Elevated level of PLRG1 is critical for the proliferation and maintenance of genome stability of tumor cells

Hyunji Choi1, Moonkyung Kang2, Kee-Ho Lee3 & Yeon-Soo Kim1,2,*

1Graduate School of New Drug Discovery and Development, Chungnam National University, Daejeon 34134, 2R&D Center, artiCure Inc., Daejeon 34134, 3Division of Radiation Cancer Research, Korea Institute of Radiological and Medical Sciences, Seoul 01812, Korea

Pleiotropic regulator 1 (PLRG1), a highly conserved element in the spliceosome, can form a NineTeen Complex (NTC) with Prp19, SPF27, and CDC5L. This complex plays crucial roles in both pre-mRNA splicing and DNA repair processes. Here, we provide evidence that PLRG1 has a multifaceted impact on cancer cell proliferation. Comparing its expression levels in cancer and normal cells, we observed that PLRG1 was upregulated in various tumor tissues and cell lines. Knockdown of PLRG1 resulted in tumor-specific cell death. Depletion of PLRG1 had notable effects, including mitotic arrest, microtubule instability, endoplasmic reticulum (ER) stress, and accumulation of autophagy, ultimately culminating in apoptosis. Our results also demonstrated that PLRG1 downregulation contributed to DNA damage in cancer cells, which we confirmed through experimental validation as DNA repair impairment. Interestingly, when PLRG1 was decreased in normal cells, it induced G1 arrest as a self-protective mechanism, distinguishing it from effects observed in cancer cells. These results highlight multifaceted impacts of PLRG1 in cancer and underscore its potential as a novel anti-cancer strategy by selectively targeting cancer cells. [BMB Reports 2023; 56(11): 612-617]

INTRODUCTION

Cancer arises from accumulation of genetic and epigenetic alterations that transform normal cells into malignant entities. Its dynamic nature leads to the development of pleiotropic tumors through diverse mechanisms (1). Traditional approaches to cancer treatment, such as surgery, radiation therapy, and chemotherapy, have relied on the observation that cytotoxic chemotherapy, have relied on the observation that cytotoxic agents can effectively eliminate rapidly proliferating cells, despite causing significant harm to normal cells (2). Consequently, there is an urgent need to identify factors that contribute to tumor initiation and progression, with the goal of developing targeted and efficient treatments. Despite the heterogeneity of cancer, dysregulated apoptosis and loss of cell cycle control are common features observed in almost all malignancies (3). Targeting mitotic dysregulation to induce irreversible cell death during the mitotic phase of the cell cycle or generating cancer cell-specific apoptosis holds great promise as a strategy for effective cancer treatment (4).

Pleiotropic regulator 1 (PLRG1) is a crucial component of the NineTeen Complex (NTC), which plays a role in pre-mRNA splicing, DNA damage response and repair, and cell cycle regulation. It has been reported that inhibiting PLRG1 can disrupt NTC assembly (5). Studies on vertebrate development have reported that PLRG1-deficient embryos are not viable and that deficiencies in PLRG1 can result in an increase of apoptotic cells and reduced survival rates in the heart and central nervous system (6). Furthermore, PLRG1 has been implicated in regulating phase separation mediated by USP42, which in turn controls gene expression related to cell function in lung cancer (7). Additionally, PLRG1 collaborates with RNA helicase DHX37 to promote progression in liver cancer (8). Despite these findings, the precise association and effects of PLRG1 on cancer have remained unclear.

In this study, we provide evidence linking PLRG1 to cancer cells and highlight its potential as a new therapeutic target. Our findings demonstrate that PLRG1 is significantly upregulated in various cancer compared to that in normal cells. Suppressing PLRG1 led to cell death in cancer cells. Moreover, dysregulation of PLRG1 contributed to tumor suppression through multiple mechanisms, including increased production of reactive oxygen species (ROS), cell cycle arrest, microtubule instability, DNA damage and non-repairability, and endoplasmic reticulum (ER) stress specifically in cancer cells. Importantly, these effects are specific to cancer cells without inducing cell death in normal cells. Data presented in this study indicate that targeting PLRG1 holds potential to promote apoptosis of cancer cells and offers multiple beneficial effects for cancer treatment.
RESULTS

PLRG1 is overexpressed in various cancer compared to that in normal cells
To examine the association of PLRG1 with tumors, immunohistochemistry (IHC) was employed to analyze PLRG1 expression in non-tumor and tumor tissues of human through microarray. Results demonstrated that PLRG1 expression levels were notably elevated in tumor tissues compared to those in non-tumor tissues across various tissue types (Fig. 1A). Based on IHC scores obtained, samples were categorized as negative (0-1), mild (2-3), moderate (4-8), or strongly positive (9-12). Most non-tumor tissue samples had negative or mild scores, while tumor tissues exhibited moderate to strongly positive scores (Fig. 1B). To explore whether the difference in PLRG1 expression between non-tumor and tumor tissues was also evident at the cellular level, we compared PLRG1 protein levels in cancer cell lines with those in normal cell lines (Fig. 1C). Results revealed significantly higher protein levels of PLRG1 in cancer cell lines than in normal cell lines. These findings indicate that tumor tissues and cells exhibit higher expression of PLRG1 than normal ones.

Depletion of PLRG1 leads to decreased viability of cancer cells
To investigate the impact of PLRG1 on cancer cell growth, we employed shPLRG1 for gene silencing in normal and cancer cell lines, respectively. In normal cell lines BJ-5ta and hTERT RPE-1, the efficacy of the shRNA system was confirmed at the RNA level as protein expression of PLRG1 was deficient. We observed a significant reduction in RNA expression of PLRG1 in the shPLRG1 group compared to that in the shScramble group (Supplementary Fig. 1A). After applying shRNA, we evaluated cell growth using WST-1 analysis and observed that shPLRG1 resulted in slower cell growth compared to shScramble at the 6-day time point (Fig. 1D and Supplementary Fig. 1C).

In cancer cell lines, shPLRG1 effectively reduced protein levels of PLRG1 (Supplementary Fig. 1B). Knocking down of PLRG1 in various cancer cells yielded different outcomes compared to normal cells, with cancer cells showing reduced cell viability in shPLRG1 group (Fig. 1D and Supplementary Fig. 1C). Moreover, in vivo experiments using a xenograft mouse model yielded consistent results (9). These findings suggest that a decrease in cell viability occurs specifically when PLRG1 expression is inhibited in cancer cells.

Inhibition of PLRG1 causes mitotic defects only in cancer cells
To gain insights into changes in gene expression resulting from inhibiting PLRG1 in cancer cells, we performed NGS analysis.

![Fig. 1. Expression of PLRG1 in microarray and reduced cell viability in cancer cells by PLRG1-knockdown. (A) IHC showing PLRG1 differentially expressed in various tumor tissues versus non-tumor tissues using human tissue microarray. Scale bars, 100 μm. (B) Scatter plot showing the IHC scores from non-tumor and tumor tissues. (C) Western blot of PLRG1 expression in cancer cell lines compared to normal cell lines. (D) The graph shows the proliferation rate of cell lines determined using WST1 activity at 6 days against 3 days after working with shScramble or shPLRG1. White bars, shScramble. Black bars, shPLRG1. The data represent the mean ± SD; ***P < 0.001 vs non-tumor or shScramble.](http://bmbreports.org)

![Fig. 2. Mitotic arrest, ER stress and apoptosis signaling activation after PLRG1 knockdown. (A) Flow cytometry for cell cycle in BJ-5ta and Huh7 after day 3 of shRNA operation. The graph shows the percentage of cell cycle phases. (B) The hand image for the western blot of proteins associated with cell cycle, autophagy, and apoptosis, p-p53, p53, p21, CDK1, CDK2, DDT1, Bec2, and Bax after day 3 of shRNA application (top and middle) and cleaved caspase-3, p62, and LC3B after day 4 of shRNA operation (bottom). (C) Relative expression of ER stress markers ATF, usXB1, and sXB1 in shPLRG1 compared with shScramble. (D) Representative dot plots using Annexin V/PI staining for apoptosis after day 4 of shRNA operation. The graph of apoptotic cell percentage. Left panel, BJ-5ta cells. Right panel, Huh7 cells. The data represent the mean ± SD, **P < 0.01 and ***P < 0.001 vs shScramble.](http://bmbreports.org)
Considering significant protein expression of PLRG1 and reduced viability in Huh7 cells, we chose this representative cancer cell line for subsequent experiments. We identified 29 differentially expressed genes (DEGs) with increased expression in shPLRG1 group than in shScramble group. These genes then underwent enrichment analysis for GO biological functions and KEGG pathways (Supplementary Fig. 2). Among genes affected by PLRG1 changes, DNA damage-inducible transcript 3 (DDIT3) and p21 were identified as highly involved in multiple signaling pathways, including apoptosis. Notably, p21 plays a crucial role in regulating cell cycle (10). To investigate effects of inhibiting PLRG1 on cell cycle, we performed propidium iodide (PI) staining. In BJ-5a cells, PLRG1 knockdown resulted in G1 arrest, while in Huh7 cells, arrests at S and G2/M were observed (Fig. 2A). To further validate the impact of PLRG1 on cell cycle, additional experiments were performed using different cell lines. Depleting PLRG1 in hTERT RPE-1 resulted in G1 arrest like in BJ-5a cells, while PLRG1 knockdown in cancer cell lines H460 and Hela led to G2/M arrest (Supplementary Fig. 3A). In both cancer and normal cell lines, inhibition of PLRG1 led to increased levels of p21, p-p53, and p53 proteins associated with all cell cycle phases (Fig. 2C). In BJ-5a cells, G1 arrest was induced by PLRG1 knockdown, while CDK2 known to regulate G1 and S phases decreased. However, there was no significant change in CDK1 known to be involved in G2/M arrest. In contrast, shPLRG1 in Huh7 cells resulted in decreased levels of both CDK1 and CDK2 as it induced S and G2/M arrests (Fig. 2B). In summary, inhibiting PLRG1 in cancer cells can lead to defects in S and G2/M of the cell cycle, while G1 arrest is observed in normal cells.

**PLRG1 knockdown enhances accumulation of ER stress and apoptosis signal pathway**

DDIT3 plays a role in apoptosis by regulating Bcl2. It is a key gene in ER stress (11). Consistent with NGS results, PLRG1 inhibition in Huh7 cells upregulated the expression of DDIT3, leading to a decrease in anti-apoptotic factor Bcl2 and an increase in pro-apoptotic factors Bax and cleaved caspase-3. In contrast, in BJ-5a cells, despite PLRG1 inhibition, there were no significant differences in expression of DDIT3, Bcl2, Bax, or cleaved caspase-3 compared to shScramble-treated cells, highlighting contrasting events occurring in cancer cells (Fig. 2B). Overall, these findings suggest that knockdown of PLRG1 in cancer cells can induce cell death by promoting expression of DDIT3.

ATF4, a marker of ER stress, was increased upon PLRG1 inhibition in all cell lines (Fig. 2C). ER stress-induced cells can resolve stress by converting unspliced-XBP1 (uXBP1) into its spliced form (sXBP1) (12). In BJ-5a cells, sXBP1 levels were increased in shPLRG1-treated cells compared to those in shScramble-treated cells, whereas in Huh7 cells, the transition to the spliced form was not smooth due to an increase in uXBP1 and a decrease in sXBP1 (Fig. 2C). Prolonged ER stress can lead to excessive autophagy (13). While PLRG1 knockdown in normal cell lines showed no decrease in p62 or increase in LC3B-II, PLRG1 inhibition in cancer cell lines significantly reduced p62 levels and increased LC3B-II levels (Fig. 2B). Collectively, these events in cancer cells ultimately can lead to apoptosis.

To confirm apoptosis, Annexin V/PI staining was performed on day 4. Compared to shScramble-treated cells, PLRG1 knockdown in BJ-5a cells did not show an increase in Annexin V/PI staining, whereas Huh7 cells did. In other words, normal cells did not display signs of apoptosis, while 57% of cancer cells showed positive staining for apoptosis (Fig. 2D). Likewise, downregulation of PLRG1 in hTERT RPE-1 did not demonstrate apoptosis, whereas, the incidence of apoptosis was increased significantly in H460 and Hela cells (Supplementary Fig. 3B). These results suggest that, unlike normal cells, PLRG1 inhibition does not alleviate ER stress in cancer cells. It can lead to excessive autophagy due to its continuous accumulation, resulting in cell death.

**Depletion of PLRG1 causes microtubule instability**

Considering that prolonged mitotic arrest can trigger mitotic catastrophe (14), we investigated whether PLRG1 inhibition could lead to mitotic catastrophe in cancer cells by examining the progression of mitosis. To visualize spindle microtubules and assess different stages of mitosis, including interphase, prophase, metaphase, anaphase, telophase, and cytokinesis, we performed α-tubulin staining. In cells treated with shScramble, a well-formed spindle structure was observed and the mitotic process proceeded normally. However, in cells treated with shPLRG1, the spindle structure was disrupted and condensed around the nucleus (Fig. 3A). To assess the impact of PLRG1 inhibition on the centrosome responsible for microtubule pro-
Depletion of PLRG1 induces ROS generation and DNA damage in cancer

In addition to microtubule destabilization, the accumulation of DNA double-strand breaks and genomic instability can lead to mitotic catastrophe (14). To assess whether PLRG1 knockdown could induce DNA damage, we performed γH2AX staining and quantification of γH2AX-positive foci per cell. In BJ-5ta cells, both shScramble and shPLRG1 groups showed no γH2AX staining, whereas PLRG1 inhibition in Huh7 intensified γH2AX staining and increased the number of γH2AX foci compared to control (shScramble treatment) (Fig. 4A). To further confirm DNA damage, we conducted comet assay on days 2 and 3 following shRNA application. In BJ-5ta cells, regardless of PLRG1 inhibition, there was no increase in tail length, indicating the absence of DNA damage on both days. In shScramble-treated Huh7 cells, there was no elongation of the comet tail on day 2 or 3. However, cells with PLRG1 knockdown exhibited a longer tail on day 3 compared to those on day 2, indicating the presence of DNA damage (Fig. 4B and Supplementary Fig. 4A). These findings suggest that PLRG1 knockdown can specifically induce DNA damage in cancer cells, while normal cells remain unaffected.

Given PLRG1’s involvement in DNA repair (5), we investigated its impact on DNA recovery under oxidative stress. On day 2, H2O2-induced oxidative stress was administered, followed by 24 hours of recovery time. In all cell lines treated with shScramble, DNA damage was evident on day 2, indicated by an elongated comet tail, which returned to a round shape by day 3, signifying DNA recovery. In normal cell lines in which PLRG1 was downregulated, the comet tail was elongated on day 2, like shScramble-treated cells, but it returned to a circular shape after 24 hours, indicating successful DNA repair. However, in PLRG1-inhibited Huh7 cells, DNA failed to recover, leading to worsened damage and separation of the comet head and tail (Fig. 4C and Supplementary Fig. 4B).

Considering that oxidative stress can induce DNA damage, microtubule instability, ER stress, and apoptosis (15-17), we examined ROS generation to confirm oxidative stress following PLRG1 inhibition. Twenty-four hours after shRNA delivery, ROS levels were measured using fluorescent imaging and flow cytometry with H2DCFDA. H2O2 was used as a positive control. In BJ-5ta cells, fluorescence and flow cytometry indicative of ROS were not observed in any groups. However, in Huh7 cells, fluorescence was detected with a positive shift in ROS level by flow cytometry in shPLRG1-treated cells compared to that in shScramble-treated cells (Fig. 4D, E). Collectively, these results suggest that DNA damage and ROS generation contribute to mitotic cell death, at least partially, as a result of PLRG1 depletion.

**DISCUSSION**

Previous reports have indicated that inhibiting PLRG1, either as a part of the NTC or as a co-factor of USP42 or DHX37, can lead to chromosome misalignment in mitosis and induce cancer cell death (7, 8, 18). These findings highlight the potential of PLRG1 itself as a promising target for cancer treatment. In this study, our objective was to explore the association between PLRG1 and cancer and provide evidence of its diverse effects on cancer cells. Our findings revealed that PLRG1 expression was elevated in cancer cells compared to that in normal cells and that downregulation of PLRG1 level in cancer cells consistently triggered apoptosis across various cell types. These results suggest that PLRG1 has the potential to be a new cancer treatment target, applicable beyond specific cancer types.
that contributed to cancer cell death upon PLRG1 knockdown. We conducted a comprehensive analysis of functions of these genes. Upregulation of p21, a key regulator of the cell cycle, was found to be p53-dependent (19), indicating defects in cell cycle progression during G2/M (Fig. 2A). Like our data, it has been reported that siPLRG1-treatment can lead to errors in chromosome alignment and mitotic progression in Hela cells (18). Moreover, PLRG1 can modulate the expression of CCND1 and PLRG1 knock-out in MEF, resulting in failure to progress through the cell cycle (6, 8). These results suggest that PLRG1 is a necessary factor in regulating all cell cycles. Further identification of the molecular mechanisms is required.

Impaired cell cycle regulators not only can directly affect cell cycle progression, but also can lead to microtubule and genome instability, ultimately resulting in cell death (20). Interestingly, normal cells exhibited a trace amount of PLRG1 protein (Supplementary Fig. 1D). Depletion of PLRG1 in normal cells only impaired the G1 phase due to an increase in p21, without inducing DNA damage or apoptosis (Figs. 2, 4, and Supplementary Fig. 3A). DNA in G1, which is not actively replicating, might be less susceptible to radical attacks due to its densely packaged chromatin structure. Moreover, G1 facilitates enhanced DNA recovery. Therefore, even when PLRG1 is inhibited in normal cells, there is minimal DNA damage and a proper recovery ability (21). This is further supported by a previous study showing that defects in p21-dependent G1 arrest have a protective function against apoptosis (22).

DDIT3 is a gene associated with ER stress. It is involved in inducing apoptosis through accumulation of intensified stress via upregulation of DDIT3 and a decrease in conversion to spliced form of XBP1 (23). Additionally, DDIT3 regulates the Bcl2-Bax pathway to enhance autophagy and also induce p53-dependent apoptosis (23, 24). Therefore, further investigations are needed to understand the relationship between autophagy and apoptosis in the context of PLRG1 deletion. Nevertheless, experiments conducted in cancer cells have demonstrated that PLRG1 depletion can lead to signaling pathways that promote cell death (Fig. 2B).

The occurrence of DNA breaks is influenced by microtubule instability and cell cycle arrest (25). Interestingly, in cancer cells depleted of PLRG1, γH2AX foci and olive tail moment in comet assay were significantly increased (Fig. 4A, B). These findings suggest that PLRG1 directly regulates DNA damage. Additionally, it has been reported that loss of the TREX complex in yeast can result in DNA damage, indicating that NTC, which is crucial for TREX stability, might be dependent on PLRG1 (26, 27). PLRG1 is recognized as an essential cofactor for NTC function in promoting DNA repair (28). Further investigations were conducted on DNA recovery. When Huh7 cells with PLRG1 knockdown were subjected to oxidative stress, it was observed that DNA repair was impaired, leading to increased olive tail moment (Fig. 4C). This observation aligns with previous studies showing a significant increase in DNA damage when Hela cells are treated with siPLRG1 and hydroxyurea (28). Importantly, depletion of PLRG1 resulted in generation of ROS specifically in cancer cells (Fig. 4D, E). Oxidative stress can impact various physiological conditions, including microtubule instability, cell cycle arrest, DNA damage, ER stress, and apoptosis (15-17). Although the precise molecular mechanism underlying the association between PLRG1 and ROS has not been fully characterized, it implies that effects of stress can be directly enhanced in multiple ways in cancer cells.

Initially, PLRG1 was identified as a subunit of the spliceosome essential for RNA splicing. However, recent studies have revealed that PLRG1's regulation extends beyond its role in splicing. It has diverse effects on cancer, increasing the potential for therapeutic benefits. The experiment with a normal cell line demonstrated that targeting PLRG1 showed minimal impact on normal cells, while downregulation of PLRG1 expression induced tumor-specific cell death followed by various cellular damages in cancer cells. Thus, further characterization of functional interactions between PLRG1 and possible molecular partners will help us decipher regulatory links associated with tumor-specific cell death. It might also uncover functions in the control of cell fate by PLRG1 in cancer.

MATERIALS AND METHODS

Detailed information on Materials and methods is available in the supplementary section.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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Depletion of PLRG1 underlies cancer cell death
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