa MR cells were treated with 20 μM cisplatin. (A) The cells were collected at 0 and 48 h and subjected to flow cytometry. The mean values of the sub-G_1 population obtained from three independent experiments and S.E. are shown *$p < 0.05$ (Student's *t*-test). (B) The cells were collected at 0, 24, 48, and 72 h after drug treatment. Whole-cell extracts were prepared and subjected to immunoblotting using specific antibodies recognizing both uncleaved and cleaved forms of the proteins. β-actin was the loading control.

FIGURE 3 Suppression of DNA damage response in *TopBP1*-knockdown cells after cisplatin treatment. SAS and HeLa MR cells transfected with siCont or siTopBP1were treated with 20 μM cisplatin and cultivated for 0, 24, and 48 h. Whole-cell extracts were prepared and subjected to SDS-PAGE, followed by immunoblotting using the indicated antibodies. β-actin was the loading control.

FIGURE 4 Enhancement of apoptotic cell death in ATR-inhibited cells in response to cisplatin treatment. SAS cells were exposed to various concentrations of an ATR inhibitor VE-821 (ATR-i) or an

ATM inhibitor KU-60019 (ATM-i) combined with 20 μM cisplatin treatment. (A) The cells were collected at 48 h and subjected to flow cytometry. The mean values of the sub-G_1 population obtained from three independent experiments and S.E. are shown *$p < 0.05$ (Student's *t*-test). (B) Whole-cell extracts prepared from cells collected at 24 h after cisplatin treatment were subjected to SDS-PAGE, followed by immunoblotting using the indicated antibodies. β-actin was the loading control.

FIGURE 5 Increased phosphorylation levels of RPA2 in *TopBP1*-knockdown cells after cisplatin treatment. SAS and HeLa MR cells transfected with siCont or siTopBP1 were treated with 20 μM cisplatin and cultivated for 0, 12, 24, and 48 h. Whole-cell extracts were prepared and subjected to SDS-PAGE, followed by immunoblotting using antibodies recognizing phosphorylated RPA2, RPA2 (A), and γH2AX (B). β-actin was the loading control.