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박사학위 논문

두경부 편평세포암종에서 PLK1과 HSF1의 DOUBLE KNOCKDOWN에 의한 항암효과

조선대학교 대학원

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지도교수 안 상 건

이 논문을 치의학 박사학위신청 논문으로
제출함

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Table of Contents

List of Figures

ABSTRACT

Antitumor Effects of PLK1 and HSF1 Double Knockdown in Head & Neck Squamous Cell Carcinoma

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Elevated level of PLK1 was reported in many different types of cancer cells and PLK1 has been considered as a novel prognostic marker for several tumor types. Recent studies have also reported the aberrant expression of HSF1 in human cancer of various origins correlates with the development of cancer, invasiveness, and metastasis. In this study, we investigated the effect of PLK1 and HSF1 double knockdown in oral squamous cell carcinoma using the small interfering RNA technique. The high level of PLK1 and HSF1 was observed in human oral squamous cell carcinoma compared with normal tissue. Silencing of both PLK1 and HSF1 showed a synergic reduction of cell viability, induction of cell death, and loss of adhesion function compared with PLK1 single depletion. Silencing of PLK1 reduced the activity of the G2/M phase regulator Cdc2/CyclinB complex. Interestingly, decreased level of PLK1 led to the inhibition of Hsps expression via the down-regulation of HSF1. These findings provide the potential value of PLK1 and HSF1 as a target for oral cancer therapy.

Ⅰ. Introduction

The polo-like kinase 1 (PLK1) is a mammalian serine/threonine protein kinase which is an important regulator involvingmany cell signaling related events, including the regulation of entry into mitosis, centrosome maturation, bipolar spindle formation, regulation of anaphase-promotion complex/cyclosome, and signal transduction at the DNA damage checkpoint.[1-3] Recent findings suggest that the expression of PLK1 is elevated in many types of human tumors, including the head and neck squamous cell carcinomas, papillary carcinomas, prostate cancer, pancreatic cancer, breast cancer, colorectal cancer, non-small cell lung cancer, melanomas, as well as ovarian and endometrial carcinomas.[3-7] In addition, PLK1 expression has also been considered a novel prognostic marker of several tumor types, and has recently been revealed to lead to mitotic arrest, induction of apoptosis and suppression of tumor growth as a result of its inhibition in vitro and in vivo.[8-11] Hence, it was proposed that PLK1 could be considered as a target for novel cancer therapeutic approaches.

Heat Shock Factor 1 (HSF1) is a family of transcription factors remarkably conserved, from yeast to humans. HSF1 is responsible for the expression of a large class of heat shock proteins (Hsps) which serve to protect cells from the damage incurred as a result of cellular insults such as oxidative stress, hyperthermia, and anticancer drugs.[12-19] Recent studies have reported that an increase in HSF1 levels was observed in aggressively malignant prostate carcinoma cell lines as well as in human cancer tissue.[20,21] Additionally, the expression of HSPs by HSF1 is also openly dysregulated in cancer tissue and cell lines. The aberrant expression of HSP27, HSP70 and HSP90 reported in human malignant cancer of various origins correlates with the development of cancer, invasiveness, metastasis, resistance to chemotherapy, and radiation therapy.[22-25] Recently, several studies have yielded new cancer research opportunities based on stress response molecules. HSF1 disruption significantly increased in sensitivity to apoptosis induced by chemotherapy in cancer cells using siRNA-mediated silencing.[26] The disruption of the HSF1 gene in cancer may eliminate the heat shock response, abolish thermotolerance, and increase

susceptibility to anticancer drug-induced apoptosis. However, the potential inhibition of cancer through HSF1 modulation has not yet been exploited. We attempted to examine the phenotype and effects of PLK1 and HSF1 silencing in oral squamous carcinoma cells and observed the relationship between PLK1 and HSF1. We demonstrated that the loss of PLK1 function results in a synergistic increase in sensitivity to apoptosis when suppressed HSF1 together, despite affecting cancer cell viability and phenotype.

Ⅱ. Materials and Methods

1. Cell cultures

The oral squamous carcinoma cells FaDu and HEp-2 were maintained in MEM medium containing fetal bovine serum, 100U/ml penicillin-streptomycin (Invitrogen, CA, USA) and were subsequently incubated at 37 °C in an atmosphere containing 95% O_2 and 5% CO_2.

2. MTT assay

In brief, 1 × 10^5 cells/well was cultured in 12 well plates. After 24 h, the siRNA and liposome was added to the FaDu and HEp-2 cell lines, followed by adding MTT (5 g/L) at 24 h and 48 h after transfection. The cells were incubated for 4 h followed by the removal of the medium and addition of 100 μL of DMSO for 5 min. The absorbance of the reaction solution was measured at 570 nm and all experiments were performed in triplicate.

3. Immunohistochemistry

Excised human oral squamous carcinoma was fixed in 10% buffered formalin and embedded in paraffin. Next, immunohistochemical staining was performed with the avidin-biotin complex method using the anti-PLK1 and HSF1 antibodies. Lastly, the immune reactions were visualized with 3,3′-diaminobenzidine and counterstained with Mayer's hematoxylin.

4. PLK1HSF1 silencing

The Ambion software was used to design RNAi sequences targeting human PLK1 (Accession no. NM 005030) and HSF1 (Accession no. XM128055). PLK1 RNAi oligomer was purchased from Ambion Inc. (Austin TX, USA). In RNA interference studies using siRNA-based plasmid constructs (IMGENEX Inc, San Diego, USA), cells were grown to 50% confluency in 6-well plates and subsequently transfected along with pSuppressor-HSF1 (3 μg), using the Lipofectamine 2000 reagent. After a 48 h transfection period, the cells were used in an immunoblot assay.

5. Western blotting

The cells were lysed in lysis buffer the protein concentrations were measured with the BCA protein assay kit (Pierce, Rockford, USA). The total protein (50 μg) was resolved by 12% SDS-PAGE and transferred onto PVDF membranes. After blocking in TBST (20 mmol/L Tris, 137 mmol/L NaCl, 1 g/L Tween 20, pH 7.6) with 5% skim milk, for 2 h at room temperature, the membranes were incubated with the PLK1, CDKs, and HSF1 primary antibodies (diluted 1:1000; Santa Cruz Biotechnology, USA) for 2 h. Next, the membranes were washed three times with a TBST solution, followed by incubation for 1 h with HRP-linked secondary antibodies (1:1000; Santa Cruz Biotechnology) at room temperature. Finally, the membranes were visualized using the ECL detection reagent.

6. Kinase analysis

The kinase activity of PLK1 and Cdc2/cyclin B was measured using CycLex PLK1 and CycLex Cdc2/cyclin B kinase assay kits (CycLex Co., Ltd., Nagano, Japan) according to the manufacturer's instructions. The absorbance was measured in cell lysates usinga microculture plate reader at 450nm. The results represented the amount of phosphorylated substrate.

7. Cell death detection assay

Cell death was quantified using a Cell Death Detection ELISA plus Assay Kit (Roche, Germany) according to the manufacturer's instructions. In brief, cells were harvested, washed in PBS, and labeled with anti-DNA-POD as well as anti-histone-biotin according to the manufacturer's protocol. The absorbance was measured with a microculture plate reader at 405nm and represents the amount of DNA and histone. In addition, the cell morphology of the experimental and control groups were compared under an inverted microscope.

Ⅲ. Results and Discussion

Oral cancer is one of the fastest growing malignancies and particularly dangerous because it has a high risk of producing second tumors.[28] There are several types of oral cancers; however, 90% are squamous cell carcinomas. Histologically, oral SCC tissues are anaplastic carcinomas showing many irregular, roughened, or verrucous areas of mucosal thickening and malignant epithelial cell islands.[29] The primary goal of the present study was to provide new avenues for the exploration of therapies, based on the molecules and genes involved in the process of oral cancer, using interfering RNA technology.

PLK1 plays an important role in cellular mitotic events and is closely associated with tumor proliferation.[1-8] On the other hand, HSF1 has a broader role in cellular signaling beyond its well-known role in stress response and its association with the development of cancer.[21,26,27] By means of investigating PLK1 and HSF1 expression of the human oral SCC tissues, we performed an immunohistochemistry assay in five oral SCC tissues. As shown in Fig1, this result is consistent with the high levels of PLK1 observed in head and neck squamous cell cancer (HNSCC) mentioned previously, whereas we had first determined that HSF1 is over-expressed in oral SCC.

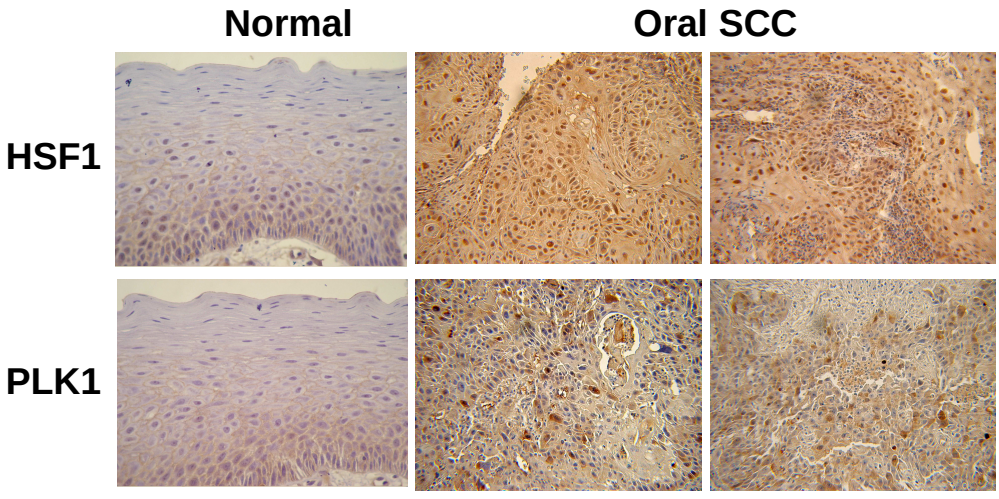
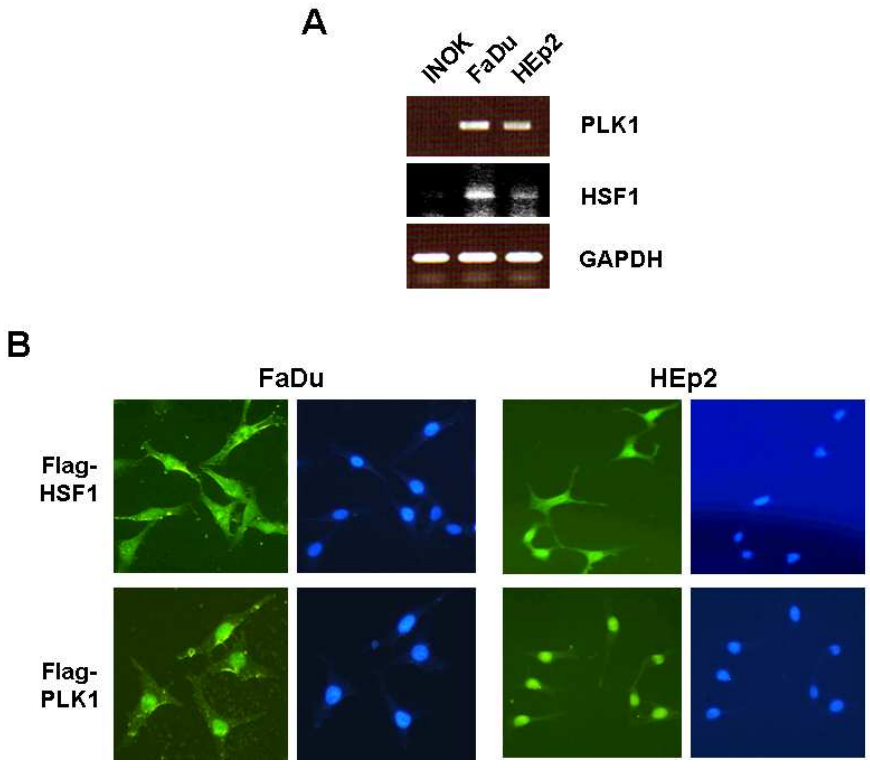


Fig. 1. The PLK1 and HSF1 immunohistochemistry of paraffin-embedded human oral squamous cell carcinomas.

In addition, we also tested human oral SCC cell lines for expression of PLK1 and HSF1 by RT-PCR analysis and found that the oral carcinoma cell lines, FaDu and HEp-2 overexpressed the PLK1 and HSF1 compared with immortalized oral INOK cells (Fig.2A).

To evaluate the subcellular localization of PLK1 and HSF1, we performed an indirect immunofluorescence staining assay. This was achieved by transfecting the FaDu and HEp-2 cells with the empty plasmid or the expression plasmid for Flag tagged PLK1 or HSF1, and followed by fractionation into cytoplasmic and nuclear fractions for assessment of the kinase activity in PLK1. For the immunofluorescent staining, the FaDu and HEp-2 cells, which expressed Flag-PLK1 or Flag-HSF1, were fixed and analyzed with monoclonal anti-Flag antibody. Consistent with previous observations, HSF1 was detected in the cytoplasm and nucleus for both cells (Fig. 2B, upper panel). However, PLK1 was primarily localized in the nucleus (Fig. 2B, lower panels). As shown in Fig. 2C, the PLK1 activity was also increased in the nucleus in relation to the cytoplasm for both cells. These results suggest that PLK1 may regulate the function of cell cycle proteins or transcription factors such as HSF1 in response to stress.



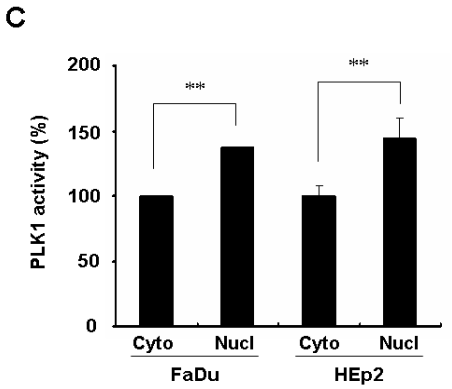


Fig. 2. The expression and localization of PLK1 and HSF1 in oral cancer cells. (A) Total RNA (5 μg) prepared from INOK, FaDU and HEp-2cells was subjected to RT-PCR analysis for PLK1 and HSF1 mRNA expression using the specific primers. The PCR-amplified products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Amplification of GAPDH was used as an internal control. (B) FaDU and HEp-2cells were transiently transfected with FLAG-PLK1 and FLAG-HSF1 expression plasmid. Following transfection, the cells were fixed and incubated with monoclonal anti-FLAG antibodies, which were revealed by fluorescein FITC-conjugated anti-mouse IgG (green). (C) The kinase activity of PLK1 was measured using a polo-like kinase 1 ELISA assay as shown explained in the "Experimental Procedures" section.

To specifically deplete PLK1 and HSF1 in oral cancer cells, we employed the recently developed siRNA technology which targets a sequence in human PLK1 and HSF1. For the generation of gene disruption, pSuppressor-HSF1 and oligomer-PLK1 were transfected into cells, and cultured for 48 h. As shown in Fig. 3A and B, the level of PLK1 and HSF1 protein was reduced by at least 40-60% after 48 h of transfection, suggesting that the vector- and oligonucleotide-based siRNA approach can efficiently deplete HSF1 and PLK1 in oral cancer cell lines. In HEp2 cells, we also showed that the down-regulation of PLK1 by siRNA was conformed by immunostaining (Fig. 3C). Similar results were observed in FaDu cells (data not shown). To validate the specificity of siRNA in cells, we examined the PLK1 activity. A kinase activity analysis indicated that 50% or 70% of PLK1 suppression activity was inhibited in both cells respectively (Fig.3B).

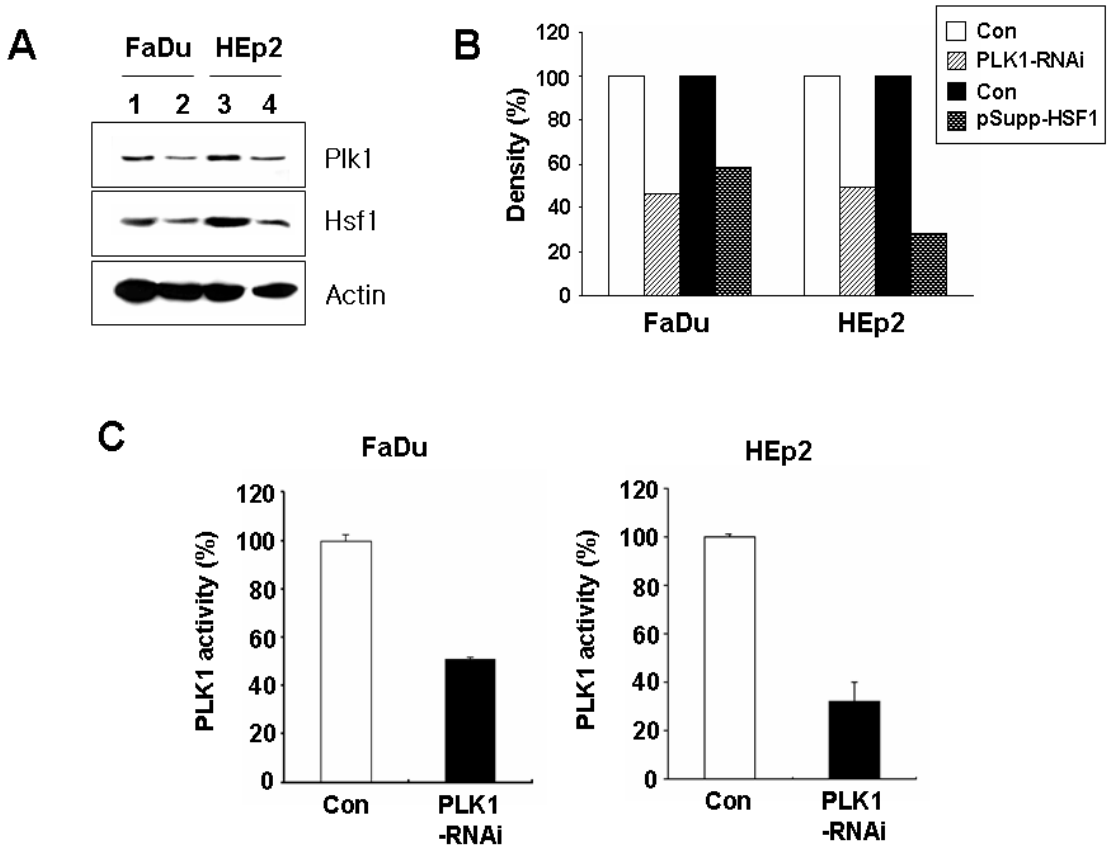

A
FaDu
HEp2
1
2
3
4
Plk1
Hsf1
Actin
B
Density (%)
120
100
80
60
40
20
0
FaDu
HEp2
Con
PLK1-RNAi
Con
pSupp-HSF1
C
FaDu
HEp2
PLK1 activity (%)
120
100
80
60
40
20
0
Con
PLK1
-RNAi
D
PLK1
DAPI
Control
RNAi

Fig. 3. The inhibition of PLK1 activity and HSF1 expression by siRNA. (A) Protein depletion was determined by Western blot analysis of the cell lysates with an antibody specific for the PLK1 and HSF1. line 1, 3: Control, line 2,4: PLK1-RNAi and pSuppressor-HSF1. (B) Twenty-four hours after transfection with PLK1-siRNA oligomer, the cell lysates were prepared and the PLK1 activity was measured at 450nm using ELISA. (C) HEp2 cells transfected with PLK1 specific siRNA were photographed on fluorescence microscopy.

We first determined whether PLK1 and HSF1 depletion influences the cell viability of FaDu and HEp-3 cells using the MTT assay. The transfection with the pSuppressor-HSF1 showed a slight inhibition of the cell growth rate. On the other hand, the transfection with PLK1-siRNA strongly inhibited the cell growth and viability (Fig. 4). In addition, we examined the viability of both PLK1 and HSF1-depleted cells. The cells that were co-transfected with pSuppressor-HSF1 and PLK1 siRNA oligomer resulted in a synergistically significant reduction in cell viability in both cells. As shown in Fig. 4C, the transfection of the control and pSuppressor-HSF1 showed no significant influence on cell morphology, whereas PLK1 depletion dramatically changed the cell morphology as well as a loss in adhesion function. Moreover, the PLK1- and HSF1-depleted cells also resulted in a greater change in phenotype than the PLK1-depleted cells alone, in addition to the fact that most cells were undergoing cell death.

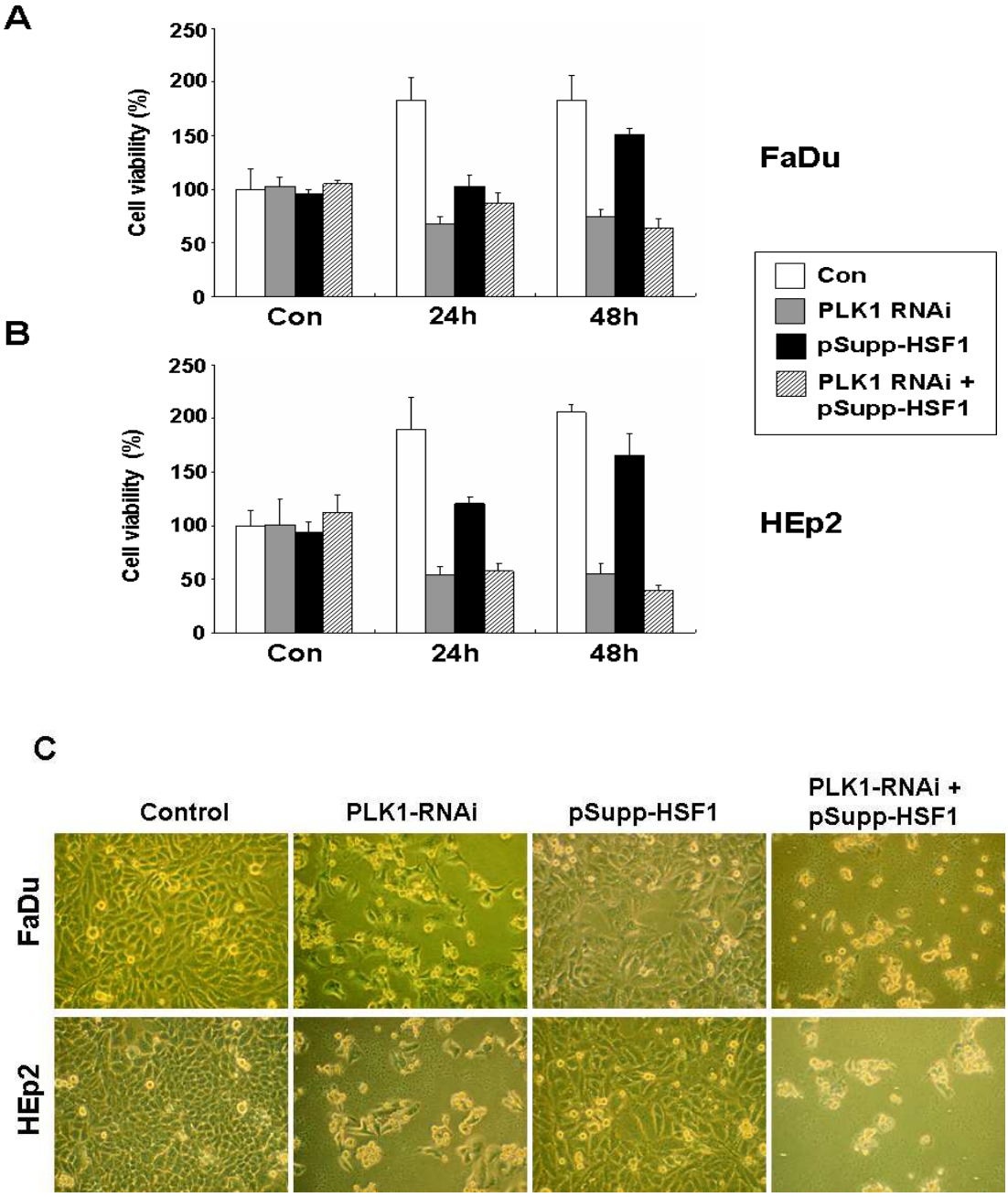


Fig. 4. The effects of PLK1 and HSF1 siRNA transfection on growth of cancer cells. The effects of PLK1 and HSF1 depletion on cell viability and growth was measured using theMTT analysis. The cells were transfected with PLK1 or HSF1 siRNA alone or with PLK1 and HSF1 double siRNA. (A) FaDu cells. (B) HEp-2cells. (C) PLK1 or PLK1 and HSF1 depletion caused changes in cell morphology and induced cell death in both cells.

To confirm the above observation, we analyzed the effect of PLK1 and HSF1 depletion on cell death using a cell death detection ELISA assay. The FaDu and HEp-2 cells were transiently co-transfected with pSuppressor-HSF1 and PLK1 siRNA for 24 h. As shown in Fig. 5, the depletion of PLK1 resulted in cell death for a number of cells compared to the control transfection, whereas HSF1 depletion had little effect on cell death. We also examined cell death for PLK1-and HSF1-depleted cells. We found that the cell death rate higher in these cells than in PLK1-depleted cells. In addition, the PLK1 and HSF1-specific siRNA affected cell proliferation and growth and caused the apoptotic mechanism to be triggered.

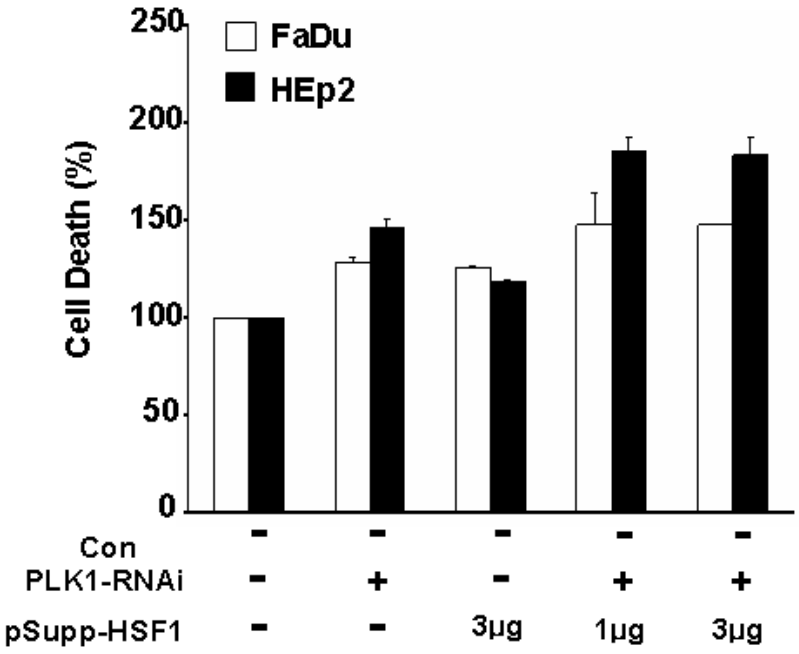


Fig. 5. The determination of cell death by cell death detection ELISA assay. FaDu and HEp-2cells were transfected with PLK1 or HSF1 siRNA alone or PLK1 and HSF1 double siRNA, for 24 h, The extent of cell death was assessed with the cell death detection ELISA assayThe fragmentation of DNA in cell death was quantified by a sandwich-enzyme-immunoassay, using the anti-histone-biotin and anti-DNA-POD antibodies. The results shown are from a representative experiment which was repeated three times with similar results.

Past studies have shown that PLK1 phosphorylates various substrate proteins, including cyclin B1 and Cdc25C. At the G_2/M phase, PLK1 both phosphorylated cyclin B1 and promoted the nuclear entry of an active Cdc2-cyclin B1 complex.[30] In addition, Plk1 was capable of phosphorylating Cdc25C, which directly activates the Cdc2-cyclin B1 complex during G_2/M phase.[31-32]

To determine the effects of PLK1 depletion on the cell cycle, we transfected PLK1-siRNA (0.5 and 2 μM) for 24 h, in Fadu and HEp2 cells and extracted the total cellular proteins. Next, we performed a Western blot analysis. Following exposure to siRNA, PLK1 protein levels dropped by 50% and 70% compared to the control groups at 0.5 and 2 μM, respectively (Fig. 6A). We then tested whether the CDKs were affected in PLK1-depleted cells and found that the cdk2 and cdk4 increased slightly after a 24 h transfection period in the PLK1-depleted cells. In contrast, we found that the cdc2/cyclinB complex activity was 40% lower than the control cells at PLK1-depleted cells. In addition, the activity of the Cdc2/cyclinB complex decreased in comparison to both PLK1 and HSF1-depleted cells (>10%, Fig.6B) however, there was no observed difference for the HSF1-depleted cells. As a major regulator of the cell cycle, PLK1 directly targets many key cell cycle regulators, such as p53 or Cdc25C. Recent studies have shown that PLK1 has the ability to phosphorylate p53 and simultaneously inhibit its transcriptional activity as well as its pro-apoptotic function, through direct interaction.[33,34] In contrast to reports that Plk1 negatively regulated p53 in human neuroblastoma, our results suggested that Plk1 depletion may inhibit p53 expression via an alternative pathway. FaDu cells have been reported to contain significantly higher levels of p53 protein than normal control cells and in addition showed point mutations. Specifically, a mutation of CGG to CTG occurred at codon 248. In contrast, the HEp-2 showed no mutations in p53 cDNA.[35] Therefore, PLK1 depletion triggered a p53-independent cell cycle program through the inhibitory phosphorylation and down-regulation of the cell cycle kinase, CDK1.

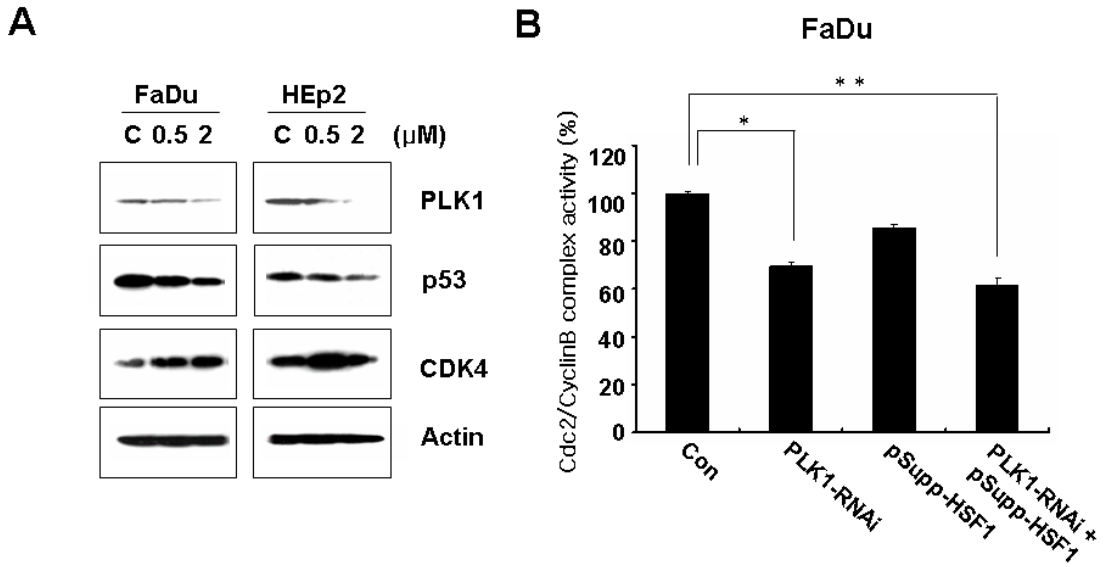


Fig. 6. PLK1 depletion inhibits Cdc2/Cyclin B complex activity. (A) FaDu and HEp-2cells were transfected with siRNA against PLK1. Twenty-four hours after transfection, the cells were harvested. About 50 μg of cell lysate was directly resolved by SDS/PAGE, and subjected to a Western blot with the use of the antibodies (p53 and Cdk4/6). (B) The cells were transfected with PLK1 or HSF1 siRNA alone or PLK1 and HSF1 double siRNA. The cell lysates were prepared and assessed for Cdc2/Cyclin B complex activity. The details of each experiment are provided in the "Materials and Methods" section.

The HSF1 protein plays an important role in the cellular events following various stress stimuli.[12,13] We investigated whether the HSF1 pathway was affected in PLK1-depleted cells. The cells were transfected with PLK1-siRNA, heat shocked at 42°C for 1 h, and allowed to recover at 37°C for 6 h. Next, the cells were harvested and the cell lysates were subjected to direct Western blotting. As shown in Fig. 7, HSF1 levels increased as a result of heat treatment in the control cells, whereas the depletion of PLK1 significantly inhibited the expression of HSF1. Our results also revealed that PLK1- siRNA resulted in the inhibition of the enhanced HSP70 expression, the major target of HSF1,in response to heat shock. The expression of Hsp90 was increased by heat stress, whereas the heat-induced Hsp90 was also significantly inhibited in the HEp-2cells with depleted PLK1. In this study, the silencing of PLK1 by siRNA, led to an inhibition of the heat stress response,

including Hsp70 and Hsp90, thus, suggesting that PLK1, as a kinase, regulates HSF1 activation. It is proposed that a close link existed between the induction of stress response and PLK1 in cancer development.[20,22-26]

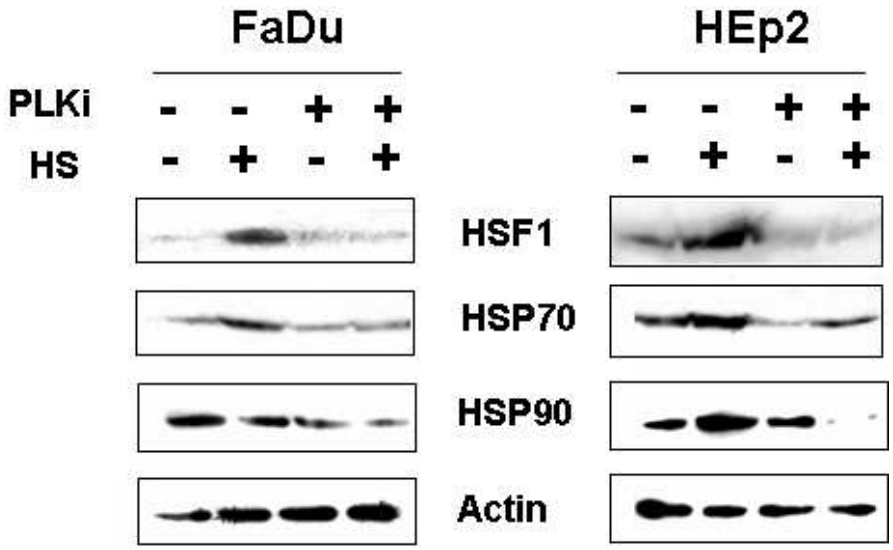


Fig. 7. The silencing effect of siRNA-PLK1 on HSF1 and Hsp70 protein expression. After transfection of cells (FaDu and HEp-2) with PLK1 siRNA, cells treated with or without heat shock (42°C, 1 h) and recovered at 37°C for 6 h. The cell lysates were directly resolved by SDS/PAGE, and subjected to a Western blot with the use of the anti-Hsp70 and Hsp90 antibodies.

Many other factors including Bcl-2, SOD and Catalase also contribute to cell survival in cancer cells.[36,37] We observed that PLK1- siRNA was also found to block the high levels of bcl-2 in HEp-2cells (data not shown) and that PLK1 depletion may induce oral cancer cell apoptosis through the down-regulation of bcl-2 as well as the HSF1/HSPs pathway. Further research would be necessary to demonstrate the relationship between PLK1, Bcl-2, p53 and stress response.

In conclusion, the results of this study, using siRNA-PLK1 and -HSF1, has provided evidence for the strong inhibition of cell proliferation, cell arrest, and induction of apoptosis in oral cancer cells, hence demonstrating its potential as a target for oral cancer therapy.

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논문제목	한글: 사람 편평세포암에서 PLK1과 HSF1의 double known에 의한 항암효과 영문: ANTITUMOR EFFECT OF PLK1 AND HSF1 DOUBLE KNOCKDOWN IN HUMAN SQUAMOUS CELL CARCINOMA				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

2008년 6월 일

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