Full Length Research Paper

Polo-like kinase 1 siRNA-607 induces mitotic arrest and apoptosis in human nasopharyngeal carcinoma cells

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Polo-like kinase (Plk) 1 is overexpressed in many human malignancies including nasopharyngeal carcinoma, indicating its potential as a therapeutic target. Recently, using a simple cellular morphology-based strategy, we have identified several novel effective siRNAs against Plk1 including Plk1 siRNA-607. In this study, we further investigated the effects of Plk1 siRNA-607 in human nasopharyngeal carcinoma cell line, HNE-1. Real time RT-PCR and Western blot indicated that Plk1 siRNA-607 transfection resulted in a significant inhibition in Plk1 expression in the HNE-1 cells. Furthermore, cell cycle, cell growth and apoptosis analysis clearly indicated that Plk1 siRNA-607 caused a dramatic mitotic cell cycle arrest followed by massive apoptotic cell death, and eventually resulted in a significant decrease in growth and viability of the nasopharyngeal carcinoma cells. Given that Plk1 has been widely accepted as a novel efficient target for cancer therapy, these results suggested that Plk1 siRNA-607 could be further developed for the treatment of human nasopharyngeal carcinoma.

Key words: Nasopharyngeal carcinoma, Plk1, RNA silencing, cell cycle, apoptosis.

INTRODUCTION

Nasopharyngeal carcinoma is a highly prevalent cancer in Southeast Asia especially in Southern China (Parkin et al., 2005; Chou et al., 2008; Tao and Chan, 2007). It poses one of the serious health problems in China with an annual incidence of more than 20 cases per 100,000 and approximately 100,000 new cases diagnosed annually (Tao et al., 2007). Despite recent advances in naso-pharyngeal carcinoma treatment, the prognosis for patients with nasopharyngeal carcinoma remains poor (Tao et al., 2007). Thus, more efforts are needed to develop novel therapeutic approaches for the treatment of this disease.

Plk1 is the best-characterized member of the human polo-like kinase (Plk) family which is highly conserved from yeasts to mammals (Glover et al., 1998; Golsteyn et al., 1994; Strebhardt and Ullrich, 2006; Chopra et al., 2010; Bu et al., 2008; Lan et al., 2010). Numerous studies have shown that Plk1 plays critical roles during mitosis and regulates many mitotic events, including centrosome maturation, spindle formation, and execution of cytokinesis (Strebhardt et al., 2006; Chopra et al., 2010; Bu et al., 2008; Lan et al., 2010). Importantly, Plk1 was also found to be up-regulated in a variety of human tumors, and thereby has prognostic value for several types of cancers including nasopharyngeal carcinoma (Strebhardt et al., 2006; Bu et al., 2008; Takai et al., 2005; Knecht et al., 1999). Moreover, a recent study has shown that Plk1 also mediates invasion process through extracellular matrix such as vimentin and beta1 integrin (Rizki et al., 2007). Consequently, Plk1 has emerged as an attractive cancer therapy target with several approaches being evaluated, including small interfering RNA (siRNA) and small molecule inhibitors (Strebhardt et al., 2006; Chopra et al., 2010; Bu et al., 2008). Consistent

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Abbreviations: **Plk1**, Polo-like kinase 1; **siRNA**, short interfering RNA; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **PCR**, polymerase chain reaction.

with this notion,several studies have shown that depletion of Plk1 by RNA silencing inhibits cellular growth and induces apoptosis in some cancer cells but not in normal cells (Liu and Erikson, 2003; Spänkuch-Schmitt et al., 2002; Reagan-Shaw and Ahmad, 2005; Liu et al., 2006). Additionally, several pharmacological small-molecule inhibitors also showed promising results in clinical trials (Strebhardt et al., 2006; Chopra et al., 2010; Lan et al., 2010).

Recently, we proposed and demonstrated a simple cellular morphology-based strategy for rapid screening effective siRNAs against Plk1 (Lan et al., 2010). Plk1 siRNA-607 is one of the four newly identified effective siRNA against Plk1. In this study, we further investigated its effects in human nasopharyngeal carcinoma cell line, HNE-1. Our results show that Plk1 siRNA-607 significantly inhibited the Plk1 expression, and subsequently resulted in mitotic arrest followed by massive apoptotic cell death, eventually leading to a significant decrease in cell viability.

MATERIALS AND METHODS

Cell culture

Human nasopharyngeal carcinoma cell line, HNE-1, was maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) and penicillin (100 IU/ml)/streptomycin (100 mg/ml). Cells were maintained at 37°C in a water-saturated atmosphere of 5% CO_2 in air.

Design and synthesis of Plk1 siRNA

Plk1 mRNA reference sequence, NM_005030, was used as template to design siRNA against Plk1. Several novel candidate siRNA sequences were chosen to undergo preliminary investigation. siRNAs were chemically synthesized by Shanghai GenePharma. The Plk1 siRNA-607 sequence was 5'-AUGAAGAUCUGGAGGU GAAdTdT-3', and the negative control siRNA sequence was 5'-UUCUCCGAACGUGUCACGUdTdT-3'.

siRNA transfection

The Plk1 siRNA and the negative control siRNA were transfected into cells using Lipofectamine RNAiMAX reagent (invitrogen) according to the manufacturer's instructions. Cells were collected and subjected to subsequent analysis 24 to 72 h after transfection.

Cell growth and viability assay

Trypan blue exclusion assay was used to determine the effects of Plk1 depletion on the cellular growth and viability. The method has been described in details previously (Bu et al., 2008).

RNA isolation and RT-PCR

Total RNA was prepared from the indicated cells using the TRIZOL reagent (Invitrogen) according to the manufacturer's instructions.

cDNA was generated from 1 µg of total RNA using SuperScript II reverse transcriptase and random primers following the manufacturer's conditions (Invitrogen). Real time PCR was carried out by using the SYBR® Premix Ex Taq™ (Perfect Real Time, TAKARA) following the manufacturer's instructions. The relative expression level of the Plk1 gene compared with that of the housekeeping gene, GAPDH, was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primer sequences used were as follows: Plk1, 5'-GGCAACCTTTTCCTGAATGA-3' and 5'-AATGGACCACACATCCACCT-3'; GAPDH, 5'-ACCTGACCTGCCG TCTAGAA-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. PCR products were also separated by a 2.0% agarose gel electro-phoresis and checked by ethidium bromide staining.

Immunoblotting analysis

Following the transfection of cells with control or Plk1 siRNA, the cells were collected, and subjected to immunoblotting analysis. The method was also described in details previously (Bu et al., 2008). The primary antibodies used in this study are the monoclonal anti-Plk1(A-1, Zymed, San Francisco, CA), polyclonal anti-Phospho-Histone H3 (ser10) (Upstate, Lake Placid, NY), and polyclonal anti-actin (20 to 33, Sigma) antibody.

Cell cycle analysis

Both floating and attached cells were collected by trypsin digestion and low speed centrifugation, washed with cold PBS, and subjected to cell cycle analysis on a FACScan flow cytometer as described in details previously (Bu et al., 2008).

Apoptosis analysis

An annexin V-FITC apoptosis detection kit (BD Pharmingen) was used to detect early apoptotic activity according to the manufacturer's instructions, with slight modifications. The cells were harvested, washed twice with ice-cold PBS, and resuspended in binding buffer. Annexin V-FITC and propidium iodide (PI) were then added and incubated for 15 min at room temperature in the dark. Cells were finally analyzed for apoptosis by flow cytometry.

RESULTS

Design and synthesis of Plk1 siRNA

In a preliminary experiment, several novel siRNAs against Plk1 were investigated for their ability to inhibit Plk1 expression and cell proliferation in malignant cells (unpublished data). Based on these observations, Plk1 siRNA-607 was chosen for further investigation in this study. The siRNA was purified by high-performance liquid chromatography (HPLC), and checked for purity and integrity by PAGE (20%) under denaturing conditions (Figure 1).

Plk1 siRNA-607 inhibits proliferation and decreases viability in HNE-1 cells

At first, we determined the effect of Plk1 siRNA-607 on the viability of the human nasopharyngeal carcinoma
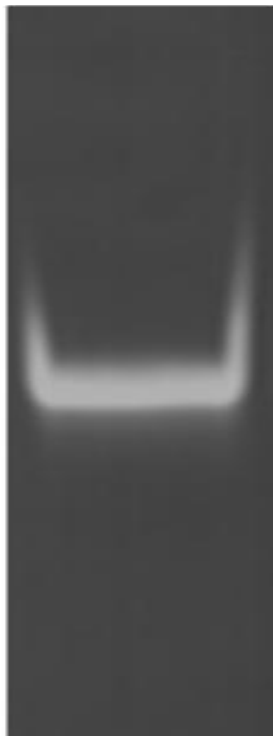
Figure 1. Synthesis of Plk1 siRNA-607. The siRNA was purified by high-performance liquid chromatography (HPLC), and checked for purity and integrity by PAGE (20%) under denaturing conditions.

cells, HNE-1. Real time RT-PCR and Western blot analysis demonstrated that the Plk1 expression was significantly inhibited at both mRNA and protein levels after transfection, whereas the expression of GAPDH or actin was unchanged. The expression level of Plk1 was efficiently reduced by at least 90% 48 h after transfection (Figure 2). As indicated in Figure 3A, Plk1 siRNA-607 strongly inhibited the growth rate of HNE-1 cells whereas control siRNA did not affect the cellular proliferation. We also examined the cellular viability by Trypan blue exclusion assay. As indicated in Figure 3B, compared with the control siRNA which showed very little effect on cell viability, Plk1 siRNA-607 significantly reduced the cell viability in HNE-1 cells.

Morphology changes in Plk1 siRNA-607-transfected HNE-1 cells

Simultaneously, we also observed the morphology change in Plk1 siRNA-607-transfected HNE-1 cells. The control cells showed a typical polygonal and intact appearance, and a normal growth phenotype, whereas the Plk1-depleted HNE-1 cells displayed significant abnormal

morphological changes (Figure 4). Twenty-four hours after Plk1 siRNA-607 transfection, cells partially detached from the culture plates and floated on the media with round shape, suggesting the cell cycle arrest at mitosis phase. Forty-eight hours after transfection, cells became less confluent, and completely detached from the culture plates forming floating aggregates, suggesting the significant growth inhibition and cell death (Figure 4).

Plk1 siRNA-607 induces mitotic cell cycle arrest in HNE-1 cells

To further investigate the mechanism of morphology changes in Plk1-depleted cells, we analyzed the effect of Plk1 siRNA-607 on cell cycle distribution using flow cytometry. As shown in Figure 5A, Plk1 siRNA-607 induced an obvious increase in the percentage of cells with 4N DNA content. Twenty-eight hours after transfection, approximately 67% of Plk1 siRNA-607-transfected HNE-1 cells had a 4N DNA content compared with only 7.5% in control siRNA-transfected cells. The phosphorylation of histone H3 at ser10, a mitosis marker, was then checked to confirm the phenotype of mitotic arrest in Plk1 siRNA-607-transfected cells. As shown in Figure 5B, a significant accumulation of ser10 phosphorylated histone H3 was observed in Plk1 siRNA-607-transfected HNE-1 cells, suggesting that Plk1 siRNA-607 causes mitotic arrest in HNE-1 cells. Consistently, the expression of mitotic cyclin, cyclin B1, also significantly accumulated in Plk1 siRNA-607-transfected HNE-1 cells.

Plk1 siRNA-607 induces apoptosis in HNE-1 cells

Noticeably, forty-eight hours after transfection, about 15% of Plk1 siRNA-607-transfected cells displayed sub-G1 DNA content, strongly suggesting the induction of apoptosis in HNE-1 cells (Figure 5A). To further confirm this phenotype, annexin V-FITC and PI double staining with flow cytometry was used to check apoptosis in Plk1-depleted cells. As expected, approximately 17 and 21% of Plk1 siRNA-607-transfected cells displayed early apoptotic (FITC$^+$/PI$^-$) and late apoptotic/secondary necrotic phenotype forty-eight hours after transfection, whereas only 4.1 and 4.3% of control cells had this phenotype (Figure 6).

DISCUSSION

Plk1 is highly expressed in a variety of human tumors and also has prognostic value in several types cancers, indicating its involvement in carcinogenesis and its potential as a therapeutic target (Strebhardt and Ullrich, 2006; Chopra et al., 2010; Bu et al., 2008). In nasopharyngeal carcinoma, Knecht et al. (1999) reported that
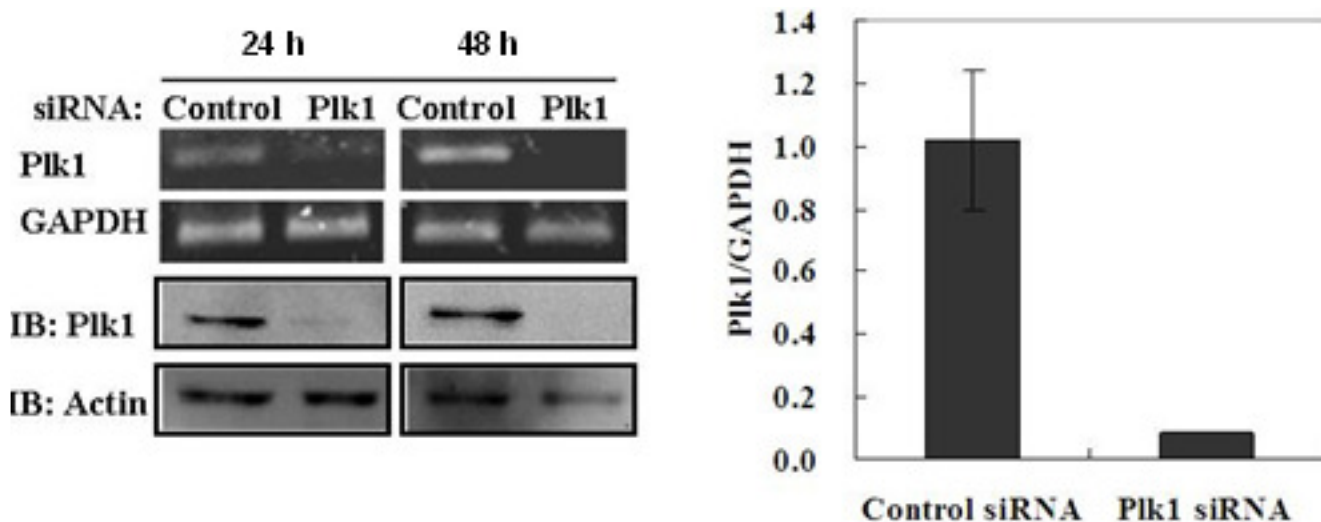
Figure 2. Plk1 siRNA-607 inhibits Plk1 expression. HNE-1 cells were transiently transfected with the control siRNA and Plk1 siRNA-607, respectively. Twenty-four and four-eight hours after transfection, total RNA and whole cell lysates were prepared and subjected to real time RT-PCR and immunoblotting, respectively. For RT-PCR, GAPDH was used as an internal control. For IB, equality of protein loading was confirmed by the expression of actin.
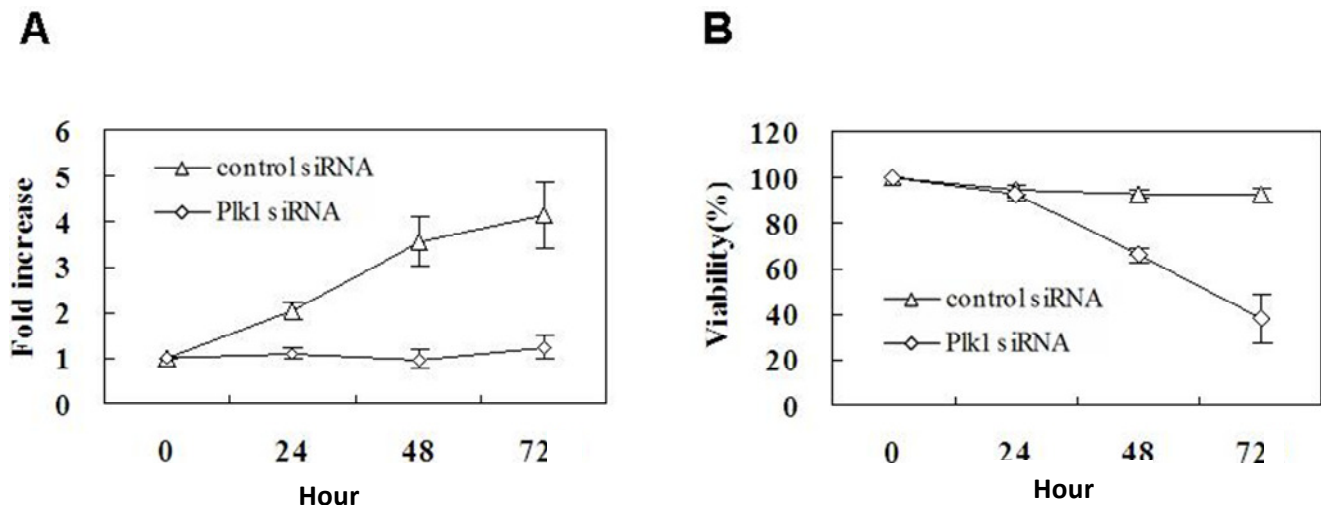
A

B

Figure 3. Plk1 siRNA-607 inhibits cell growth. (A) The effect of Plk1 siRNA-607 on cell growth. Following siRNA transfection, cells were collected and counted at the indicated time points. The Y axis represents average folds of increase in cell numbers. The error bar represents the standard error of three independent experiments. (B) The effect of Plk1 siRNA-607 on cell viability. Cell viability was measured using Trypan blue exclusion analysis as described in material and methods. Cell viability data are expressed as the percent viable cells out of the total number of cells. The error bar represents the standard error of three independent experiments.

Plk1 mRNA is overexpressed in human head and neck tumors including nasopharyngeal carcinoma and the overexpression of Plk1 mRNA correlates to prognostic parameters and the survival of patients with head and neck cancer. In this study, we designed and synthesized a novel Plk1 siRNA, Plk1 siRNA-607, and investigated its effects in human nasopharyngeal carcinoma cell line, HNE-1.

According to our results, the newly designed Plk1 siRNA, Plk1 siRNA-607, significantly inhibited the Plk1 expression at both mRNA and protein levels, and subsequently caused growth retardation and apoptosis in HNE-1 cells. Furthermore, we also found that mitotic cell cycle arrest was the early events and the first major phenotype in Plk1 siRNA-607-transfected HNE-1 cells. Additionally, as shown by the appearance of a sub-G1 population in cell cycle profiles, and the increase of annexin V-FITC positive cells, apoptosis was the second
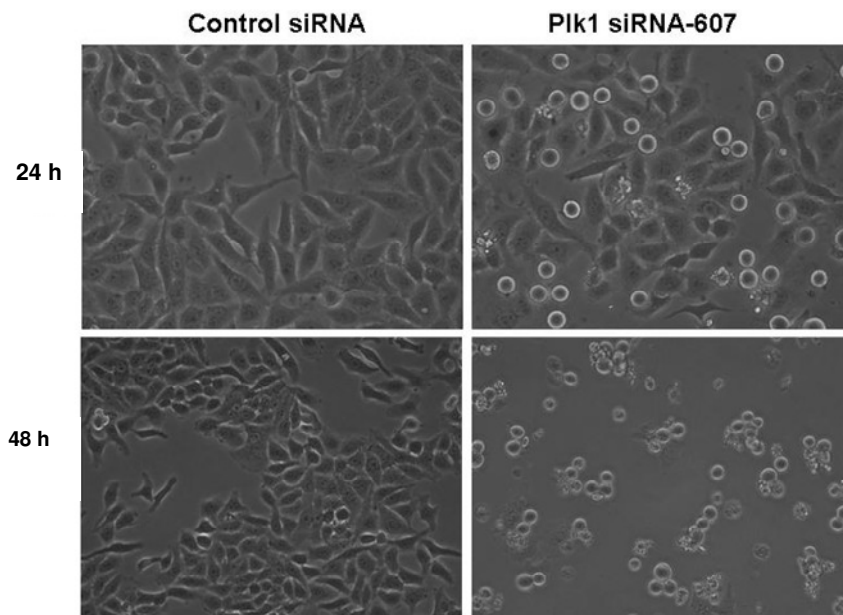
Figure 4. Morphology changes in HNE-1 cells. HNE-1 cells were transiently transfected with the control siRNA and Plk1 siRNA-607, respectively. Cellular morphology were observed under phase-contrasted microscope at the indicated times after siRNA transfection (200×).
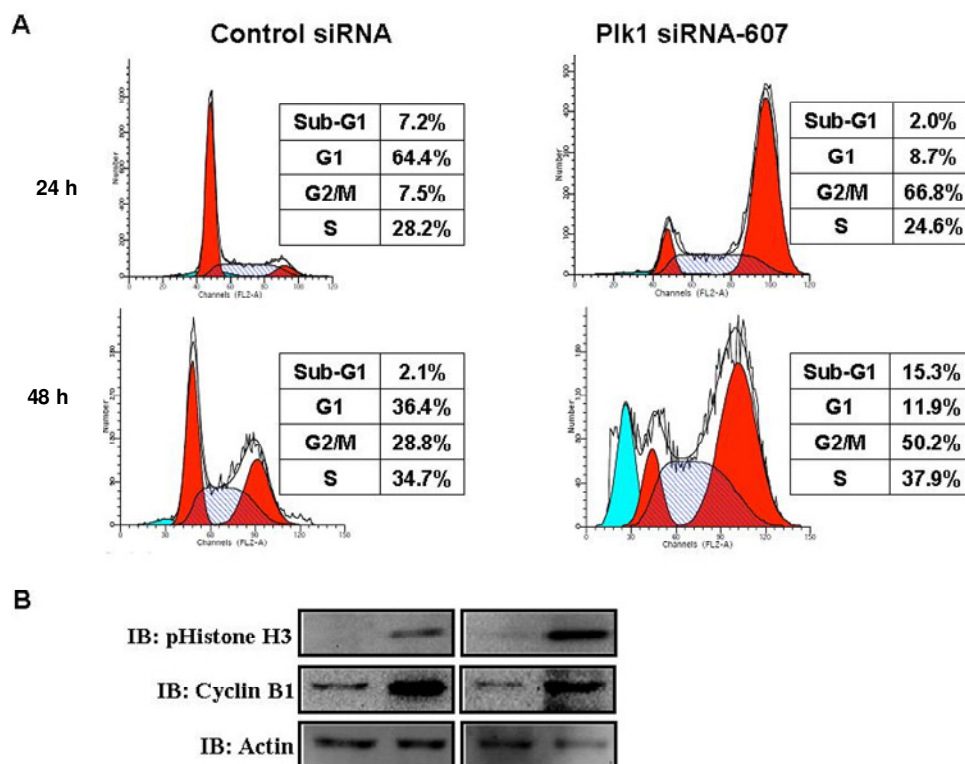

Figure 5. Plk1 siRNA-607 induces mitotic cell cycle arrest. (A) FACS profiles. HNE-1 cells were transiently transfected with the control siRNA and Plk1 siRNA-607, respectively. Four-eight hours after transfection, cells were collected, fixed by ethanol, stained with propidium iodide and subjected to FACS analysis. The positions and percentages of G1, G2/M, and sub-G1 populations are shown. (B) Western blot analysis. Following siRNA transfection, whole cell lysates were prepared and subjected to immunoblotting. Equality of protein loading was confirmed by the expression of actin.
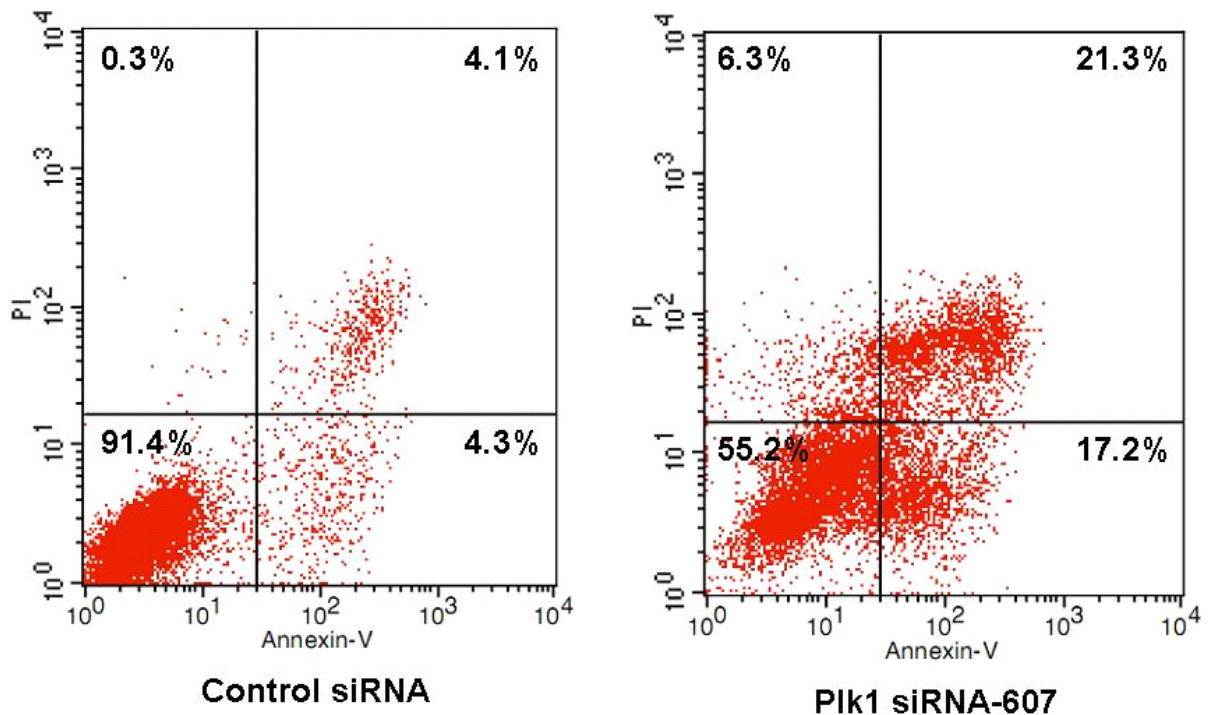
Figure 6. Plk1 siRNA-607 induces apoptosis. HNE-1 cells were transiently transfected with the control siRNA and Plk1 siRNA-607, respectively. Forty-eight hours after transfection, cells were collected, stained with annexin V-FITC and propidium iodide (PI) and subjected to apoptosis analysis. Quadrants: Lower left, viable (intact) cells (FITC⁻/PI⁻); l ower right, early apoptotic cells (FITC＋/PI—); upper right, late apoptotic and secondary necrotic cells (FITC＋/PI＋).

major phenotype observed in Plk1 siRNA-607-transfected HNE-1 cells. These results are consistent with our study and other previous reports observed in other cancer cells (Bu et al., 2008; Reagan-Shaw and Ahmad, 2005; Liu and Erikson, 2002). Notably, the author of this study and other author have also shown that depletion of Plk1 causes defects in mitosis processes such as failure of cytokinesis, and disruption of mitotic cell cycle progression via the Cdc25C and cdc2/cyclin B1 positive feedback loop (Bu et al., 2008; Reagan-Shaw and Ahmad, 2005; Roshak et al., 2000). Thus, further studies are needed to clarify whether Plk1 depletion results in these mitotic defects via the similar molecular mechanism in nasopharyngeal carcinoma cells.

Recently, Shi et al. (2010) also obtained similar results in another nasopharyngeal carcinoma cell line, C666-1. These results demonstrated that targeting Plk1 using siRNA caused significant inhibition of proliferation, mitotic catastrophe, and apoptosis. The effect of targeting Plk1 using siRNA could be further enhanced by ionizing radiation both *in vitro* and *in vivo*. Combined with our present and other previous reports, it is not unreasonable to speculate that Plk1 may serve as a potential target in the treatment of human nasopharyngeal cancer (Shi et al., 2010; Strebhardt and Ullrich, 2006; Chopra et al., 2010; Bu et al., 2008; Lan et al., 2010).

In summary, our study clearly indicated that Plk1 siRNA-607 can significantly inhibit Plk1 expression, and induces mitotic arrest and apoptosis in HNE-1 cells. Given that Plk1 has been widely accepted as a novel efficient target for cancer therapy, we believe that Plk1 siRNA-607 could be further developed for the treatment of human nasopharyngeal carcinoma.

ACKNOWLEDGMENTS

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