

Inhibition of Polo-Like Kinase 1 Promotes Hyperthermia Sensitivity via Inactivation of Heat Shock Transcription Factor 1 in Human Retinoblastoma Cells

Tatsuya Yunoki,^{1,2} Yoshiaki Tabuchi,³ Atsushi Hayashi,² and Takashi Kondo¹

¹Department of Radiological Sciences, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

²Department of Ophthalmology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

³Division of Molecular Genetics Research, Life Science Research Center, University of Toyama, Toyama, Japan

Correspondence: Tatsuya Yunoki, Department of Radiological Sciences, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan; yunokiki@med.u-toyama.ac.jp.

Submitted: June 20, 2013

Accepted: November 9, 2013

Citation: Yunoki T, Tabuchi Y, Hayashi A, Kondo T. Inhibition of polo-like kinase 1 promotes hyperthermia sensitivity via inactivation of heat shock transcription factor 1 in human retinoblastoma cells. *Invest Ophthalmol Vis Sci.* 2013;54:8353–8363. DOI:10.1167/iovs.13-12618

PURPOSE. Hyperthermia (HT) has been recognized as an effective focal treatment in retinoblastoma. However, one of the problems with HT therapy is that cells acquire acquisition. The purpose of this study was to evaluate whether the inhibition of polo-like kinase 1 (PLK1) would promote HT sensitivity in human retinoblastoma cells.

METHODS. We examined the effects of PLK1 knockdown by small interfering RNA (siRNA) or by the inhibition of PLK1 activity with PLK1 inhibitor (BI-2536) on the sensitivity to HT (44°C, 1 hour) in human retinoblastoma Y79 and WERI-Rb-1 cells by evaluating apoptosis and cell proliferation using flow cytometry, Western blotting, real-time quantitative polymerase chain reaction, and WST-8 assay. Furthermore, we investigated the effects of activating heat shock transcription factor 1 (HSF1) through a combination of PLK1 knockdown and HT using Western blotting and immunocytochemistry.

RESULTS. The combination of PLK1 inhibition and HT enhanced sensitivity to HT synergistically. Furthermore, PLK1 knockdown inhibited HT-induced phosphorylation of HSF1, the nuclear translocation of HSF1 from the cytoplasm, and nuclear granule formation of HSF1. Heat shock transcription factor 1, inactivated by the silencing of PLK1, reduced the expression of heat shock proteins (HSPs), such as HSP70 and HSP40, as well as the expression of Bcl-2-associated athanogene 3 (BAG3).

CONCLUSIONS. Polo-like kinase 1 inhibition may attenuate the thermoresistance of HT through the inactivation of HSF1 concomitant with reductions in HSPs and BAG3. The combination of PLK1 inhibition and HT may become an option for HT therapy in patients with retinoblastoma.

Keywords: retinoblastoma, PLK1, HSF1, hyperthermia

Retinoblastoma is the most common intraocular malignant tumor of childhood. It occurs in approximately 1 out of 14,000 to 20,000 newborns.¹ Retinoblastoma left untreated is fatal and the patients die of intracranial extension and disseminated disease within a few years.² The retinoblastoma *Rb1* gene is located at chromosome 13q14, and the RB protein encoded by the *Rb1* gene functions as a tumor suppressor by controlling the cell cycle.³ Retinoblastoma is an inherited cancer caused by mutations or deletions of the *Rb1* gene.⁴ Although in the past retinoblastoma was regarded as a fatal childhood cancer, most cases have been well managed through early detection and treatment.⁵ Previously, external beam radiotherapy was the main treatment, but more recently there has been a tendency to avoid radiation therapy whenever possible due to associated problems such as a high risk of secondary cancers and the orbital growth failure.⁶ In recent years, it has been reported that chemotherapeutic drugs such as cisplatin, carboplatin, and etoposide are effective for retinoblastoma.⁷ Shields et al.⁸ use the term “chemoreduction,” which means tumor reduction by systematic chemotherapy, and they have demonstrated that focal treatments, such as photocoagulation, cryopexy, and hyperthermia (HT), after chemoreduction, are effective treatments for retinoblastoma.^{8,9} Among them, HT has been recognized as one of the most effective modalities for use as a focal treatment.

Local HT has been used to treat patients with various cancers and has been clinically demonstrated to be an effective treatment. The combination of HT with chemotherapy, radiotherapy, or both has been clinically used for patients with cancer in various organs, and the antitumor effects of these combinations have been verified by clinical trials.^{10–12} Hyperthermia has been reported to be effective for retinoblastoma in vitro^{13,14} and in small tumors, namely tumors less than 1.5-disc diameter or 3.0 mm in diameter clinically.^{15,16} However, tumors that are large in height or basal diameter are more difficult to control with HT therapy, and in such cases there is a high risk of HT complications, such as focal iris atrophy, peripheral focal lens opacity, retinal traction, retinal vascular obstruction, and transient localized serous retinal detachment.¹⁵ Furthermore, it is considered that tumors with well-differentiated characteristics do not adequately respond to hyperthermia in retinoblastoma.¹⁷

Local HT has been used to treat patients with various cancers and has been clinically demonstrated to be an effective treatment. The combination of HT with chemotherapy, radiotherapy, or both has been clinically used for patients with cancer in various organs, and the antitumor effects of these combinations have been verified by clinical trials.^{10–12} Hyperthermia has been reported to be effective for retinoblastoma in vitro^{13,14} and in small tumors, namely tumors less than 1.5-disc diameter or 3.0 mm in diameter clinically.^{15,16} However, tumors that are large in height or basal diameter are more difficult to control with HT therapy, and in such cases there is a high risk of HT complications, such as focal iris atrophy, peripheral focal lens opacity, retinal traction, retinal vascular obstruction, and transient localized serous retinal detachment.¹⁵ Furthermore, it is considered that tumors with well-differentiated characteristics do not adequately respond to hyperthermia in retinoblastoma.¹⁷

Hyperthermia is thought to combat cancers by reducing their blood supply and inducing apoptosis.^{18,19} In some cancer cells, however, the cytoprotective effect of an increase in heat shock proteins (HSPs) renders HT less effective, at least in part.^{20,21} Heat shock proteins have cytoprotective functions against various stresses and work as molecular chaperones. Therefore, we think it is important to inhibit the expression of HSPs in order to increase HT's effects for retinoblastoma refractory to HT, such as large tumors.

The induction of HSPs is mainly mediated by the activation of heat shock transcription factor 1 (HSF1), which binds to conserved regulatory sequences called heat shock elements (HSE) that are located in the promoter regions of *HSP* genes.²² Heat shock transcription factor 1 largely localizes to the cytoplasm as an inactive monomer. Under stresses such as heat shock, HSF1 forms an active trimer and translocates from the cytoplasm to nucleus. This active HSF1 binds to the HSE of DNA, thereby activating the transcription of HSPs.²² It has been reported that phosphorylation of HSF1 and HSF1 nuclear translocation are regulated by polo-like kinase 1 (PLK1).^{23,24}

Polo-like kinase 1 is an important regulator of mitosis and plays a role in G2/M phase progression by regulating CDK1, cyclin B1, and *cdc25C*.^{25,26} Overexpression of PLK1 has been reported in many cancer cells,²⁷⁻²⁹ and several studies have shown that the depletion of PLK1 using RNA interference inhibits cancer cell proliferation and induces apoptosis.^{30,31} It has been demonstrated that PLK1 depletion using RNA interference or PLK1 inhibitor enhances the effects of chemotherapy and radiotherapy without affecting normal cells.³²⁻³⁴ In addition, a PLK1 inhibitor (BI2536) has been used in a multicenter, multitumor phase II trial.³⁵ However, to our knowledge, there has been no report that PLK1 is involved in HT sensitivity, even though PLK1 plays a role in the activation of HSF1. In this study, we examined the effects of PLK1 knockdown (PLK1-targeted small interfering RNA [siRNA]) and PLK1 inhibitor (BI-2536) on sensitivity to HT in human retinoblastoma cells.

METHODS

Cell Culture and Heat Treatment

The human retinoblastoma Y79 and WERI-Rb-1 cell lines were obtained from the Riken Bioresource Center (Tsukuba, Japan). The cells were cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (FBS) at 37°C in humidified air with 5% CO₂. When the cells were treated with HT, plastic vessels containing the cells were immersed in a water bath at 42°C, 43°C, or 44°C for 1 hour. The temperature was monitored with a digital thermometer (No. 7536; Yokogawa, Tokyo, Japan) during heating. After HT, the cells were incubated for 1 to 24 hours at 37°C.

Silencing of PLK1 Using Small Interfering RNA (siRNA)

The four siRNAs used for PLK1 knockdown were designed by Nippon EGT Co., Ltd. (Toyama, Japan). The sequences of the siRNAs are listed in Table 1. A luciferase siRNA (CGUACGCG GAAUACUUCGA) was used as a negative control siRNA. Cells were incubated in Opti-MEM I Reduced Serum Medium (Life Technologies Japan Ltd., Tokyo, Japan) containing 50 nM siRNA and Lipofectamine RNAiMAX (Life Technologies Japan, Ltd.) at 37°C. Twenty-four hours after transfection, the medium

TABLE 1. Nucleotide Sequences of siRNAs for PLK1

| Name | Sequence-TT | Position | GC% |
|----------|----------------------|----------|-----|
| siPLK1-1 | AGAUCACCCUCCUAAAUA | 1477 | 37 |
| siPLK1-2 | UCACCCUCCUAAAUAUUU | 1480 | 32 |
| siPLK1-3 | CACGCCUCAUCCUCUACAA | 1372 | 53 |
| siPLK1-4 | ACCGAGUUUAUUCAUCGAGA | 562 | 42 |

Human PLK1 nucleotide database: GenBank accession number, NM_005030.

was exchanged for RPMI-1640 medium supplemented with 10% FBS, and the cells were maintained at 37°C for 24 hours.

Compound Treatment

Polo-like kinase 1 inhibitor BI-2536 (Selleck Chemicals, Houston, TX) was prepared in dimethyl sulfoxide (DMSO) and diluted to the final concentration in culture medium. The control group was treated with the same amount of DMSO employed as vehicle for drug treatment. After 1 hour of compound treatment, cells were exposed to HT.

Western Blotting

Cells were dissolved in a lysis buffer (50 mM NaCl, 1% Nonidet P-40, and 50 mM Tris-HCl, pH 8.0) containing a protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). After electrophoresis on SDS-PAGE, the proteins were transferred electrophoretically onto polyvinylidene fluoride membranes. The primary antibodies used were as follows: a rabbit monoclonal anti-Bcl-2-associated athanogene 3 (BAG3) antibody (Funakoshi Co., Tokyo, Japan), mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and PLK1 antibodies (Millipore Co., Temecula, CA), rabbit polyclonal anticlaved caspase-3, HSF1, *cdc25c*, and CDK1 antibodies (Cell Signaling Technology, Inc., Beverly, MA), a rabbit monoclonal anti-cyclinB1 antibody (Cell Signaling Technology, Inc.), a rabbit monoclonal antiphospho-HSF1 antibody (Gene Tex, Inc., Irvine, CA), a mouse monoclonal anti-Histone H1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-HSP70 and HSP40 antibodies (MBL Co., Ltd., Nagoya, Japan), and a rabbit polyclonal anti-HSP27 antibody (MBL Co., Ltd.). Immunoreactive proteins were visualized by a luminescent image analyzer (LAS 4000mini; GE Healthcare Co., Tokyo, Japan) using an enhanced chemiluminescence detection system. Glyceraldehyde 3-phosphate dehydrogenase served as a loading control. The nuclei and cytoplasm were separated by using a Nuclear/Cytosol Fractionation Kit (BioVision, Inc., Mountain View, CA) according to the manufacturer's protocol.³⁶ The proteins in the nuclear and cytoplasmic fractions were used for Western blotting.

RNA Isolation

Total RNA was extracted from cells using a NucleoSpinRNA (Takara Bio, Inc., Shiga, Japan) and was treated with rDNase (RNase-free DNase kit; Takara Bio, Inc.) for 15 minutes at room temperature to remove residual genomic DNA. The RNA concentration was measured by a Nanodrop (Thermo Fisher Scientific, Inc., Waltham, MA). Ribonucleic acid integrity was determined by a Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA). Ribonucleic acid samples that had RIN (RNA integrity number) values above 9.0 were considered acceptable.

TABLE 2. Nucleotide Sequences of Primers and a Probe for Target Genes

| Genes | Orientation | Nucleotide Sequence (5'→3') | GenBank Accession No. |
|-------|-------------|-----------------------------|-----------------------|
| PLK1 | Sense | CGAGGACAACGACTTTCGTGT | NM_005030 |
| | Antisense | GGTTGCCAGTCCAAAATCCC | |
| HSF1 | Sense | GGGAACAGCTTCCACGTGTT | NM_005526 |
| | Antisense | TGGAACTCCGTGTCGTCTCT | |
| GAPDH | Sense | AAGGACTCATGACCACAGTCCAT | NM_002046 |
| | Antisense | CCATCACGCCACAGTTTCC | |

Real-Time Quantitative Polymerase Chain Reaction (qPCR) Assay

Real-time qPCR was performed on a Real-Time PCR system Mx3005P (Agilent Technologies, Inc.) using SYBR PreMix ExTaq (Takara Bio, Inc.) according to the manufacturer's protocols. Reverse transcriptase reaction was carried out with total RNA by using a random 6 mers and an oligo dT primer (PrimeScript RT reagent kit; Takara Bio, Inc.). Real-time qPCR was performed by using the specific primers listed in Table 2. Each mRNA expression level was normalized with respect to the mRNA expression of GAPDH.

Cell Cycle Analysis

Cells were exposed to HT, and then cultured at 37°C. After 24 hours of culturing, the cells were fixed in 70% ice cold ethanol for at least 24 hours at -20°C and subsequently treated with 0.25 mg/mL RNase A. After the staining with propidium iodide (PI) at a concentration of 0.05 mg/mL, the samples were finally run on an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA).³⁷

Analysis of Cell Death

Cells were exposed to HT, and then cultured at 37°C. After 12 hours of culturing, the cells were harvested to examine cell death. We performed chromatin condensation analysis using a Nuclear-ID Green Chromatin Condensation Kit (Enzo Life Sciences, Inc., Farmingdale, NY) according to the manufacturer's protocol.³⁸ The samples were run on an Epics XL flow cytometer (Beckman Coulter).

Cell Viability and Cell Proliferation Analysis by WST-8 Assay

Cell Count Reagent SF (Nacalai Tesque, Inc.) was used to count the viable cells in the cell proliferation assay. The two retinoblastoma cell lines were treated with HT at 44°C for 1 hour with or without transfection of PLK1 siRNA, or were treated with various concentrations of a PLK1 inhibitor, BI-2536. After incubation for 12 or 24 hours, the cells were seeded with a volume of 100 µL into 96-well plates, and 10 µL WST-8 reagent was added to each well. Absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA) after 3 hours of incubation with the WST-8 reagent. Absorbance is proportionally related to the number of viable cells.

Immunocytochemistry

Y79 cells were washed with PBS and fixed in methanol for 10 minutes at room temperature. Then, the cells were seeded in an eight-well chamber slide, and incubated with Blocking One Histo (Nacalai Tesque, Inc.) for 1 hour at room temperature. Next, cells were incubated with the first antibody against HSF1 (Cell Signaling Technology, Inc.) for 24 hours at 4°C and treated

with the Chromeo 488-labeled secondary antibody (Active Motif, Carlsbad, CA) for 1 hour at 25°C. Finally, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes/Invitrogen, Eugene, OR) for 5 minutes for nuclear visualization and thoroughly washed before observation under a fluorescent microscope (Olympus BX-61 microscopic image analyzer; Olympus Co., Ltd., Tokyo, Japan).

Statistical Analysis

Data are shown as the means ± SDs. Student's *t*-test was used for statistical analysis and values of *P* less than 0.05 were considered to indicate statistical significance.

RESULTS

HT Induced Apoptosis in Retinoblastoma Cells Under Various Temperature Conditions

First, we examined the effects of HT on apoptosis in Y79 and WERI-Rb-1 cells in order to determine the appropriate HT conditions for the induction of apoptosis. When we monitored apoptosis using chromatin condensation as an apoptotic marker, the percentages of apoptotic cells were $4.72 \pm 1.1\%$ in the control Y79 cells and $4.98 \pm 1.1\%$ in the control WERI-Rb-1 cells. When Y79 and WERI-Rb-1 cells were incubated at 42°C for 1 hour, apoptosis did not increase significantly during the recovery periods after HT, but it did increase significantly during the recovery following 1-hour incubation at 43°C or 44°C (Fig. 1). The percentages of apoptotic cells at 43°C in the Y79 cells were $6.2 \pm 1.6\%$ at 6 hours after HT, $7.7 \pm 0.8\%$ at 12 hours after HT ($P < 0.05$), and $7.1 \pm 0.9\%$ at 24 hours after HT ($P < 0.05$); in the WERI-Rb-1 cells, they were $6.4 \pm 0.7\%$ at 6 hours after HT, $7.6 \pm 0.5\%$ at 12 hours after HT ($P < 0.05$), and $7.6 \pm 0.8\%$ at 24 hours after HT ($P < 0.05$). The percentages of apoptotic cells at 44°C in the Y79 cells were $8.2 \pm 1.1\%$ at 6 hours after HT ($P < 0.05$), $11.3 \pm 0.9\%$ at 12 hours after HT ($P < 0.05$), and $10.1 \pm 2.2\%$ at 24 hours after HT ($P < 0.05$); in the WERI-Rb-1 cells, they were $8.3 \pm 1.2\%$ at 6 hours after HT ($P < 0.05$), $13.0 \pm 1.3\%$ at 12 hours after HT ($P < 0.05$), and $11.5 \pm 0.9\%$ at 24 hours after HT ($P < 0.05$). The percentages of apoptosis at 44°C were thus greater than those at 43°C. Furthermore, the increase in apoptosis at 44°C reached a plateau at 12 hours after HT. Therefore, we concluded that the conditions most appropriate for the evaluation of HT-induced apoptosis in Y79 and WERI-Rb-1 cells were the observation at 12 hours after HT at 44°C for 1 hour.

PLK1 Knockdown Using siRNA in Y79 and WERI-Rb-1 Cells

We performed Western blotting to select the most effective siRNA for PLK1. Forty-eight hours after siRNA transfection, siPLK1-1 markedly decreased the protein level of PLK1 in Y79 and WERI-Rb-1 cells (Figs. 2A, 2B). On the other hand, the siRNA for luciferase, a negative control, did not affect PLK1

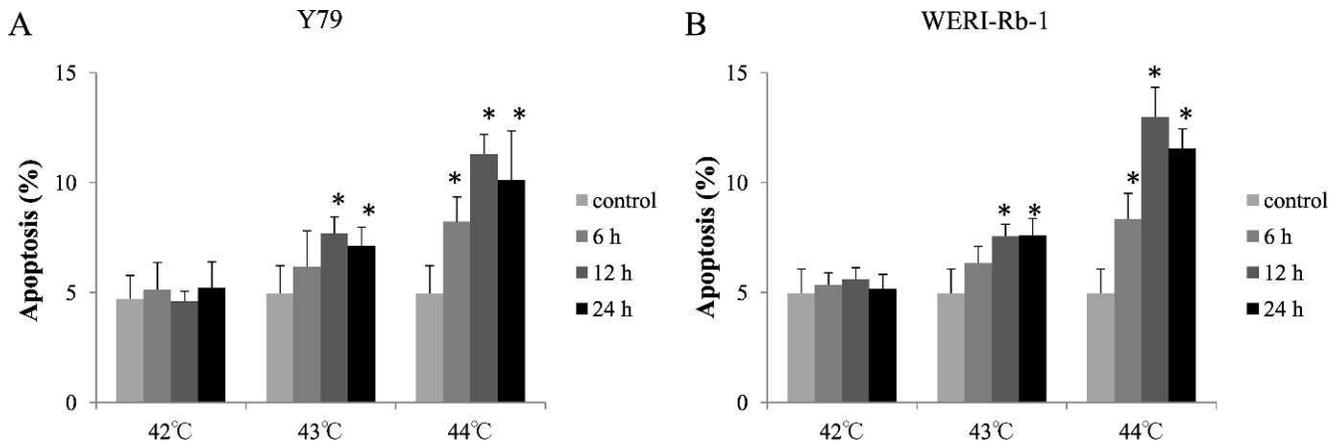


FIGURE 1. Effects of HT on apoptosis in human retinoblastoma Y79 (A) and WERI-Rb-1 (B) cells. The cells were incubated at different temperatures for 1 hour and then cultured at 37°C for 6 to 24 hours. The apoptosis was monitored using chromatin condensation. Data are presented as means ± SDs ($n = 4$). * $P < 0.05$ versus control (Student's t -test).

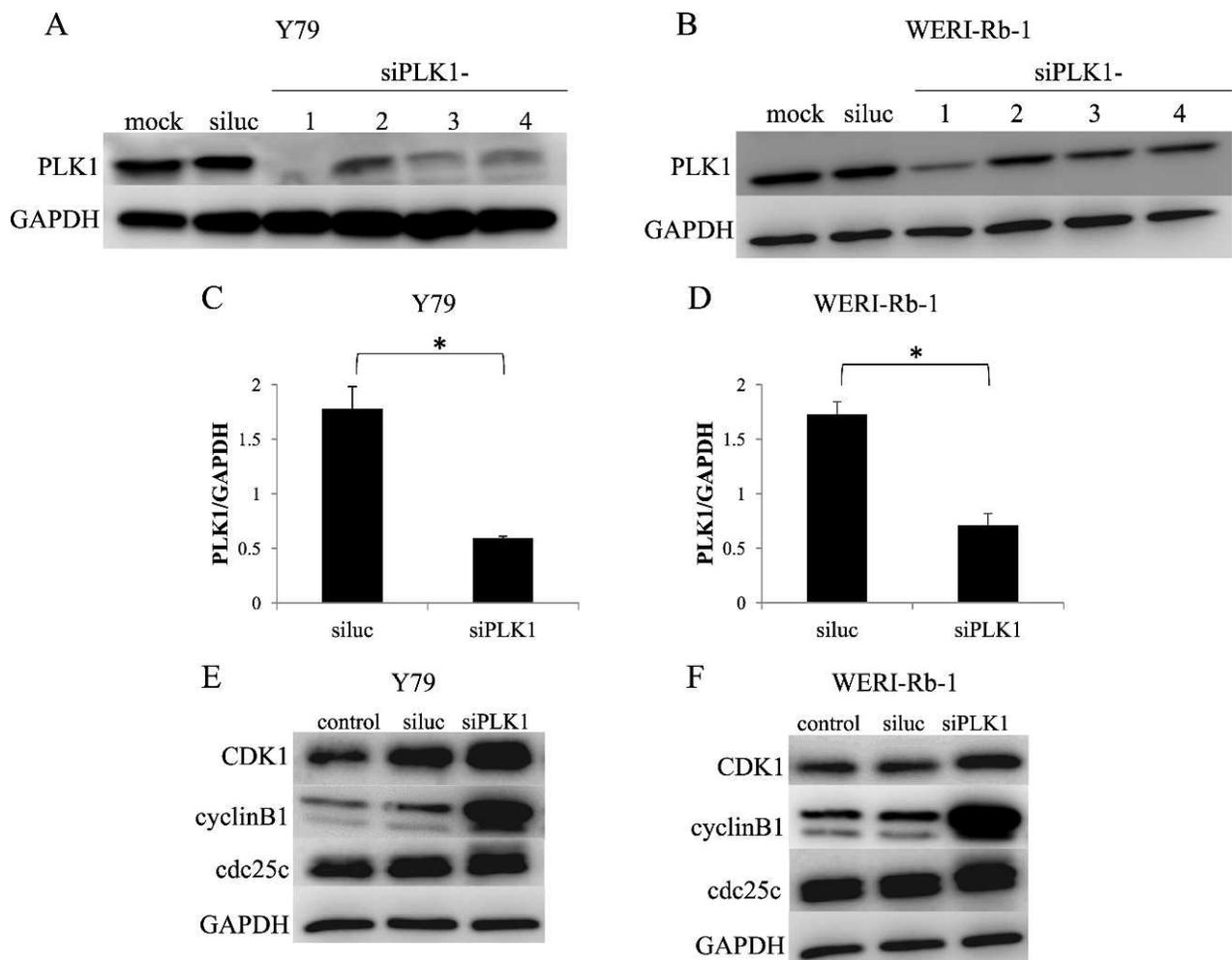


FIGURE 2. PLK1 knockdown using siRNAs in Y79 (A) and WERI-Rb-1 (B) cells. Cells transfected with each siRNA (siPLK1-1 to -4; 50 nM) were maintained at 37°C. Forty-eight hours after transfection, the cells were harvested for Western blotting. Western blotting was performed using a specific primary antibody for PLK1 or GAPDH. Glyceraldehyde 3-phosphate dehydrogenase served as a loading control. The sequences of the four siRNAs are listed in Table 1 (mock, siRNA transfection reagent only; siluc, siRNA for luciferase [50 nM]). (C, D) The effects of siRNA for PLK1 (siPLK1-1) on the mRNA expression level of PLK1 in Y79 and WERI-Rb-1 cells. Real-time quantitative PCR assay was performed with specific primers for PLK1 or GAPDH. The PLK1 mRNA level was normalized to the GAPDH expression level. Data are presented as mean ± SDs ($n = 4$). * $P < 0.05$ versus siluc (control) (Student's t -test). (E, F) Polo-like kinase 1 knockdown affects the expression of CDK1, cyclinB1, and cdc25c, which are known downstream of PLK1. Western blotting was performed using specific primary antibodies for CDK1, cyclinB1, cdc25c, and GAPDH. Glyceraldehyde 3-phosphate dehydrogenase served as a loading control.

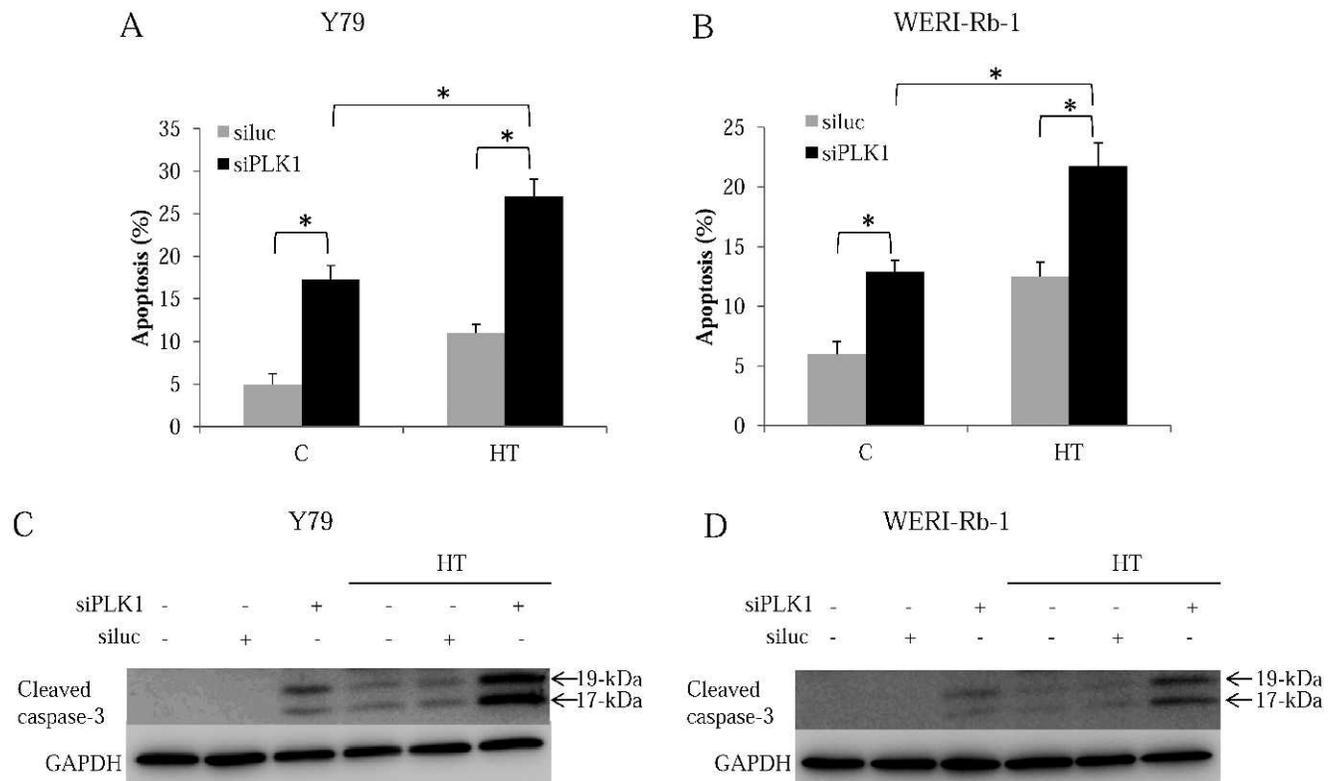


FIGURE 3. Polo-like kinase 1 knockdown induced apoptosis, and this effect was enhanced by HT. Cells transfected with siPLK1 (50 nM) were maintained at 37°C. Forty-eight hours after transfection, the cells were treated with HT at 44°C for 1 hour, and then cultured at 37°C for 12 hours. The apoptosis was monitored using chromatin condensation (A, B). Analyses were performed using flow cytometry. Data are presented as means \pm SDs ($n = 4-6$) (C, control [nontreatment]). * $P < 0.05$ (Student's *t*-test). (C, D) Western blotting was performed using a specific primary antibody for cleaved caspase-3 or GAPDH. Glyceraldehyde 3-phosphate dehydrogenase served as a loading control.

expression. Therefore, we chose the siPLK1-1 siRNA for use in the subsequent experiments. In addition, an effective knockdown of the PLK1 mRNA level was verified using the real-time qPCR assay using siPLK1-1 (Figs. 2C, 2D). We next examined whether the PLK1 knockdown would affect the expression levels of CDK1, cyclinB1, and cdc25c, which are required for cell cycle progression and are known downstream from PLK1.^{25,26} Polo-like kinase 1 knockdown induced the accumulation of CDK1, cyclinB1, and cdc25c. A similar protein expression pattern was found for the downstream targets of PLK1, such as CDK1, cyclinB1, and cdc25c (Figs. 2E, 2F). These results suggest that CDK1, cyclinB1, and cdc25c are targets of PLK1 and that PLK1 knockdown affects PLK1 downstream proteins in retinoblastoma cells, as reported previously.^{34,39}

Knockdown of PLK1-Induced Apoptosis and Its Enhancement by HT

We next examined whether PLK1 knockdown would induce apoptosis in Y79 and WERI-Rb-1 cells under the control and HT conditions. The results are shown in Figure 3. When apoptosis was monitored by chromatin condensation, the percentages of apoptosis were $4.96 \pm 1.26\%$ in the Y79 control cells transfected with the siRNA for luciferase and $5.97 \pm 1.08\%$ in the WERI-Rb-1 control cells transfected with the siRNA for luciferase. Treatment of the cells transfected with siPLK1 significantly increased the percentage of apoptosis to $17.20 \pm 1.67\%$ in the Y79 cells and to $12.90 \pm 0.94\%$ in the WERI-Rb-1 cells. Under HT-treated cells, the percentage of apoptosis was

$11.01 \pm 1.10\%$ in the Y79 cells and $12.48 \pm 1.23\%$ in the WERI-Rb-1 cells, and PLK1 knockdown enhanced HT-induced apoptosis ($27.03 \pm 2.05\%$ in the Y79 cells and $21.73 \pm 1.96\%$ in the WERI-Rb-1 cells) (Figs. 3A, 3B). We further performed Western blotting to confirm the induction of apoptosis detected by the cleavage of caspase-3 in the two retinoblastoma cell lines. Although the expression levels of the 17- and 19-kDa activated fragments of caspase-3 were very low in the control cells, significant elevations of these fragments were observed in the knockdown of PLK1 cells. Furthermore, the combination of PLK1 knockdown and HT enhanced these fragments of caspase-3 (Figs. 3C, 3D).

PLK1 Inhibition Using BI-2536 Decreased Cell Viability in a Dose-Dependent Manner: BI-2536 Induced Apoptosis, and Its Effects Were Enhanced by HT

We next analyzed the effects of BI-2536, a PLK1 inhibitor, in the two retinoblastoma cell lines. The cells were treated with BI-2536 at various concentrations, and cell survival was determined using the WST-8 assay. BI-2536 treatment decreased the viability of Y79 and WERI-Rb-1 cells after 12 and 24 hours of treatment in a dose-dependent manner (Figs. 4A, 4B). Furthermore, when apoptosis was monitored by chromatin condensation, BI-2536 (10 nM)-induced apoptosis was enhanced by HT in the two retinoblastoma cell lines, as was siRNA-mediated PLK1 inhibition (Figs. 4C, 4D).

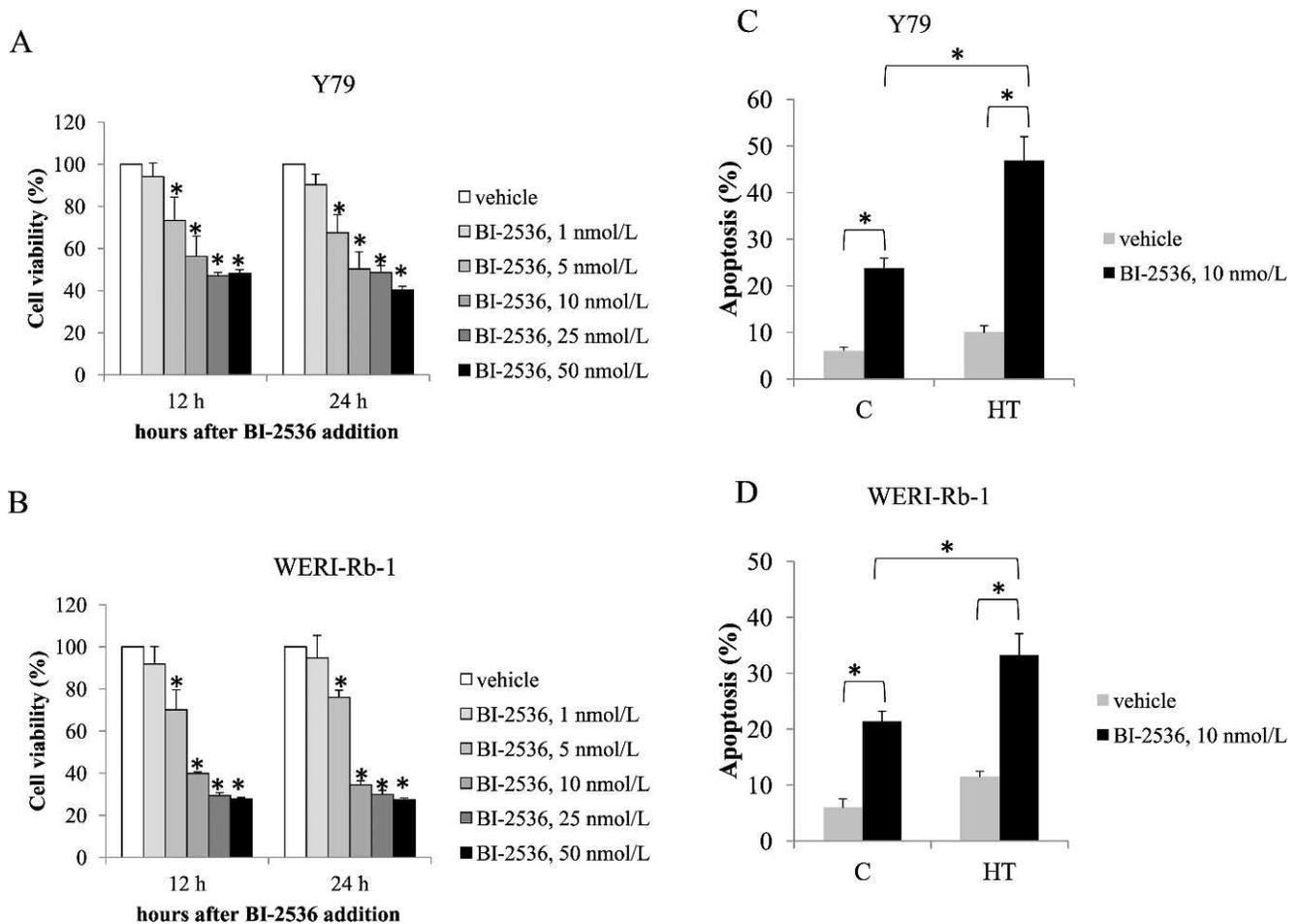


FIGURE 4. Polo-like kinase 1 inhibition using BI-2536 decreased cell viability. BI-2536 induced apoptosis, and its effect was enhanced by HT. (**A, B**) For the examination of cell viability, the Y79 and WERI-Rb-1 cells were inoculated at a density of 20×10^4 cells/mL and treated with various concentrations of a PLK1 inhibitor, BI-2536. After incubation for 12 or 24 hours, cell viability was measured by the WST-8 assay. Data are presented as means \pm SDs ($n = 4$), $*P < 0.05$ versus vehicle (DMSO only) (Student's *t*-test). (**C, D**) The Y79 and WERI-Rb-1 cells transfected with siPLK1 (50 nM) were maintained at 37°C . Forty-eight hours after transfection, cells were treated with vehicle (DMSO only) or BI-2536 (10 nM) for 1 hour, and then exposed to HT at 44°C for 1 hour. Twelve hours after HT, a chromatin condensation assay was performed using a flow cytometer. Data are presented as means \pm SDs ($n = 4$). $*P < 0.05$ (Student's *t*-test).

Effects of PLK1 Knockdown on Cell Cycle Analysis and Cell Proliferation, and Its Combined Effects With HT

We next examined whether the silencing of PLK1 would affect cell proliferation in Y79 and WERI-Rb-1 cells under the control and HT conditions. The WST-8 assay was used to count the viable cells in the cell proliferation assay. The results are shown in Figures 5A and 5B. Polo-like kinase 1 knockdown inhibited the viable cell proliferation of Y79 and WERI-Rb-1 cells compared with the control cells. Furthermore, the inhibition of viable cell proliferation was enhanced by the combination of PLK1 knockdown and HT. We next performed cell cycle analysis using flow cytometry. The results are shown in Figures 5C through 5H. Treatment of the cells transfected with siPLK1 increased the percentages of G2/M and sub-G1 phases significantly, under both the control and HT conditions in the two retinoblastoma cell lines. Although the combination of PLK1 knockdown and HT did not enhance G2/M arrest in comparison with the PLK1 knockdown alone, it did significantly increase the percentage of cells in the sub-G1 phase, a marker for apoptosis in the two retinoblastoma cell lines.

PLK1 Knockdown Inhibits the HT-Induced Activity of HSF1

We examined whether the silencing of PLK1 would affect the expression of HSF1 using real-time qPCR. There were no significant differences in the HSF1 mRNA expression levels after siRNA transfection for PLK1 under the control and HT conditions in Y79 and WERI-Rb-1 cells (Figs. 6A, 6B). Next, we performed Western blotting to monitor the activity of HSF1 in Y79 cells. Although the expression level of phosphorylated HSF1 (p-HSF1) was low under the nonstress conditions, it significantly increased in Y79 cells treated with HT at 44°C for 1 hour. The peak expression of p-HSF1 was observed from 1 to 3 hours after the HT treatment. On the other hand, the treatment of the cells with siRNA for PLK1 strongly suppressed the phosphorylation of HSF1 under either the non-HT- or HT-induced condition (Fig. 6C). We next investigated the role of PLK1 in HSF1 nuclear translocation in Y79 cells. Under the nonstress condition, expression of HSF1 was observed predominantly in the cytosolic fraction with or without knockdown of PLK1. Under the HT-induced condition, the expression of HSF1 showed an increased localization in the nucleus, but HSF1 nuclear translocation was decreased by the

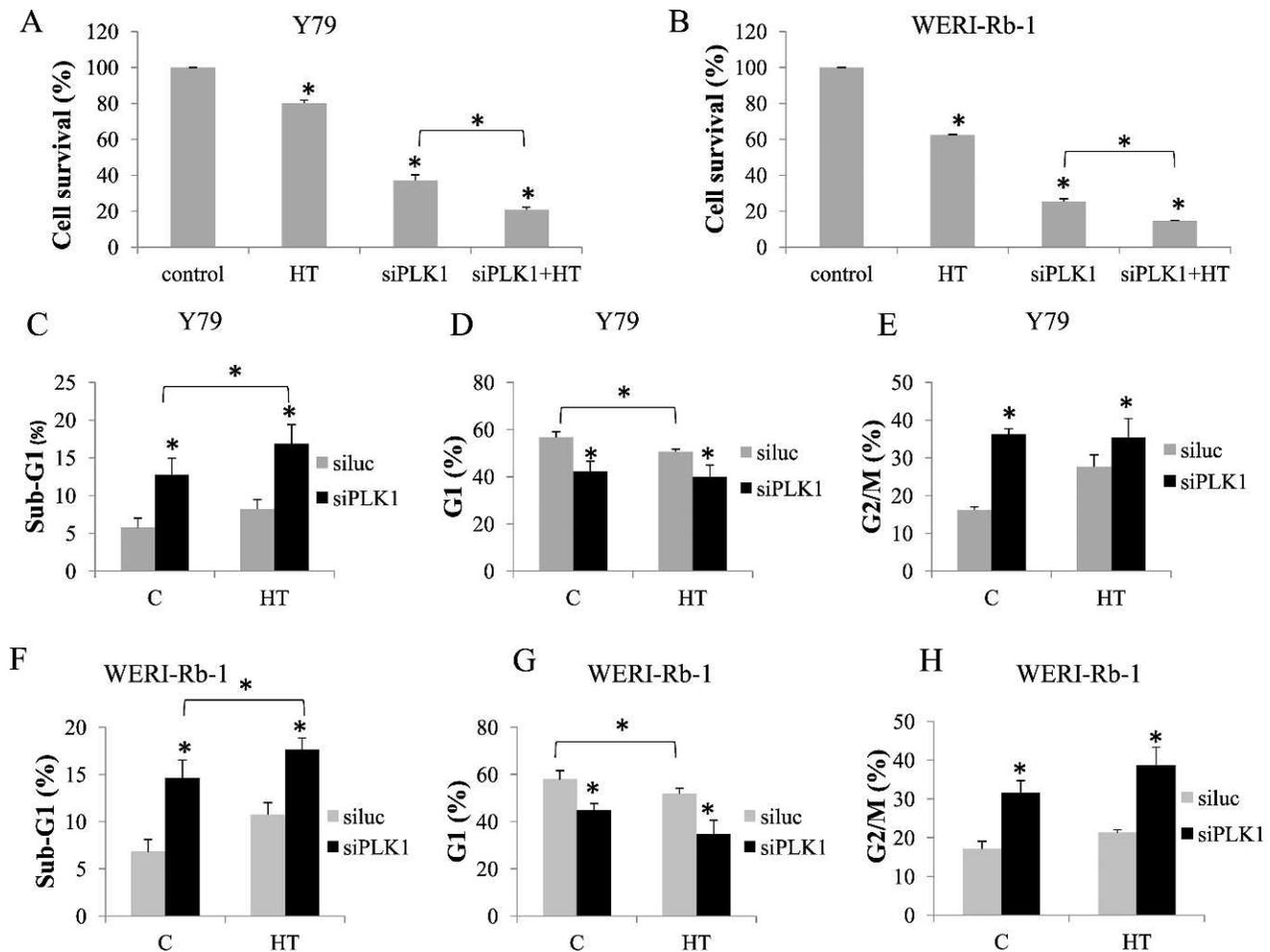


FIGURE 5. Effects of PLK1 knockdown on cell proliferation and cell cycle analysis, and the combined effects of PLK1 knockdown and HT. The Y79 and WERI-Rb-1 cells were transfected with siRNA for PLK1 (50 nM). Forty-eight hours after transfection, cells were treated with HT at 44°C for 1 hour, and then cultured at 37°C for 24 hours. (A, B) The WST-8 assay was used to count for the viable cells in the cell proliferation assay. Data are presented as the mean \pm SD ($n = 6$). * $P < 0.05$ (Student's *t*-test). (C–H) Y79 and WERI-Rb-1 cells were harvested and fixed after 24 hours of HT treatment. The percentages of cells in the different cell cycle phases were detected using flow cytometry. Data are presented as mean \pm SD ($n = 4$ –6). * $P < 0.05$ (Student's *t*-test).

silencing of PLK1 (Fig. 6D). To confirm the effect of PLK1 knockdown on the activity of HSF1, we performed immunocytochemistry in Y79 cells. The cells were transfected with siRNA for PLK1, HT was performed at 44°C for 1 hour, and the cells were cultured at 37°C for 1 hour. Under the nonstress condition, almost no formation of nuclear HSF1 granules was observed. On the other hand, under the HT-induced condition, the changes of HSF1 granules in the nucleus increased. However, the HT-induced nuclear granules of HSF1 were decreased by PLK1 knockdown (Fig. 6E).

PLK1 Knockdown Inhibits HT-Induced Proteins Such as HSPs and BAG3

We next investigated whether the HT-induced expression of HSPs and BAG3 would be affected by PLK1 knockdown in Y79 cells and WERI-Rb-1 cells (Fig. 7). The cells were transfected with siRNA for PLK1, HT was performed at 44°C for 1 hour, and the cells were cultured at 37°C for 6 hours. Although HT induced the expression of HSP70, HSP40, and BAG3 in control cells and cells transfected with siRNA for luciferase, PLK1 knockdown remarkably inhibited the HT-induced expression of those HSPs and BAG3 in the two retinoblastoma cell lines.

These results suggest that PLK1 plays an important role in the induction of HSPs and BAG3. However, PLK1 knockdown did not affect the expression of HSP27.

DISCUSSION

Hyperthermia has been considered a possible modality of cancer treatment. Hyperthermia in combination with chemotherapy and/or radiotherapy has been used for various types of cancer, and the anticancer effects of these combinations have been verified by clinical trials.^{10–12} However, one of the problems with HT therapy is that cells acquire thermoresistance.^{20,21} Heat shock proteins, molecular chaperones with cytoprotective functions, are induced by various stresses, including heat stress. It has been considered that HSPs play a role in the acquisition of thermoresistance,^{20,21} and the expression of HSPs is mainly regulated by HSF1.²² In the current study, we focused on PLK1 because there have been several reports that PLK1 regulates the activity of HSF1.^{23,24} PLK1 also acts as an important regulator of mitosis,²⁵ and several studies have shown that the expression of PLK1 is elevated in various types of cancer cells.^{27–29} Further, it has

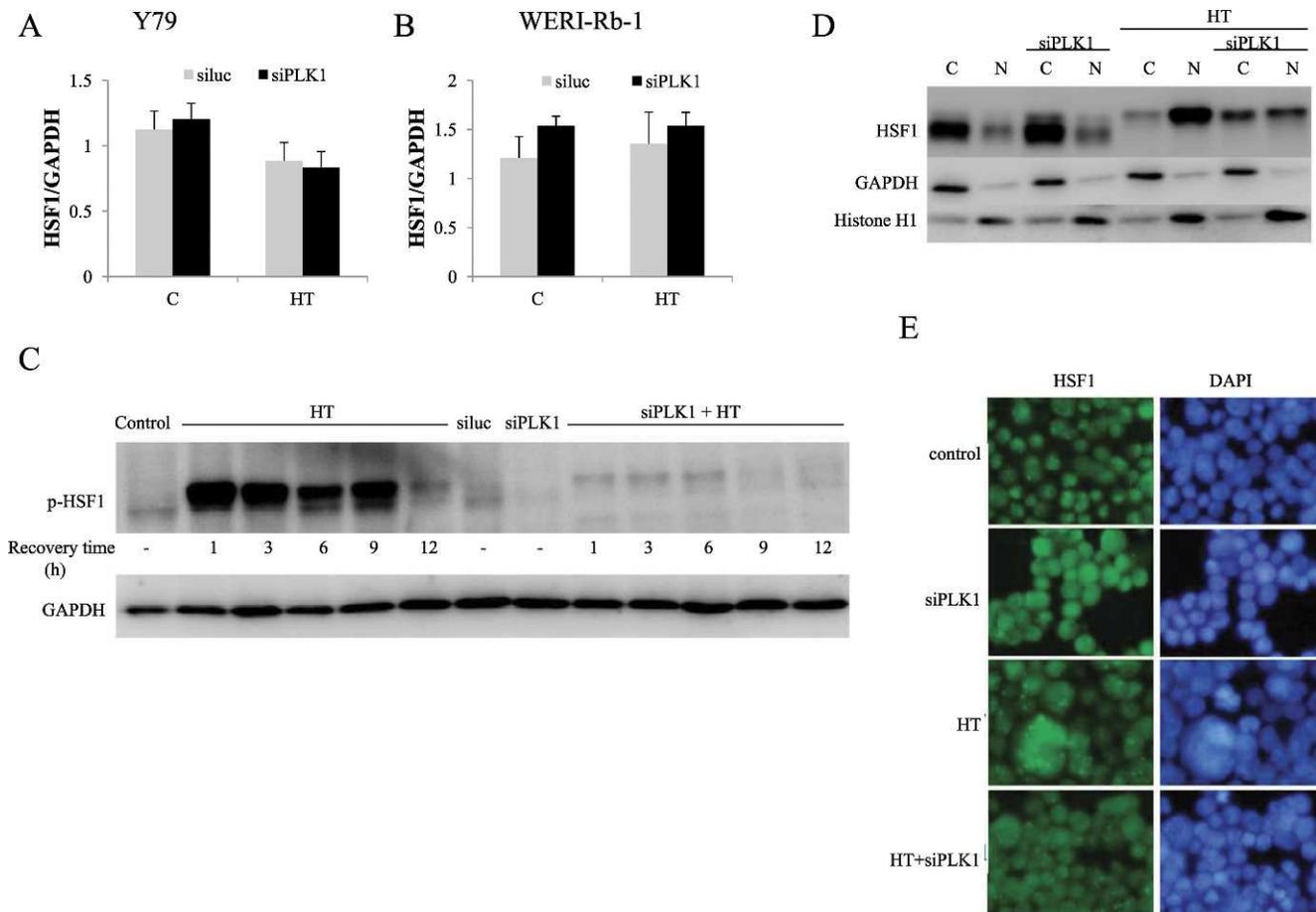


FIGURE 6. PLK1 knockdown inhibits the HT-induced activity of HSF1. Cells were transfected with siRNA for PLK1 (50 nM) and then HT was performed at 44°C for 1 hour and then cultured at 37°C. (A, B) Total RNA was extracted from cells at 3 hours after HT treatment. The effects of siRNA for PLK1 on the HSF1 mRNA expression level were investigated under the control and HT conditions in Y79 and WERI-Rb-1 cells. Real-time quantitative PCR assay was performed with specific primers for HSF1 or GAPDH. The HSF1 mRNA level was normalized to GAPDH expression level. Data are presented as mean ± SDs (*n* = 4). (C) After HT treatment, the Y79 cells were cultured at 37°C for 1, 3, 6, 9 or 12 hours. Then, the time course of changes in the expression of p-HSF1 was examined by Western blotting. Western blotting was performed using a specific primary antibody for p-HSF1 or GAPDH. Glyceraldehyde 3-phosphate dehydrogenase served as a loading control. (D) The nuclei and cytoplasm were separated by using a Nuclear/Cytosol Fractionation Kit at 1 hour after HT treatment in Y79 cells. The localization of HSF1 was analyzed by Western blotting. Western blotting was performed using specific primary antibodies for HSF1, GAPDH, and Histone H1. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control of cytoplasm and Histone H1 was used as a loading control of nuclei. (E) Immunocytochemistry was performed at 1 hour after HT treatment in order to confirm the activity of HSF1 in Y79 cells. A specific primary antibody for HSF1 was used, and nuclei were stained with DAPI. C, cytosolic fractions. N, nuclear fractions.

been demonstrated that inhibition of PLK1 promotes the sensitivity of cancer cells to chemotherapy or radiotherapy,³²⁻³⁴ and clinical trials employing PLK1 inhibition have been performed for multiple tumors.³⁵ However, there have been no reports of the use of PLK1 to enhance HT sensitivity in retinoblastoma. In this study, we demonstrated for the first time that the combination of inhibition of PLK1 and HT enhanced apoptosis and inhibited cell proliferation via the inactivation of HSF1 in human retinoblastoma cells. In addition, siRNA-mediated PLK1 knockdown and PLK1 inhibitor-mediated PLK1 inhibition promoted HT sensitivity similarly.

Previous reports have shown that overexpression of HSF1 was observed in human cancer cells of various origins.⁴⁰⁻⁴² Furthermore, the HSF1-induced overexpression of HSPs, such as HSP27, HSP70, and HSP90, has been reported in various cancers, and it has been demonstrated that these overexpressions are related to the development of cancer, invasiveness, metastasis, resistance to chemotherapy, and radiotherapy.⁴³⁻⁴⁵ Several authors have reported that inhibition of HSF1 enhances

the effect of HT treatment concomitant with inhibition of HSPs.⁴⁶⁻⁴⁸ With respect to human retinoblastoma, Kase et al.⁴⁵ and Jiang et al.⁴⁹ demonstrated that HSP27, HSP70, and HSP90 were all significantly expressed in patients with this cancer, and these HSPs were related to cell proliferation and resistance to chemotherapy. In addition, several clinical reports demonstrated that HT is effective as a focal treatment, but these effects were limited to small tumor retinoblastoma tumors.^{15,16} Accordingly, we consider that the targeting of HSF1 and HSPs is a good approach to HT treatment in retinoblastoma. However, in this study we targeted PLK1, not HSF1, in order to enhance HT sensitivity, for the following reasons. First, PLK1 inhibition does not have many influences on normal cells even though it has strong anticancer activity.³¹⁻³³ Second, PLK1 inhibitor has been used already in clinical trials.³⁵ Third, the potential of PLK1 as a therapeutic target for HT has not been reported.

In the current study, the silencing of PLK1 inhibited HT-induced phosphorylation of HSF1, nuclear translocation of HSF1, and the formation of nuclear HSF1 granules. Previous reports have shown that phosphorylation of HSF1 and HSF1

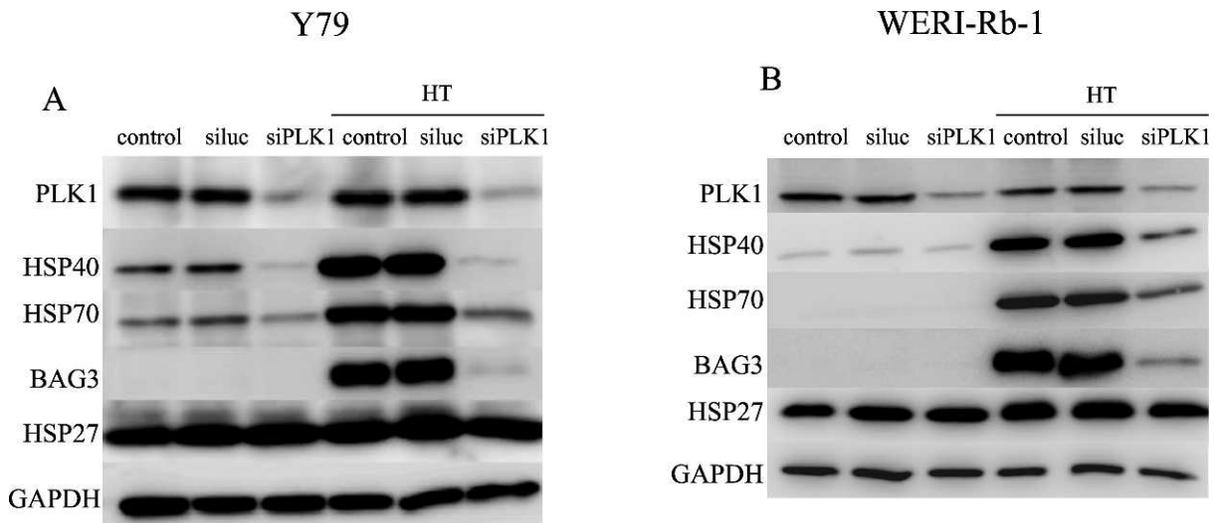


FIGURE 7. PLK1 knockdown inhibits HT-induced proteins such as HSPs and BAG3 in Y79 (A) and WERI-Rb-1 (B) cells. Cells were transfected with siRNA for PLK1 (50 nM) and subjected to HT at 44°C for 1 hour. Then, the cells were cultured at 37°C for 6 hours and harvested for Western blotting. Western blotting was performed using specific primary antibodies for PLK1, HSP70, HSP40, HSP27, BAG3, and GAPDH. Glyceraldehyde 3-phosphate dehydrogenase served as a loading control.

nuclear translocation are regulated by PLK1.^{23,24} Kim et al.⁵⁰ showed that inhibition of PLK1 suppresses the expression level of HSF1 after heat shock. Moreover, Holmberg et al.⁵¹ showed that granule formation of HSF1 is required for some of the molecular mechanisms underlying the inducible phosphorylation and transcriptional activation of HSF1. In keeping with the previous reports, we here observed that PLK1 modulated activation of HSF1. Therefore, an inhibition of PLK1 may produce a synergistic effect in HT through an inactivation of HSF1. Furthermore, we think that an enhancement of HT sensitivity may be involved in the inhibition of HT-induced HSPs, especially HSP70. Chen et al.⁵² reported that PLK1 mediated the phosphorylation of HSP70 during mitosis. Inhibition of HSP70, a main player in thermoresistance, may be suppressed effectively by the inhibition of PLK1 and resulting inactivation of HSF1. However, HSP27 was not changed significantly by PLK1 knockdown. Additional work will be needed in regard to HSP27 and other HSPs. In addition, the silencing of PLK1 strongly inhibited the HT-induced expression of BAG3. Bcl-2-associated athanogene 3 is a family of cochaperones that interact with the adenosine triphosphatase (ATPase) domain of HSP70 through the BAG domain.⁵³ The expression level of BAG3 is increased in response to various stresses, including heat stress.^{54,55} The induction of BAG3 is at least partly mediated by HSF1.⁵⁶ We recently reported that the silencing of BAG3 enhanced HT sensitivity in human oral squamous cell carcinoma.⁵⁷ In the present work, the enhancement of HT sensitivity by PLK1 knockdown may have been related to the inhibition of BAG3. At present, the detailed molecular mechanisms underlying the relationship between PLK1 and thermoresistance are not well known. Further investigations will be needed to clarify this issue. Furthermore, there are several limitations to this study. HT under in vitro experimental conditions ensures a more homogeneous heating than under clinical conditions. Additionally, HSPs are thought to be involved in the protection of retinoblastoma cells against heat damage. On the other hand, HSPs have antitumor immunity.^{58,59} These opposite immunogenic and antiapoptotic effects of HSPs may affect the therapeutic effects of this combination therapy. Accordingly, additional studies, including in vivo experiments, are needed to

clarify the effect of combination therapy with HT and PLK1 inhibition.

In conclusion, the inhibition of PLK1 may attenuate the thermoresistance of HT through an inactivation of HSF1 concomitant with reductions in HSPs and BAG3. The combination of PLK1 inhibition and HT may become an option for HT therapy in patients with retinoblastoma.

Acknowledgments

Supported in part by a Grant-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

Disclosure: **T. Yunoki**, None; **Y. Tabuchi**, None; **A. Hayashi**, None; **T. Kondo**, None

References

- MacCarthy A, Draper GJ, Steliarova-Foucher E, Kingston JE. Retinoblastoma incidence and survival in European children (1978–1997). Report from the Automated Childhood Cancer Information System project. *Eur J Cancer*. 2006;42:2092–2102.
- Chen Z, Yang A, Xu C, Xing Y, Gong W, Li J. c-Jun N-terminal kinase is involved in the regulation of proliferation and apoptosis by integrin-linked kinase in human retinoblastoma cells. *Graefes Arch Clin Exp Ophthalmol*. 2011;249:1399–1407.
- Lillington DM, Kingston JE, Coen PG, et al. Comparative genomic hybridization of 49 primary retinoblastoma tumors identifies chromosomal regions associated with histopathology, progression, and patient outcome. *Genes Chromosomes Cancer*. 2003;36:121–128.
- Classon M, Harlow E. The retinoblastoma tumour suppressor in development and cancer. *Nat Rev Cancer*. 2002;2:910–917.
- Lin P, O'Brien JM. Frontiers in the management of retinoblastoma. *Am J Ophthalmol*. 2009;148:192–198.
- Kleinerman RA, Tucker MA, Tarone RE, et al. Risk of new cancers after radiotherapy in long-term survivors of retinoblastoma: an extended follow-up. *J Clin Oncol*. 2005;23:2272–2279.

7. Murphree AL, Villablanca JG, Deegan WF III, et al. Chemotherapy plus local treatment in the management of intraocular retinoblastoma. *Arch Ophthalmol*. 1996;114:1348-1356.
8. Shields CL, De Potter P, Himelstein BP, Shields JA, Meadows AT, Maris JM. Chemoreduction in the initial management of intraocular retinoblastoma. *Arch Ophthalmol*. 1996;114:1330-1338.
9. Shields CL, Shields JA, Needle M, et al. Combined chemoreduction and adjuvant treatment for intraocular retinoblastoma. *Ophthalmology*. 1997;104:2101-2111.
10. Xia H, Karasawa K, Hanyu N, et al. Hyperthermia combined with intra-thoracic chemotherapy and radiotherapy for malignant pleural mesothelioma. *Int J Hyperthermia*. 2006;22:613-621.
11. Aktas M, de Jong D, Nuyttens JJ, et al. Concomitant radiotherapy and hyperthermia for primary carcinoma of the vagina: a cohort study. *Eur J Obstet Gynecol Reprod Biol*. 2007;133:100-104.
12. Van Der Zee J, De Bruijne M, Mens JW, et al. Radiation combined with hyperthermia in breast cancer recurrences: overview of experience in Erasmus MC. *Int J Hyperthermia*. 2010;26:638-648.
13. Choi EK, Park SR, Lee JH, et al. Induction of apoptosis by carboplatin and hyperthermia alone or combined in WERI human retinoblastoma cells. *Int J Hyperthermia*. 2003;19:431-443.
14. Inomata M, Kaneko A, Kunimoto T, Saijo N. In vitro thermo- and thermochemo-sensitivity of retinoblastoma cells from surgical specimens. *Int J Hyperthermia*. 2002;18:50-61.
15. Shields CL, Santos MC, Diniz W, et al. Thermotherapy for retinoblastoma. *Arch Ophthalmol*. 1999;117:885-893.
16. Abramson DH, Scheffler AC. Transpupillary thermotherapy as initial treatment for small intraocular retinoblastoma: technique and predictors of success. *Ophthalmology*. 2004;111:984-991.
17. Francis JH, Abramson DH, Brodie SE, Marr BP. Indocyanine green enhanced transpupillary thermotherapy in combination with ophthalmic artery chemosurgery for retinoblastoma. *Br J Ophthalmol*. 2013;97:164-168.
18. Suzuki S, Kaneko A. Management of intraocular retinoblastoma and ocular prognosis. *Int J Clin Oncol*. 2004;9:1-6.
19. Liggett PE, Lavaque AJ, Chaudhry NA, Jablon EP, Quiroz-Mercado H. Preliminary results of combined simultaneous transpupillary thermotherapy and ICG-based photodynamic therapy for choroidal melanoma. *Ophthalmic Surg Lasers Imaging*. 2005;36:463-470.
20. Parsell DA, Lindquist S. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu Rev Genet*. 1993;27:437-496.
21. Ohtsuka K, Hata M. Molecular chaperone function of mammalian Hsp70 and Hsp40—a review. *Int J Hyperthermia*. 2000;16:231-245.
22. Morimoto RI. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev*. 1998;12:3788-3796.
23. Kim SA, Yoon JH, Lee SH, Ahn SG. Polo-like kinase 1 phosphorylates heat shock transcription factor 1 and mediates its nuclear translocation during heat stress. *J Biol Chem*. 2005;280:12653-12657.
24. Lee YJ, Kim EH, Lee JS, et al. HSF1 as a mitotic regulator: phosphorylation of HSF1 by Plk1 is essential for mitotic progression. *Cancer Res*. 2008;68:7550-7560.
25. Roshak AK, Capper EA, Imburgia C, Fornwald J, Scott G, Marshall LA. The human polo-like kinase, PLK, regulates cdc2/cyclin B through phosphorylation and activation of the cdc25C phosphatase. *Cell Signal*. 2000;12:405-411.
26. Schmit TL, Zhong W, Nihal M, Ahmad N. Polo-like kinase 1 (Plk1) in non-melanoma skin cancers. *Cell Cycle*. 2009;8:2697-2702.
27. He ZL, Zheng H, Lin H, Miao XY, Zhong DW. Overexpression of polo-like kinase1 predicts a poor prognosis in hepatocellular carcinoma patients. *World J Gastroenterol*. 2009;15:4177-4182.
28. Wang ZX, Xue D, Liu ZL, et al. Overexpression of polo-like kinase 1 and its clinical significance in human non-small cell lung cancer. *Int J Biochem Cell Biol*. 2012;44:200-210.
29. Ito Y, Miyoshi E, Sasaki N, et al. Polo-like kinase 1 overexpression is an early event in the progression of papillary carcinoma. *Br J Cancer*. 2004;90:414-418.
30. Guan R, Tapang P, Levenson JD, Albert D, Giranda VL, Luo Y. Small interfering RNA-mediated Polo-like kinase 1 depletion preferentially reduces the survival of p53-defective, oncogenic transformed cells and inhibits tumor growth in animals. *Cancer Res*. 2005;65:2698-2704.
31. Nogawa M, Yuasa T, Kimura S, et al. Intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer. *J Clin Invest*. 2005;115:978-985.
32. Liu X, Lei M, Erikson RL. Normal cells, but not cancer cells, survive severe Plk1 depletion. *Mol Cell Biol*. 2006;26:2093-2108.
33. Maire V, Némati F, Richardson M, et al. Polo-like kinase 1: a potential therapeutic option in combination with conventional chemotherapy for the management of patients with triple-negative breast cancer. *Cancer Res*. 2013;73:813-823.
34. Gerster K, Shi W, Ng B, et al. Targeting polo-like kinase 1 enhances radiation efficacy for head-and-neck squamous cell carcinoma. *Int J Radiat Oncol Biol Phys*. 2010;77:253-260.
35. Schöffski P, Blay JY, De Greve J, et al. Multicentric parallel phase II trial of the polo-like kinase 1 inhibitor BI 2536 in patients with advanced head and neck cancer, breast cancer, ovarian cancer, soft tissue sarcoma and melanoma. The first protocol of the European Organization for Research and Treatment of Cancer (EORTC) Network of Core Institutes (NOCI). *Eur J Cancer*. 2010;46:2206-2215.
36. Mani B, Baltzer C, Valle N, Almendral JM, Kempf C, Ros C. Low pH-dependent endosomal processing of the incoming parvovirus minute virus of mice virion leads to externalization of the VP1 N-terminal sequence (N-VP1), N-VP2 cleavage, and uncoating of the full-length genome. *J Virol*. 2006;2:1015-1024.
37. Furusawa Y, Iizumi T, Fujiwara Y, et al. Inhibition of checkpoint kinase 1 abrogates G2/M checkpoint activation and promotes apoptosis under heat stress. *Apoptosis*. 2012;17:102-112.
38. Park JB. Protective effects of veskamide, enferamide, becata-mide, and oretamide on H2O2-induced apoptosis of PC-12 cells. *Phytomedicine*. 2011;18:843-847.
39. Grinshtein N, Datti A, Fujitani M, et al. Small molecule kinase inhibitor screen identifies polo-like kinase 1 as a target for neuroblastoma tumor-initiating cells. *Cancer Res*. 2011;71:1385-1395.
40. Dudeja V, Chugh RK, Sangwan V, et al. Prosurvival role of heat shock factor 1 in the pathogenesis of pancreatobiliary tumors. *Am J Physiol Gastrointest Liver Physiol*. 2011;300:G948-G955.
41. Hoang AT, Huang J, Rudra-Ganguly N, et al. A novel association between the human heat shock transcription factor 1 (HSF1) and prostate adenocarcinoma. *Am J Pathol*. 2000;156:857-864.
42. Cen H, Zheng S, Fang YM, Tang XP, Dong Q. Induction of HSF1 expression is associated with sporadic colorectal cancer. *World J Gastroenterol*. 2004;10:3122-3126.

43. Tang D, Khaleque MA, Jones EL, et al. Expression of heat shock proteins and heat shock protein messenger ribonucleic acid in human prostate carcinoma in vitro and in tumors in vivo. *Cell Stress Chaperones*. 2005;10:46-58.
44. Ciocca DR, Vargas-Roig LM. Hsp27 as a prognostic and predictive factor in cancer. *Prog Mol Subcell Biol*. 2002;28:205-218.
45. Kase S, Parikh JG, Rao NA. Expression of heat shock protein 27 and alpha-crystallins in human retinoblastoma after chemoreduction. *Br J Ophthalmol*. 2009;93:541-544.
46. Rossi A, Ciafrè S, Balsamo M, Pierimarchi P, Santoro MG. Targeting the heat shock factor 1 by RNA interference: a potent tool to enhance hyperthermochemotherapy efficacy in cervical cancer. *Cancer Res*. 2006;66:7678-7685.
47. Tabuchi Y, Furusawa Y, Wada S, Ohtsuka K, Kondo T. Silencing heat shock transcription factor 1 using small interfering RNA enhances mild hyperthermia and hyperthermia sensitivity in human oral squamous cell carcinoma cells. *Thermal Medicine*. 2011;27:99-108.
48. Nakamura Y, Fujimoto M, Hayashida N, Takii R, Nakai A, Muto M. Silencing HSF1 by short hairpin RNA decreases cell proliferation and enhances sensitivity to hyperthermia in human melanoma cell lines. *J Dermatol Sci*. 2010;60:187-192.
49. Jiang LB, Liu XQ, Li B, et al. Heat shock proteins and survivin: relationship and effects on proliferation index of retinoblastoma cells. *Histol Histopathol*. 2008;23:827-831.
50. Kim SA, Kwon SM, Yoon JH, Ahn SG. The antitumor effect of PLK1 and HSF1 double knockdown on human oral carcinoma cells. *Int J Oncol*. 2010;36:867-872.
51. Holmberg CI, Illman SA, Kallio M, Mikhailov A, Sistonen L. Formation of nuclear HSF1 granules varies depending on stress stimuli. *Cell Stress Chaperones*. 2000;5:219-228.
52. Chen YJ, Lin YP, Chow LP, Lee TC. Proteomic identification of Hsp70 as a new Plk1 substrate in arsenic trioxide-induced mitotically arrested cells. *Proteomics*. 2011;22:4331-4345.
53. Rosati A, Ammirante M, Gentilella A, et al. Apoptosis inhibition in cancer cells: a novel molecular pathway that involves BAG3 protein. *Int J Biochem Cell Biol*. 2007;39:1337-1342.
54. Kariya A, Tabuchi Y, Yunoki T, Kondo T. Identification of common gene networks responsive to mild hyperthermia in human cancer cells. *Int J Mol Med*. 2013;32:195-202.
55. Liao Q, Ozawa F, Friess H, et al. The anti-apoptotic protein BAG-3 is overexpressed in pancreatic cancer and induced by heat stress in pancreatic cancer cell lines. *FEBS Lett*. 2001;503:151-157.
56. Franceschelli S, Rosati A, Lerosé R, De Nicola S, Turco MC, Pascale M. Bag3 gene expression is regulated by heat shock factor 1. *J Cell Physiol*. 2008;215:575-577.
57. Yunoki T, Kariya A, Kondo T, Hayashi A, Tabuchi Y. The combination of silencing BAG3 and inhibition of the JNK pathway enhances hyperthermia sensitivity in human oral squamous cell carcinoma cells. *Cancer Lett*. 2013;335:52-57.
58. Gurbuxani S, Bruey JM, Fromentin A, et al. Selective depletion of inducible HSP70 enhances immunogenicity of rat colon cancer cells. *Oncogene*. 2001;20:7478-7485.
59. Frey B, Weiss EM, Rubner Y, et al. Old and new facts about hyperthermia-induced modulations of the immune system. *Int J Hyperthermia*. 2012;28:528-542.