Overexpression of Aiolos in Nalm-6 acute lymphoblastic leukaemia cells reduces apoptosis by suppressing phosphatase and tensin homologue deleted on chromosome 10 and activating the phosphatidylinositol-3-kinase/Akt signalling pathway

RAN WANG[1], GANG GUO[2], HAO LI[1], XIANGXIN LI[1], YUAN YU[1] and DONG LI[2]

[1]Department of Hematology; [2]Cryomedicine Laboratory, Qilu Hospital, Shandong University, Ji'nan, Shandong 250012, P.R. China

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Abstract. The aim of the present study was to elucidate the molecular mechanism of Aiolos in the regulation of B-cell leukaemia. A lentiviral system was used for overexpression of the Aiolos gene in Nalm-6 cells to determine the effects of Aiolos on proliferation, apoptosis and the cell cycle. The expression and activation of phosphatase and tensin homolog deleted on chromosome ten (PTEN) and Akt were also investigated. Upregulation of Aiolos inhibited cell growth and arrested an increased number of Nalm-6 cells at the G0/G1 phase. The apoptotic cell quantities were also significantly lower in the Aiolos-transfected Nalm-6 cells. In addition, Aiolos overexpression downregulated PTEN, but increased the expression and phosphorylation of Akt in the Nalm-6 cells. The Akt inhibitor, Akti-1/2, reduced the percentage of viable Aiolos-overexpressed Nalm-6 cells, however, it had no effect on cell cycle arrest or proliferation. Aiolos upregulation in the Nalm-6 cells inhibited cell proliferation, suppressed apoptosis and arrested the cell cycle at the G0/G1 phase. Aiolos improved the survival of Nalm-6 cells via PTEN- and Akt-dependent processes.

Introduction

Acute lymphoblastic leukaemia (ALL) is a malignant disorder of lymphoid progenitor cells. ALL is the most common type of childhood malignancy and accounts for almost 20% of cases of acute leukaemia in adults. B-cell precursor ALL (BCP-ALL) is the most common type of childhood ALL (1). Survival rates have markedly improved with combination chemotherapy and treatment intensification; however, the occurrence of minimal residual disease has prompted further examination of the molecular mechanisms underlying ALL (2).

BCP-ALL is a malignancy characterised by the progressive accumulation of immature clonal B-cell precursors in the bone marrow. Numerous transcriptional regulators have critical functions in this malignant process and may regulate the expression of genes, whose products affect the fate and function of lymphoid cells (3). The Ikaros family of proteins is an example of these factors (4), which encode zinc-finger DNA-binding proteins (5-7). Aiolos, the second Ikaros family member identified, is of particular interest in the investigation of BCP-ALL (6). Aiolos is an important regulator of B-cell differentiation, proliferation and maturation to an effector state. Disruption of Aiolos increases the number of pre-B and immature B cells, and significantly reduces the number of circulating B cells (8). In addition, an Aiolos null mutation in mice causes B-cell hyperproliferation, elevates serum antibody levels, promotes auto-antibody formation and triggers lymphoma development, thereby demonstrating tumour suppressor function (8). The deregulated expression of Aiolos has been associated with adult B-cell ALL and chronic lymphocytic leukaemia (CLL) in human patients (9-11) and aberrant expression levels of Aiolos levels been reported in cases of lymphoma (12).

However, the molecular mechanisms through which Aiolos exerts its growth inhibitory effect remain to be elucidated. The Aiolos gene is crucial in lymphoid development and apoptosis; therefore, the function of Aiolos in childhood BCP-ALL and the downstream regulatory mechanisms of Aiolos require investigation. In the present study, a lentiviral system was used to achieve stable overexpression of the Aiolos gene in the Nalm-6 BCP-ALL cell line. Subsequently, the effects of Aiolos upregulation on the proliferation, apoptosis and cell cycle distribution of Nalm-6 cells *in vitro* was examined. Further experiments indicated that the Akt, protein kinase, signalling pathways were regulated by loss of phosphatase and tensin homologue deleted on chromosome ten (PTEN) caused by overexpression of Aiolos.

Correspondence to: Mr. Dong Li, Cryomedicine Laboratory, Qilu Hospital, Shandong University, 107 Wenhuaxilu Road, Ji'nan, Shandong 250012, P.R. China
E-mail: lidong73@sdu.edu.cn

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Materials and methods

Recombinant lentiviral vector production. Aiolos cDNA (NM_012481; YRgene, Changsha, China) was cloned into a pWPT-PURO-green fluorescent protein (GFP) plasmid (Shanghai Telebio Biomedical Co. Ltd., Shanghai, China), which was co-transfected with three packaging plasmids (pRsv-REV, pMD1g-pRRE and pMD2G; Shanghai Telebio Biomedical Co. Ltd.) into human embryonic kidney (HEK)293T cells (Shanghai Telebio Biomedical Co. Ltd.). The HEK293T cells were plated at $7x10^5$ on six-well plates (Corning Costar; Corning, Inc., New York, NY, USA) in 2 ml of DMEM (Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% foetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA) and no antibiotics, and incubated at 37˚C with 5% CO_2, overnight. Subsequently, 10 μg pRsv-REV, 15 μg pMDlg-pRRE and 7.5 μg pMD2G packaging plasmids diluted in 1600 μl of Opti-MEM-I (Invitrogen Life Technologies) were combined, and 20 μg pWPT-PURO-GFP-Aiolos lentiviral DNA diluted in 200 μl of Opti-MEM-I was added to the tube. Finally 200 μl $CaCl_2$ (2.5 mol/l) was added to the tube, directly into the media to bring the final reaction volume to 2000 μl. The tube was agitated and then incubated at room temperature for 30 min. The reaction mixture (300 μl) was added to each well of the HEK293T cells plated the previous day, and incubated at 37˚C, 5% CO_2, overnight. The following day the media was aspirated and replaced with 2 ml fresh DMEM containing 10% FBS and 1% PenStrep (Invitrogen Life Technologies), and incubated overnight. The virions, released into the media, were collected 48 and 72 h after transfection. To concentrate the viral particles, the media were ultracentrifuged twice at 106,000 x g (1.5 h/round) and the final pellet was dissolved in RPMI 1640 (Invitrogen Life Technologies), 10% FBS and 1% glutamine (Invitrogen Life Technologies). Lenti-GFP was constructed similarly, but without the Aiolos insert.

Cell culture and transfection. The BCP-ALL cell line Nalm-6 was purchased from the American Type Culture Collection. (Manassas, VA, USA) and cultured in standard RPMI 1640 medium (Gibco-BRL) containing 10% foetal bovine serum (Gibco-BRL) and 1% penicillin-streptomycin (Gibco-BRL) at 37˚C in 5% CO_2. The Nalm-6 cells were divided into the following three groups: Untransfected control (UT), Lenti-GFP group and Aiolos-transfected (Lenti-Aiolos) group. The viral concentrate was diluted in polybrene (5 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) to infect the Nalm-6 cells at a multiplicity of infection of 100. Successful transduction was confirmed by visualisation of enhanced GFP after 4 days using a fluorescence microscope (IX71; Olympus Corp., Tokyo, Japan) and Phenix micro-image analysis software version 2.2 (Phenix Optical Holding Stock Co., Ltd, Jiangxi, China). The cells were maintained and allowed to grow at 37˚C for an additional 6 days, following which the expression levels of Aiolos were confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. The virus-infected cells were selected with 8 μg/ml puromycin (Invitrogen Life Technologies, Carlsbad, CA, USA). The antibiotic-resistant clones were pooled and used for subsequent assays.

Treatment with Akt inhibitor. Akti-1/2, previously known as Akt-I-1/2, was purchased from Sigma-Aldrich and reconstituted in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to a stock concentration of 50 mM. The final concentration of DMSO in the cultures was maintained at 0.1%. For all the experiments, the cells ($2x10^5$/ml) were treated with 0.5 μM Akti-1/2 at 37˚C for 48 h and treatment was continued for subsequent experiments. The control cells were treated with equal quantities of the solvent. All experiments were performed in triplicate.

Cell growth curve. The Nalm-6 cells of the UT, Lenti-GFP and Lenti-Aiolos groups were seeded into 24-well plates (Corning Costar) in triplicate at a density of $2x10^5$ per well at 37˚C seven days after transfection. The number of live cells was determined by trypan blue staining (Sigma-Aldrich) and was measured daily for 5 days using a haemocytometer (Jingmai Biotech., Ji'nan, China) under an inverted microscope (IX71; Olympus Corp., Tokyo, Japan).

Cell cycle analysis. For cell cycle analysis, a total of $1x10^6$ cells were first fixed with1 ml ice-cold 70% ethanol and then incubated with RNase A at 37˚C for 30 min. The cells were stained with propidium iodide (PI; 50 μg/ml PI in 0.1% sodium citrate and 0.1% Nonidet P-40; Sigma-Aldrich) for 30 min at 4˚C. The cells were then analysed using a fluorescence-activated cell sorting (FACS)Calibur instrument equipped with Cell Quest software version 5.0 (BD Biosciences, Franklin Lakes, NJ, USA). The results are expressed as the percentage of the cells in each cell cycle phase and error bars indicate the standard deviation of the mean (SEM).

Detection of apoptosis. Annexin V/PI labelling was performed using the Vybrant Apoptosis Assay kit #3 (Invitrogen Life Technologies), according to the manufacturer's instructions, followed by flow cytometry. The Nalm-6 cells were scanned in fluorescein isothiocyanate (FL1-H), vs. PI (FL2-H) channels using a FACSCalibur instrument and Cell Quest software version 5.0.

Isolation of RNA and RT-qPCR analysis. Total RNA was extracted from each group of Nalm-6 cells using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. Purified total RNA (1 μg) was reverse transcribed to cDNA using the Omniscript cDNA synthesis kit (Qiagen, Hamburg, Germany) using random primers. The relative gene expression levels were measured by qPCR using gene-specific primers and SYBR Green supermix (Promega Corp., Madison, WI, USA). β-actin was used as an endogenous control. The primers used are listed in Table I.

Western blot analysis. The proteins were extracted using radioimmunoprecipitation assay buffer containing 1X phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium dexoycholate, 0.1% SDS and protease inhibitor mixture tablet (Roche Diagnostics GmbH, Mannheim, Germany). At least 20 μg/lane proteins were subjected to 12% SDS-PAGE and subsequently transferred to polyvinylidene fluoride hydrophobic membranes (EMD Millipore, Bedford, MA, USA).

Table I. Primers used in reverse transcription-quantitative polymerase chain reaction.

Gene	Forward/reverse	Sequence	Product (bp)
PTEN	Forward	5'-GTGGCGGAACTTGCAATCCT-'3	119
	Reverse	5'-CGGCTGAGGGAACTCAAAGT-'3	
Akt1	Forward	5'-ATGGACAGGGAGAGCAAACG-'3	134
	Reverse	5'-CTGGCCACAGCCTCTGATG-'3	
Akt2	Forward	5'-GGTGACAGACTGTGCCCTG-'3	81
	Reverse	5'-GGTACGCTGTCACCTAGCTC-'3	
Akt3	Forward	5'-TTGGTTCGAGAGAAGGCAAGT-'3	87
	Reverse	5'-GTGTGCCACTTCATCCTTTGC-'3	
GAPDH	Forward	5'-ATAAATTGAGCCCGCAGCC-'3	140
	Reverse	5'-ACCAAATCCGTTGACTCCGA-'3	

PTEN, phosphatase and tensin homolog deleted on chromosome ten.

Membranes were blocked with 5% nonfat dry milk (Shifeng Biotech) in Tris-buffered saline containing 0.05% Tween-20 (TBST; Sigma-Aldrich) at 4˚C for 2 h. The membranes were rinsed with TBST and incubated with rabbit monoclonal Aiolos antibody (1/20;000; cat. 39408; Abcam, Cambridge, MA, USA), rabbit monoclonal PTEN antibody (1/500; cat. 32199; Abcam), rabbit monoclonal Akt (1/10,000; cat. 32505; Abcam), rabbit polyclonal phosphorylated-Akt antibody (Thr-308, 1/2,000; cat. 66134; Abcam) and rabbit monoclonal β-actin (1/20,000; cat. 79467; Abcam). Horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit immunoglobulin G H&L-HRP; 1/10,000; cat. 16721; Abcam) and an Enhanced Chemiluminescence (ECL) Plus Immunoblotting Detection system (Beyotime, Shanghai, China) were used to detect specific binding, and images of the signals were captured using X-ray films (Kodak, Rochester, NY, USA) and WD-9413C Electrophoresis image analysis system (Liuyi, Beijing, China).

Statistical analysis. All data are presented as the mean ± standard deviation and analysed via analysis of variance, which was followed by Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were conducted using the SPSS 13.0 software program (SPSS Inc., Chicago, IL, USA).

Results

Aiolos overexpression by stable transfection in Nalm-6 cells. The Nalm-6 cells were infected with the pWPT-PURO-GFP-Aiolos lentivirus vector, which was inserted with the entire Aiolos coding sequence, to induce the upregulation of Aiolos. As a control, Nalm-6 cells were either infected with a lentivirus vector expressing GFP or were not infected. At 4 days after infection, the infection efficiency was determined based on the expression level of GFP using a fluorescence microscope. A substantial number of cells emitted bright green fluorescence, suggesting high infection efficiency (Fig. 1A-F). The cells were maintained in culture and grown for an additional 3 days. Subsequently, the mRNA and protein expression levels of Aiolos in the Nalm-6 cells of the three groups were determined by RT-qPCR and western blot assays. The RT-qPCR results demonstrated that the mRNA expression of *Aiolos* in the Nalm-6 cells of the Lenti-Aiolos group was 1.5-fold higher compared with that in the Nalm-6 cells of the UT group ($P<0.01$; Fig. 1G). This result is consistent with the increase in the protein expression of Aiolos (Fig. 1H). No significant difference was observed in the distribution of expression of Aiolos between the Lenti-GFP group and UT group.

Overexpression of Aiolos inhibits the growth of Nalm-6 cells and leads to cell cycle arrest at the G0/G1 phase. The growth properties of the Lenti-Aiolos group were measured and compared with those of the control Lenti-GFP transfected group to determine whether overexpression of Aiolos affects the proliferation of Nalm-6 cells. As shown in Fig. 2A, the growth rate of the Lenti-Aiolos group was significantly lower compared with that of the Lenti-GFP or UT group. This result suggested that the upregulation of Aiolos may inhibit the growth of Nalm-6 cells.

The resulting inhibition of the proliferation of Nalm-6 cells infected with Lenti-Aiolos compared with control Nalm-6 cells may be, in part, caused by differences in cell cycle regulation. Thus, the cell cycles of Nalm-6 cells in different groups were characterised by FACS analysis 9 days after transfection. No significant difference was detected between the Lenti-GFP and Nalm-6 cells (Fig. 2B; $P>0.05$). However, the percentage of Nalm-6 cells in the G0/G1 phase increased between 72% (UT) and 84.5% (Lenti-Aiolos; $P<0.01$), and the percentage of Nalm-6 cells in the S-phase cells decreased between 19% (UT) and 10.9% (Lenti-Aiolos; $P<0.01$). A significant difference in the percentage of Nalm-6 cells in the G2/M phase was found between the Lenti-Aiolos group and the UT group (4.5. vs. 9%, respectively; $P<0.01$). These data indicated that Aiolos upregulation arrested an increased number of Nalm-6 cells at the G0/G1 phase, which possibly contributed to the growth inhibition observed.

Aiolos overexpression suppresses the apoptosis of Nalm-6 cells. An apoptosis assay kit was used to measure cell

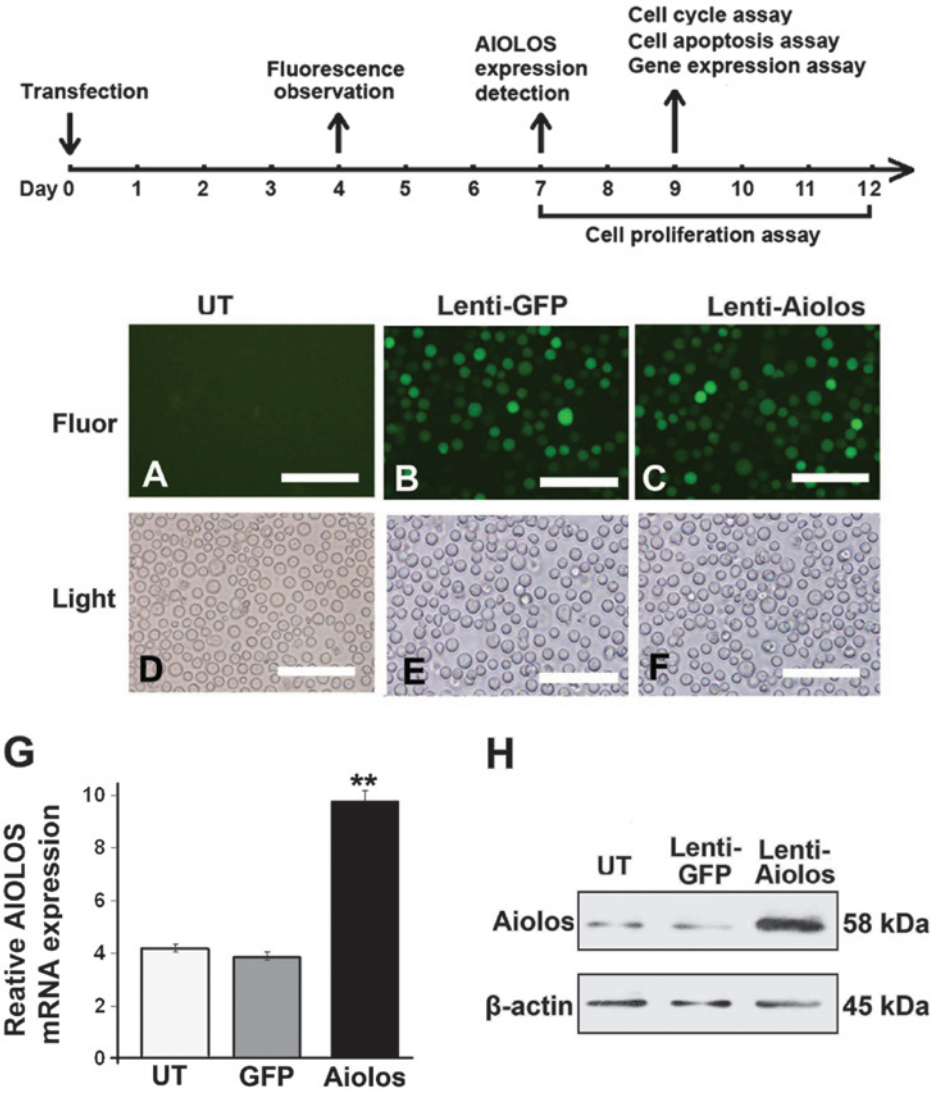

Figure 1. Timeline of the treatment of Nalk-6 cells involving the overexpression of Aiolos. (A-F) Lentiviral transduction efficiency in the Nalm-6 cells. Transduction efficiency was estimated 4 days after infection at a multiplicity of infection of100. The expression of GFP was observed under (A, B and C) fluorescence or (D, E and F) light microscopy. Scale bar: 200 μm (magnification, x200). (G) Reverse transcription-quantitative polymerase chain reaction of the relative transcription levels of Aiolos in the UT, Lenti-GFP, and Lenti-Aiolos groups. β-actin was used as an internal control. (H) Western blotting results revealed that protein expression of Aiolos was present in the Nalm-6 cells of the three groups. β-actin was used as a loading control. Error bars indicate the standard deviation of the mean of three experiments. **P<0.01 Lenti-Aiolos group vs. Lenti-GFP group. GFP, green fluorescent protein; UT, untransfected control; Lenti-GFP, lentiviral vector control; Lenti-Aiolos, Aiolos-transfected; Fluor, fluorescence.

apoptosis 9 days after transfection to determine whether overexpression of Aiolos affects the apoptosis of Nalm-6 cells. As shown in Fig. 3, the total number of apoptotic cells was significantly lower in the Aiolos-transfected Nalm-6 cells (11%) compared with the cells in the Lenti-GFP (16.5%) or UT groups (18.9%; P<0.05). A minimal difference in the percentage of early apoptotic cells was found between the Aiolos-transfected and UT Nalm-6 cells (8.05, vs. 11.2%; P>0.05), whereas a significant difference was observed in the percentage of late apoptotic cells between the cell groups (2.95, vs. 7.7%; P<0.05). The obtained data suggested that overexpression of Aiolos may suppress apoptosis in Nalm-6 cells.

Upregulated Aiolos suppresses the expression of PTEN, but activates certain phosphatidylinositol-3-kinase (PI3K)/Akt signalling pathway-associated genes in Nalm-6 cells. Microarrays were used to analyse the gene expression of Nalm-6 cells transfected with Aiolos and to further examine the underlying mechanism responsible for the aforementioned changes in biological behaviour (data not shown). Among the deregulated genes, several key genes are of note. The expression levels of PTEN and certain Akt family genes were further detected using RT-qPCR and western blot analysis. The RT-qPCR results revealed that the expression level of PTEN in the Nalm-6 cells was significantly lower in the Lenti-Aiolos group compared with the control group (P<0.05). In addition, upregulation of Akt1 (P<0.05) and Akt2 (P<0.01) was confirmed in the Lenti-Aiolos group. No significant change was observed in the expression of Akt3. Consistent with the RT-qPCR results, decreased expression levels of PTEN was observed by western blot analysis. The protein expression of Akt did not change considerably, whereas the levels of phosphorylated Akt were significantly

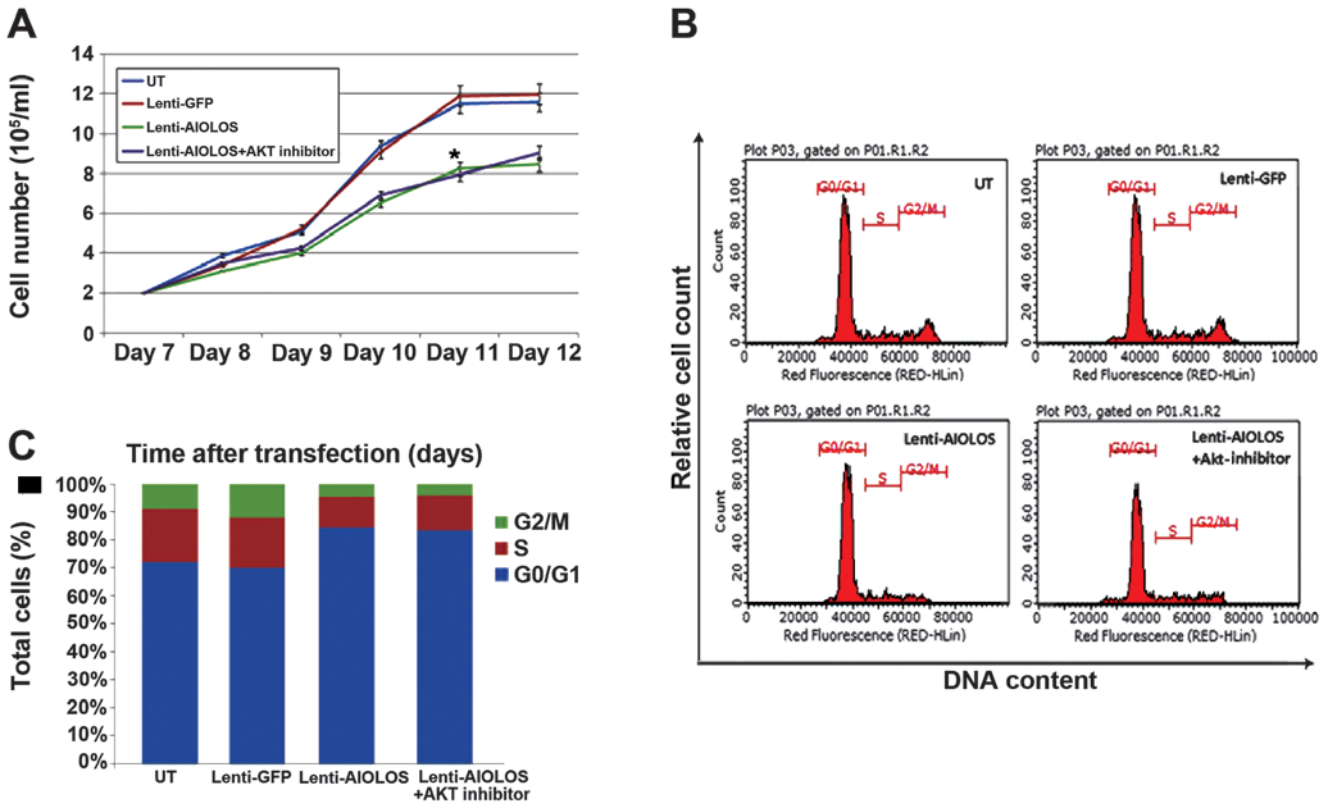

Figure 2. Effect of overexpression of Aiolos on the proliferation and cell cycle distribution of Nalm-6 cells. The numbers of Nalm-6 cells in the three groups were counted between day 7 and day 12 following transfection. Each time-point was counted in triplicate. (A) Proliferation of Nalm-6 cells in the Lenti-Aiolos group reduced by 16% on day 9 compared with that of the UT group cells (P>0.05). The reduction peaked at 29% on day 11 (*P<0.05 vs. UT group cells). The same proliferative capacity was observed in the Lenti-GFP group and the UT group (P>0.05 vs. UT group). Treatment with chemical inhibitors of Akt (Akti-1/2) did not improve the growth of the Nalm-6 cells in the Lenti-Aiolos group. (B and C) Flow cytometric analysis demonstrated that the proportions of cells in the G0/G1 phase increased and the proportion of cells in the G2/M and S phase decreased in the Nalm-6 cells of the Lenti-Aiolos group compared with those in the Lenti-GFP group (P<0.01 vs. Lenti-GFP group). Akti-1/2 did not affect the cell cycle of the Lenti-Aiolos group. Error bars indicate the standard deviation of the mean from three experiments). GFP, green fluorescent protein; UT, untransfected control; Lenti-GFP, lentiviral vector control; Lenti-Aiolos, Aiolos-transfected.

higher in the Lenti-Aiolos group compared with the control group (Fig. 4).

Akt inhibitors reverse Aiolos *overexpression-induced apoptosis, but do not affect proliferation arrest*. The cells were incubated with varying concentrations of the inhibitor, Akti-1/2, (0.1-10 μM) for 48 h and cell viability was measured to verify whether the activation of Akt was principally responsible for the decreased apoptosis in the Aiolos-overexpressed Nalm-6 cells. The results of the western blotting revealed that Akti-1/2, at a concentration of 5 μM, effectively inhibited Akt phosphorylation. In addition, Akti-1/2 effectively increased the percentage of apoptotic cells in the Aiolos-transfected Nalm-6 cells (Fig. 3). However, in the cell proliferation experiments, Akti-1/2 treatment did not improve the growth of the Nalm-6 cells in the Lenti-Aiolos group (Fig. 2A). Similarly, Akti-1/2 did not affect the cell cycle of the Lenti-Aiolos group (Fig. 2B and C).

Discussion

In the present study, the Nalm-6 BCP-ALL cell line was used for a series of functional investigations to understand the function of Aiolos in the pathogenesis of BCP-ALL. The results demonstrated that lentivirus-mediated overexpression of Aiolos suppressed cell apoptosis and arrested the cell cycle at the G0/G1 phase, which possibly contributed to growth inhibition of the Nalm-6 cells. Furthermore, these changes were possibly associated with downregulation of PTEN and phosphorylation of Akt.

At least 16 different isoforms of Aiolos exist, resulting from alternate splicing, have different cellular localisations and have the ability to alter the localisation of other Ikaros members (13). However, the AIO1 transcript comprises >80% of the Aiolos isoforms in B-cells (14). To mimic the isoforms and cellular localizations of Aiolos in B-cells, the pWPT-PURO-GFP-Aiolos plasmid, containing the entire Aiolos coding sequence, was constructed and lentivirus-mediated transduction of Nalm-6 cells was performed to create a stable transfection cell line. The expression of the Aiolos isoform has been previously investigated in lymphoid development, however, its expression in unaffected Nalm-6 cells remains to be elucidated. In the present study, untransfected Nalm-6 cells expressed low levels of Aiolos at the RNA and protein levels. The results indicated that Nalm-6 cells were successfully transduced with the lentivirus and that Aiolos was successfully overexpressed in the Nalm-6 cells.

Aiolos is undoubtedly important in the control of mature B-lymphocyte differentiation and proliferation (15). Aiolos

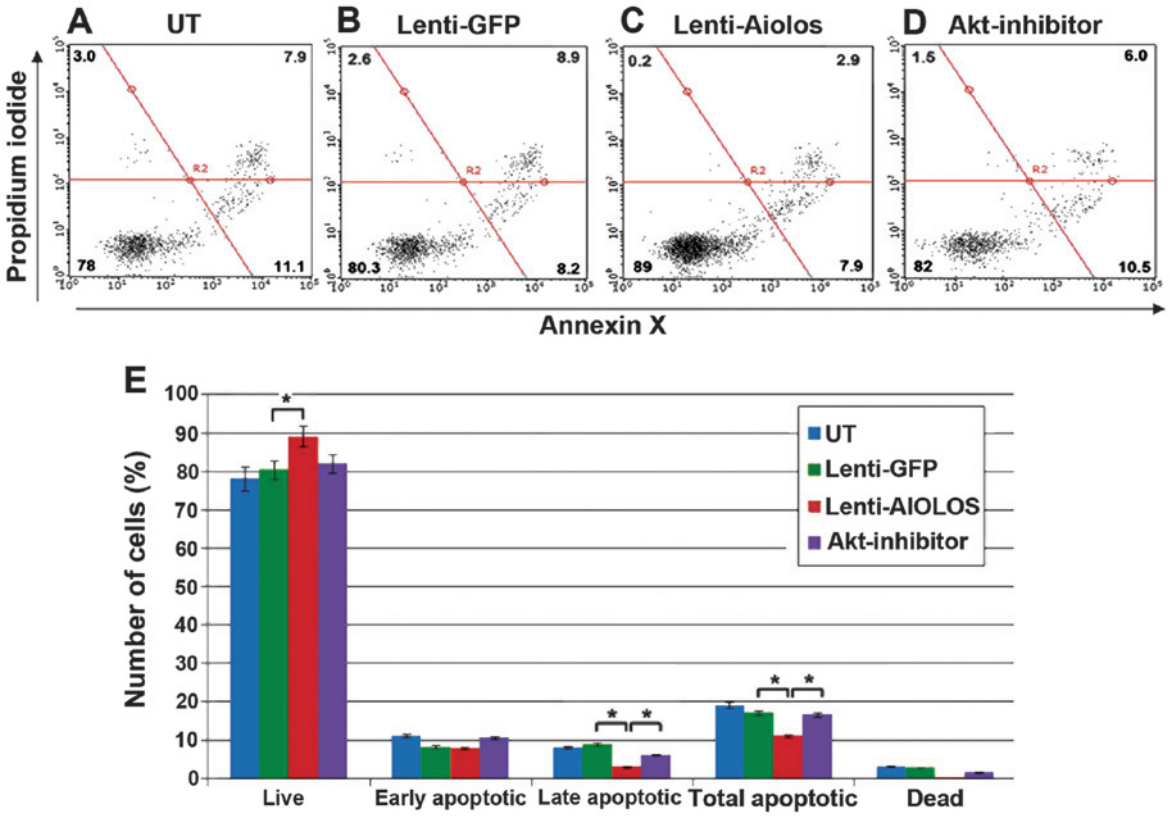

Figure 3. Effect of overexpression of Aiolos on Nalm-6 cell apoptosis. (A-C) Flow cytometric analysis demonstrated that the proportion of total apoptotic cells decreased in the Nalm-6 cells with increased expression of aiolos. (D and E) Total number of apoptotic cells decreased in Aiolos-transfected Nalm-6 cells compared with those in the Lenti-GFP group (*P<0.05). The difference between the Aiolos-transfected and the UT Nalm-6 cells in the percentage of early apoptotic cells was marginal, whereas the difference in the percentage of late apoptotic cells was significant (*P<0.05). Akti-1/2 effectively increased the percentage of apoptotic cells in the Aiolos-transfected Nalm-6 cells (*P<0.05 vs. Aiolos-transfected group). Error bars indicate the standard deviation of the mean of three experiments. GFP, green fluorescent protein; UT, untransfected control; Lenti-GFP, lentiviral vector control; Lenti-Aiolos, Aiolos-transfected.

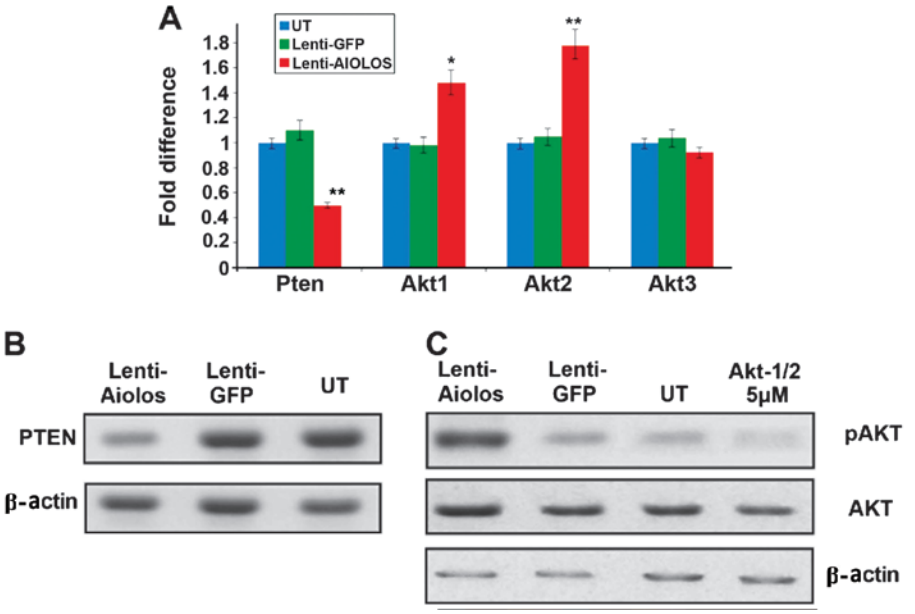

Figure 4. Upregulated Aiolos suppresses the expression of PTEN, but activated certain PI3K/Akt signalling pathway-associated genes in the Nalm-6 cells. (A) Reverse transcription-quantitative polymerase chain reaction revealed that the relative transcript levels of PTEN were markedly reduced in the Nalm-6 cells of the Lenti-Aiolos group compared with those in the Lenti-GFP group (**P<0.01). The upregulation of Akt1 (*P<0.05) and Akt2 (**P<0.01) was confirmed in the Lenti-Aiolos group compared with the control group. No significant change was observed in the expression of Akt3. β-actin was used as an internal control. Error bars indicate the standard deviation of the mean from three experiments. (B) Western blotting results revealed the expression of PTEN decreased in the Nalm-6 cells of Lenti-Aiolos group and that the (C) protein expression of Akt did not change considerably, whereas the levels of pAkt were significantly higher in the Lenti-Aiolos group compared with the control group. Treatment with 5 μM Akti-1/2 for 48 h effectively inhibited the phosphorylation of Akt. β-actin served as a loading control. PTEN, phosphatase and tensin homolog deleted on chromosome ten; PI3K, phosphatidylinositol-3-kinase; GFP, green fluorescent protein; pAkt, phosphorylated Akt; Lenti-GFP, lentiviral vector control; Lenti-Aiolos, Aiolos-transfected.

silences the expression of the surrogate light-chain gene λ5, thereby downregulating the pre-B-cell receptor (pre-BCR) (16). In addition, Aiolos directly binds to the c-Myc promoter and represses the expression of c-Myc in pre-B cells. Repression of c-Myc by Aiolos subsequently leads to the expression of p27 and the downregulation of cyclin D3 (17). P27 is a type of cyclin-dependent kinase inhibitor protein, which inhibits G1/S conversion and negatively regulates the cell cycle by inhibiting the functions of cell cycle regulators, including cyclin D3 (18,19). By contrast, pre-BCR, cyclin D3 and c-Myc are necessary for the proliferation of pre-B-cells (20-22). Consistent with results of previous studies, the present study demonstrated that overexpression of Aiolos suppressed proliferation of Nalm-6 cells. In addition, the results of cell cycle analysis indicated that upregulation of Aiolos arrested the Nalm-6 cells at the G0/G1 phase.

Previous tudies have confirmed that overexpression of Aiolos in Nalm-6 cells inhibits cell apoptosis, which is consistent with previous studies demonstrating that disruption of Aiolos accelerates premature B cell apoptosis, mediated by BCR signalling through elevation in the release of cytochrome *c* (23,24). However, different findings have been reported regarding the function of cyclin D3 in the apoptotic process of B cells. Wang *et al* (25) reported that downregulation of cyclin D3 increases the number of B-cell CLL cells undergoing apoptosis and another study suggested that repression of c-Myc and cyclin D3 is necessary to arrest human leukaemia cells at the G1 phase of the cell cycle, but neither is required for apoptosis (26). Therefore, in addition to c-Myc and cyclin D3, Aiolos potentially acts through other mechanisms to regulate the apoptotic process.

The present results demonstrate the crucial function of PTEN. The expression of PTEN was significantly reduced in Aiolos-overexpressed Nalm-6 cells. Previous studies have identified the functions of PTEN in leukaemogenesis (27-29), however, the functions of PTEN in association with Aiolos remain to be elucidated. PTEN was initially identified as a tumour suppressor, and loss or mutation of PTEN is reported to be associated with the development of various types of cancer (30,31). PTEN may exert this regulatory role by altering its own expression and activity in response to external stimuli, including cigarette smoking or cytokines (32-35). PTEN primarily acts downstream of various other pathways as a negative regulator of the PI3K pathway due to its lipid phosphatase activity. Loss of PTEN increases the level of PI3-phosphate, thereby mimicking the effect of constitutive PI3K activation (36-38). Accumulation of PI3-phosphate activates various protein kinases, including Akt kinases (39). In addition to the reduction in PTEN levels, the results of the present study demonstrated that the expression and phosphorylation of Akt increased. This was consistent with the findings of the previously mentioned studies (40). Akt is the central node in the PTEN-regulated pathway, and activated Akt can act on multiple downstream targets, which are involved in proliferation, cell metabolism and apoptosis (40). Thus, improved cell survival may be attributed to the activated Akt pathway in the Aiolos-overexpressed Nalm-6 cells.

Previous studies have demonstrated that, in addition to inhibiting apoptosis, activated Akt can promotes cell cycle progression and cell growth (41-43). However, the present study revealed that overexpression of Aiolos inhibited the growth of Nalm-6 cells and arrested the cells at the G0/G1 phase. Thus, the loss of PTEN may be insufficient to provide a proliferative advantage in BCP-ALL cells. Downregulation of c-Myc and cyclin D3 was sufficient for growth inhibition in the Aiolos-overexpressed Nalm-6 cells, despite proliferation signals, driven by the loss of PTEN. Subsequently, chemical inhibitors of Akt (Akti-1/2) were used to verify whether Akt is a key regulatory factor of apoptosis or growth regulation. The findings indicated that inhibiting the phosphorylation of Akt promoted the apoptosis of Aiolos-overexpressed Nalm-6 cells. Akti-1/2 inhibited Akt, however, it did not induce changes in proliferation or the cell cycle. The results further demonstrated that overexpression of Aiolos induced changes in PTEN, and that Akt affected BCP-ALL cell apoptosis. Certain previous studies have indicated that loss of PTEN, which positively regulates Akt signalling, leads to the expansion of leukaemic stem cell subpopulations (44). However, loss of PTEN does not exert a positive effect in promoting a short-term increase in the total number of cells.

In conclusion, the results of the present study indicated that upregulation of the expression of Aiolos in Nalm-6 cells inhibited cell proliferation, suppressed apoptosis and arrested the cell cycle at the G0/G1 phase. These findings revealed two critical functions of Aiolos in Nalm-6 cells; to limit pre-B-cell expansion via c-Myc and cyclin D3-dependent pathways and to improve pre-B cell survival via PTEN- and Akt-dependent processes. Characterising these potential genetic interactions may provide a foundation for further investigation of the pathogenesis of BCP-ALL.

Acknowledgements

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