DADS downregulates the Rac1-ROCK1/PAK1-LIMK1-ADF/cofilin signaling pathway, inhibiting cell migration and invasion

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Abstract. The aim of this study was to explore the molecular mechanisms of the diallyl disulfide (DADS)-mediated downregulation of LIM kinase-1 (LIMK1) and the consequent inhibition of the migration and invasion of human colorectal cancer cells. RNA interference technology was used to establish stable LIMK1-miRNA/SW480 cell lines. The effects of DADS and LIMK1 RNA interference on the migration and invasion of SW480 cells were observed by scratch wound healing assay and Transwell migration assay. The effects of DADS on signaling molecules of the Rac1-Rho kinase (ROCK)1/p21-activated kinase (PAK)1-LIM kinase (LIMK)1-actin depolymerizing factor (ADF)/cofilin pathway in SW480 cells were examined by RT-PCR and western blot analysis. The healing and migration rate of the SW480 cells was significantly reduced and the cell penetrating ability was significantly suppressed ($P<0.05$) following treatment with DADS (45 mg/l). The immunohistochemistry and western blot analysis results showed that DADS significantly downregulated LIMK1 protein expression and suppressed LIMK1 protein phosphorylation. Furthermore, the RT-PCR and western blot analysis results revealed that DADS suppressed Rac1, ROCK1, PAK1, LIMK1 and destrin mRNA and protein expression, as well as the protein phosphorylation of LIMK1 and cofilin 1. The data demonstrate that LIMK1 expression positively correlates with the SW480 cell migration and invasion ability. DADS downregulates the Rac1-ROCK1/PAK1-LIMK1-ADF/cofilin signaling pathway, suppressing SW480 cell migration and invasion.

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Introduction

Colorectal cancer is a common type of cancer, ranking third in the cause of cancer-related mortality with its incidence mortality rates gradually rising (1). Metastasis occurs in approximately 50% of colorectal cancer patients within 5 years, which is the leading cause of mortality. At present, surgery remains the primary option; however, recurrence rates are relatively high. Therefore, the study of the factors and mechanisms relating to the migration and invasion of colorectal cancer cells are of utmost importance in order to develop a new generation of drugs that would be more effective in suppressing tumor metastasis with low toxicity.

Tumor metastasis is a complex process that involves complex biochemical and biomechanical changes. Invadopodia (plate pseudopodia and filopodia) are formed during cell migration and invasion. Invadopodia are primarily composed of the actin network, and the regulatory gene involved has gained increasing attention (2,3). It has been found that the LIM kinase (LIMK) affects the activity of the actin cytoskeleton by regulating the cofilin family proteins, thus affecting tumor cell migration and invasion (4-7). The LIMK family, including LIMK1 and 2, is a type of serine protein kinase, and is associated with muscle actin polymerization and microtubule decomposition (5,6). The p21-activated kinase (PAK)1, 4 and the Rho kinase (ROCK) are activated by the phosphorylation of threonine residues in the LIMK ring, thus regulating the phosphorylation and dephosphorylation balance of the actin depolymerizing factor (ADF/cofilin), as well as the actin cytoskeleton (5,6). It has been found that the phosphorylation of ADF/cofilin mediated by LIMK1 is associated with tumor cell migration and invasion (7,8). However, the correlation of LIMK expression with the migration and invasion of colorectal cancer cells reamins unclear. Garlic has been shown to exert anti-tumor effects, with its main active ingredient, allyl sulfides, belonging to γ-glutamylcysteine (9). Diallyl disulfide (DADS), one of the major fat-soluble active ingredients in garlic, has been shown to significantly suppress the growth of breast cancer, gastric cancer, leukemia, lung cancer and other tumor cells (9-13). Thus, it has great potential for use in the development of novel anticancer drugs.

In our previous studies, we showed the significant suppressive effects of DADS on the proliferation and induction of SW480 and HT-29 human cell apoptosis *in vitro* and *in vivo*.

The mechanisms involved were related to cell cycle arrest, the downregulation of p53, proliferating cell nuclear antigen (PCNA), Bcl-2, cyclin E and C-myc, and the upregulation of p21(WAF1) and Bax (14-16). Proteomics studies demonstrated that DADS significantly downregulated LIMK1 and its downstream target molecules of ADF/cofilin (16). In our previous studies (17), we showed that DADS suppressed the migration and invasion of SW480 human colorectal cancer cells. These suppressive effects may correlate with LIMK1, destrin and p-cofilin 1 expression. In another study of ours, we found that DADS significantly downregulated Rac1 gene expression in colorectal cancer cells (18).

Rac belongs to the Rho GTPase family, and indirectly activates LIMK1 through PAK1. The activation of LIMK1 by PAK1 couples Rac/Cdc42 GTPase signaling with actin cytoskeletal dynamics. Therefore, in this study, we further investigated the suppressive effect of DADS on the migration and invasion of SW480 cells. We hypothesized that LIMK1 may be the target molecule of DADS for the suppression of the migration and invasion of human colorectal cancer cells. Thus, DADS primarily negatively regulates the Rac1-ROCK1/PAK1-LIMK1-ADF/cofilin signaling pathway, suppressing the migration and invasion of SW480 cells.

Materials and methods

Cells and plasmids. The SW480 human colorectal cancer cell line was kindly provided by the Cancer Institute of Xiangya Medical College, Changsha, China. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Gibco) with 5% CO_2 at 37˚C. The pcDNA™6.2-GW/EmGFPmiR LIMK1-microRNA (miRNA)-expessing plasmid was provided by Invitrogen Corp. The stable low-level expression of the LIMK1 gene in the SW480 cells was achieved by the transfection of recombinant plasmid (pcDNA6.2/LIMK1-miRNA).

Reagents. DADS and Tween-80 were dissolved at a ratio of 1:2, and stored at -20˚C after a 100-fold dilution with saline. RPMI-1640 medium was provided by Gibco. Matrigel (5 mg/ml) was provided by the BD Company. Transwell chambers (3428 type) were provided by Corning. The total RNA kit was purchased from Omega, the RT reagent kit from Takara, the PCR kit from Promega, and the ECL luminescence detection and immunohistochemistry kits were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. Rabbit polyclonal antibody against LIMK1 (ab39641) was purchased from Abcam. Rabbit polyclonal antibody against cofilin 1, phosphorylated rabbit polyclonal antibody against LIMK1 (phospho T508), phosphorylated polyclonal antibody against cofilin 1 (S3), goat anti-rabbit secondary antibody and goat anti-mouse secondary antibody were provided by Abzoom Biolabs (Dallas, TX, USA). Rabbit polyclonal antibodies against PAK1 and ROCK1 were provided by Epitomics Inc., rabbit polyclonal antibody against destrin was provided by Abcam and rat monoclonal antibody against Rac1 was provided by Millipore.

Scratch wound healing assay. The cell concentration was adjusted to 1x10^6/ml. Cell suspension of 1 ml was cultured in a 6-well plate with serum-free DMEM for 6 h, in adherent monolayer. A 10-μl Eppendorf tip was used to scratch the cells in the plates and the cells were then washed 3 times with serum-free medium. The cells were then placed in fresh serum-free medium and divided into 6 groups as follows: the control group (SW480), DADS-treated SW480 group (SW480 + DADS), vector group (Vector), DADS-treated vector group (Vector + DADS), LIMK1-miR group (LIMK1-miR) and DADS-treated LIMK1-miR group (LIMK1-miR + DADS). Samples were taken at the beginning and at 24 h after culture with 5% CO_2 at 37˚C. Images of the scratch wounds were taken and measured by Image-Pro Plus 6.0 software to calculate the mean and standard deviation. Each group was compared with the control group (SW480). The experiments were repeated 3 times. Cell migration was expressed as the migration rate: (original scratch width - new scratch width)/original scratch width x100%.

Table I. Primers used in this study.

Primers	Sequences (5'→3')
LIMK1F	GGGGCATCATCAAGAGCA
LIMK1R	GAGGACTAGGGTGGTTCAG
Rac1F	CCCTATCCTATCCGCAAACA
Rac1R	CGCACCTCAGGATACCACTT
ROCK1F	AAAACCTTATTTGTGCCTTCC
ROCK1R	CGTTTCCCAAGCCCACT
PAK1F	AAGACATCCAACAGCCAGAA
PAK1R	TGTAGCCACGTCCCGAGT
DestrinF	TGGTTGGAGATGTTGGTG
DestrinR	ACAAGCCCGATTGAGAT
Cofilin 1F	CAAGAAGGCGGTGCTCT
Cofilin 1R	ACAAAGGTGGCGTAGGG
β-actinF	ACACTGTGCCCATCTACGAGGGG
β-actinR	ATGATGGAGTTGAAGGTAGTTTCGTGGAT

Transwell migration assay. For the cell invasion assay *in vitro* we used 24-well Transwell chambers with a pore size of 8.0 μm. Cells were dived into 6 experimental groups as described above. Experimental procedures were as follows: cell density was adjusted to 1x10^6/ml. Cell suspension (100 μl) was added to the upper chamber, and 500 μl of complete medium were added to the lower chamber. The medium was disposed of 24 h after incubation at 37˚C. Cells were fixed for 10 min by 4% paraformaldehyde, the plates were then inverted and the cells were air-dried. The cells were stained in 0.1% crystal violet solution for 20 min and washed with PBS 3 times. The wells were gently wiped with a swab. Cell numbers were counted using an inverted microscope at x200 magnification with 10 fields of view, and the mean values were taken as the invasive cell number. The experiment was repeated 3 times. Invasion suppression rate = [(penetration cell number in control group - penetration cell number in treated group)/penetration cell number in control group] x100%.

RT-PCR. An RNA extraction kit was used to extract total RNA. The cDNA was produced by reverse transcription. The primers use are presented in Table I. PCR products were separated on a 2% agarose gel, images were obtained using the Bio-Rad gel

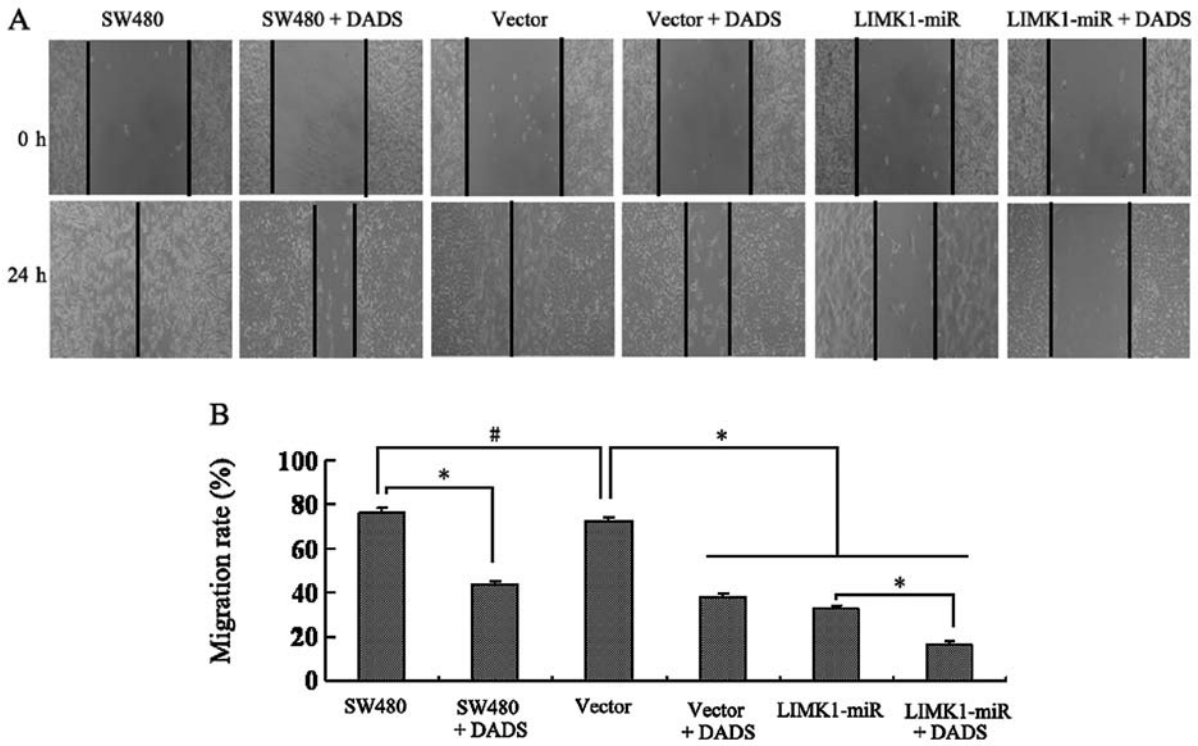

Figure 1. Effects of DADS treatment and LIMK1 gene silencing on the migration of SW480 cells. (A) Scratch wound healing assay was performed to examine the effects of DADS treatment and LIMK1 gene silencing on SW480 cell migration. (B) Mobility rate histograms of each group. *P<0.05, #P>0.05.

imaging system, and scanned by AlphaImager 2200 software. The gene expression abundance was expressed as the relative optical density (ROD), and the average optical density value was calculated with β-actin as the internal control.

Western blot analyses. Total cellular proteins were extracted. After electrophoresis, the proteins were electrotransferred onto a PVDF membrane. Goat anti-rabbit secondary antibody or goat anti-mouse secondary antibody (1:1,000) was used. β-actin was used as the loading control. Determination of the optical density of the imprinted zone was analyzed by AlphaImager 2200.

Statistical analyses. Data were analyzed using one-way Anova and the Bonferroni method (homogeneity of variance) or the t-test with SPSS 13.0 software. P<0.05 was considered to indicate a statistically significant difference.

Results

DADS suppresses SW480 cell migration. To determine whether DADS suppresses SW480 cell migration, scratch wound healing assay was performed. The scratch wounds were almost the same size in each experimental group at 0 h; however, the healing and cell migration rate was significantly reduced (P<0.05) in the DADS (45 mg/l) treatment group after 24 h (Fig. 1). Compared with the control and empty vector groups, the healing rate was slower, and the cell migration rate was significantly decreased (P<0.05) in the LIMK1 gene silenced group. Furthermore, DADS enhanced the suppressive effect (P<0.05). The results showed that LIMK1 gene expression positively correlated with SW480 cell migration ability, and DADS suppressed SW480 cell migration, which may be a result of the downregulation of LIMK1 gene expression.

DADS suppresses SW480 cell invasion. To determine whether DADS suppresses SW480 cell invasion, we performed invasion assays. The results (Fig. 2) showed that cell penetrating ability was significantly suppressed and that the number of penetrating cells number was significantly decreased (P<0.05) in all the experimental groups with treated with DADS (45 mg/l) for 24 h, compared with the non-treated groups. When LIMK1 gene expression was knocked down by miRNAs, the number of penetrating cells decreased significantly and the cell penetrating ability was significantly suppressed (P<0.05) compared to the control and empty vector groups. Following DADS treatment, cell invasion ability was significantly suppressed (P<0.05). These results show that LIMK1 gene expression positively correlates with SW480 cell invasion ability, and that DADS suppresses SW480 cell invasion, which may be associated with the downregualtion of LIMK1 gene expression.

DADS downregulates LIMK1 expression and suppresses the phosphorylation of LIMK1 protein. To determine whether DADS affects the expression of LIMK1 and phosphorylated LIMK1 (p-LIMK1) in SW480 cells, we performed western blot analyses. As shown in Fig. 3A, apart from the LIMK1-miR and LIMK1-miR + DADS groups, LIMK1 and p-LIMK1 protein expression was significantly decreased (P<0.05) in the groups treated with DADS (45 mg/l) in the absence of LIMK1-miR. However, in the LIMK1 gene silenced group, DADS had little effects on LIMK1 and p-LIMK1 protein expression (P>0.05) due to LIMK1 gene silencing.

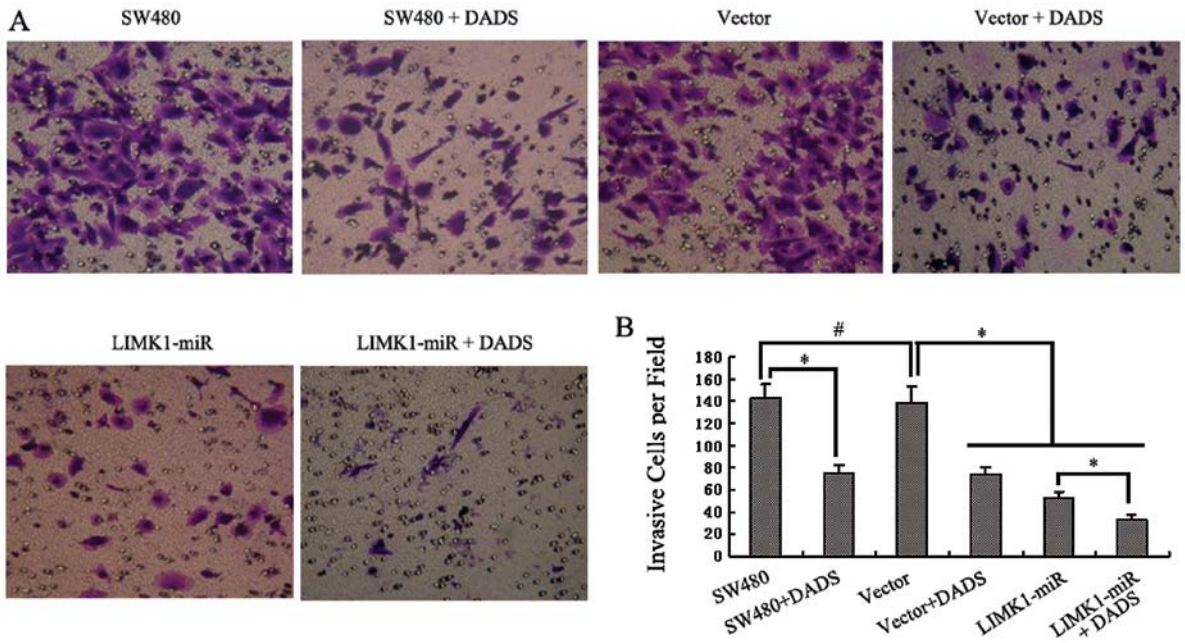

Figure 2. (A) Effects of DADS treatment and LIMK1 gene silencing on SW480 cell invasion. (B) Histograms showing the numbers penetrating cells in each group.*P<0.05, #P>0.05.

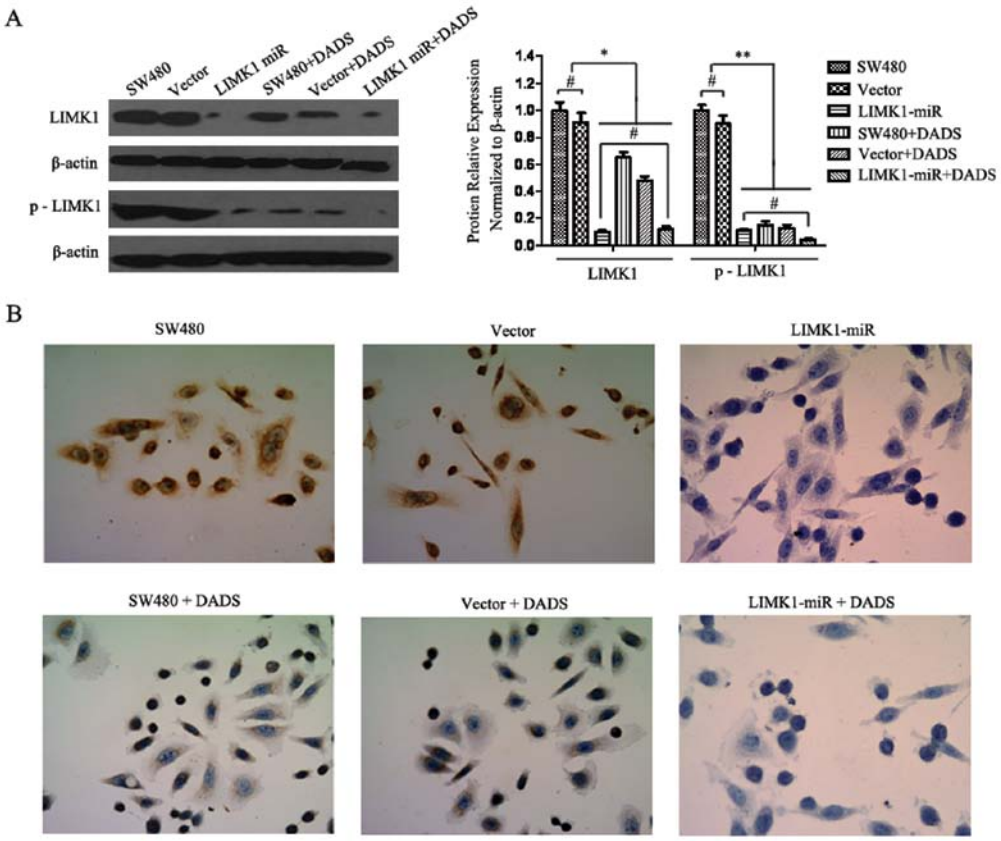

Figure 3. Effects of DADS treatment and LIMK1 gene silencing on LIMK1and p-LIMK1 protein expression in SW480 cells. (A) Western blot analysis was carried out to determine the effects of DADS treatment and LIMK1 gene silencing on p-LIMK1 protein expression. (B) Immunohistochemistry analysis was used to determine the effects of DADS treatment on LIMK1 protein expression. *P<0.05, #P>0.05.

In order to further determine whether DADS affects LIMK1 expression in SW480 cells, we performed immunohistochemical experiments. Immunohistochemical analysis revealed that DADS treatment decreased the LIMK1 and p-LIMK1 protein expression (Fig. 3B). In the presence of LIMK1-miR, however, the effect of DADS on LIMK1 and

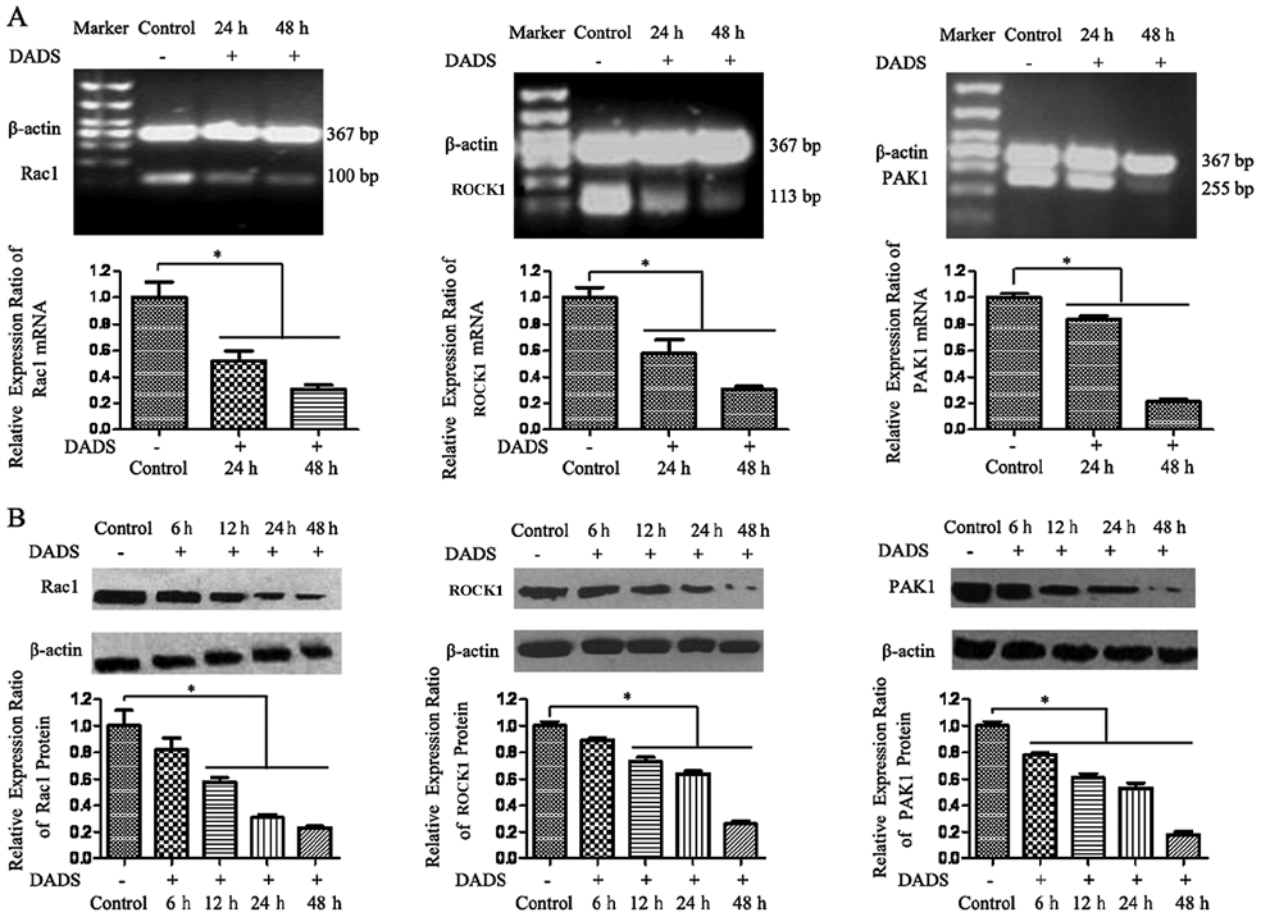

Figure 4. Effects of DADS treatment on Rac1, ROCK1 and PAK1 expression. (A) RT-PCR was used to examine the effect of DADS treatment on Rac1, ROCK1 and PAK1 mRNA expression. (B) Western blot analysis was used to examine the effect of DADS treatment on Rac1, ROCK1 and PAK1 protein expression. *P<0.05.

p-LIMK1 protein expression was undetectable (Fig. 3B). These results are consistent with the results shown in Fig. 3A. Taken together, the above results suggest that DADS downregulates LIMK1 protein expression and suppresses LIMK1 protein phosphorylation.

DADS suppresses LIMK1 expression by downregulation of Rac1-ROCK1/PAK1 expression. It was found that LIMK is regulated by various upstream signaling pathways, but mainly the Rho GTPase pathway. Our previous study using suppression subtractive hybridization showed that DADS significantly downregulated Rac1 gene expression in colorectal cancer cells (18). Therefore, we hypothesized that the downregulation of LIMK1 protein expression by DADS may be associated with the downregulation of the Rho GTPase pathway. Rac1, ROCK1 and PAK1 mRNA expression in SW480 cells was significantly decreased (P<0.01) at 24 and 48 h after the treatment with DADS (45 mg/l), as shown by RT-PCR analysis, compared with the control group (Fig. 4A).

The results obtained from western blot analyses (Fig. 4B) also demonstrated that Rac1, ROCK1 and PAK1 mRNA expression in SW480 cells was significantly decreased following treatment with DADS (45 mg/l) for 6, 12, 24 and 48 h (P<0.01) in a time-dependent manner, compared with the control group. These results suggest that DADS downregulates the expression of Rac1-ROCK1/PAK1, inhibiting LIMK1 expression.

DADS suppresses ADF/cofilin 1 expression by the downregulation of LIMIK1 expression. It has been shown that LIMK phosphorylation regulates the phosphorylation and dephosphorylation of the ADF/cofilins and affects the actin cytoskeleton structure (22). Our previous proteomics study showed that DADS significantly decreased ADF/cofilin expression (16). Therefore, we further examined the effects of DADS on ADF/cofilin expression. The results from RT-PCR analysis (Fig. 5A) showed that destrin mRNA expression was significantly suppressed (P<0.01) at 24 and 48 h after treatment of DADS (45 mg/l), compared with the control group. However, the cofilin 1 mRNA expression levels were not significantly affected by DADS treatment when comparing the levels between the groups with or without DADS treatment. Western blot analyses (Fig. 5B) revealed that compared with the control group, destrin protein expression was deceased in a time-dependent manner (P<0.05) following treatment with DADS (45 mg/l) at 6, 12, 24 and 48 h. Although cofilin 1 protein expression levels were not affected, p-cofilin 1 expression was deceased in a time-dependent manner. In addition, DADS downregulated p-LIMK1 protein expression (P<0.05) in a time-dependent manner (Fig. 5C). These results suggest that

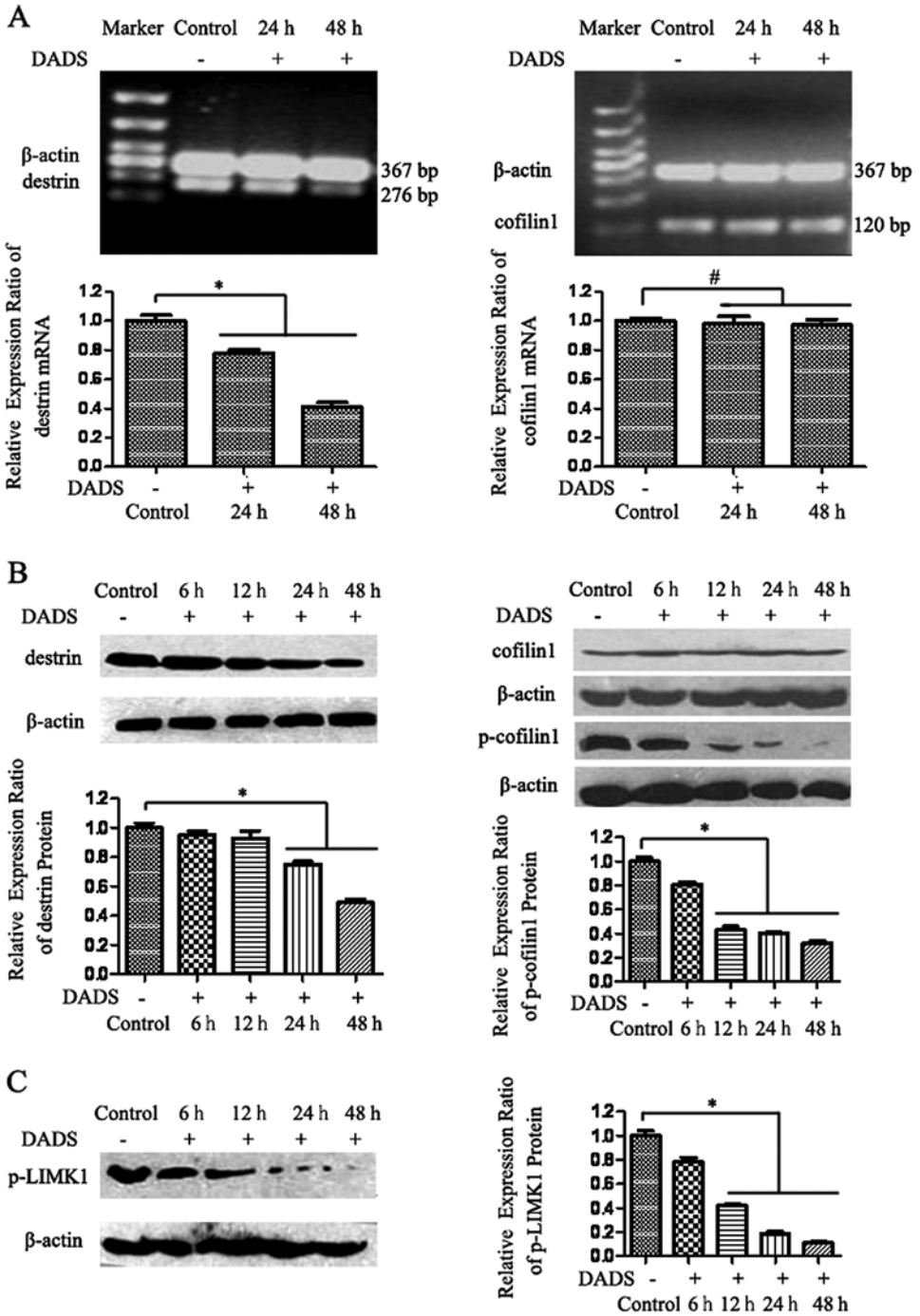

Figure 5. Effects of DADS treatment on ADF/cofilin 1 expression. (A) RT-PCR was used to examine the effects of DADS on ADF/cofilin mRNA expression. (B) Western blot analysis was used to examine the effect of DADS treatment on ADF/cofilin protein expression. (C) Western blot analysis was used to examined the effect of DADS treatment on p-LIMK1 protein expression. $^{*}P<0.05$, $^{\#}P>0.05$.

DADS inhibits ADF/cofilin 1 expression by downregulating LIMK1 expression.

Discussion

The migration and invasion ability of tumor cells are the key factors affecting distant tumor metastasis. Several studies have indicated that the reorganization of the actin cytoskeleton is the basis of tumor cell migration, adhesion and invasion (4,19,20). A number of molecules are involved in the regulation of actin polymerization and depolymerization, in which the LIMK gene plays an important role (8,21).

The LIMK family, including LIMK1 and 2, belongs to the serine protein kinase family and is associated with the regulation of actin polymerization and microtubule decomposition (5,21). LIMK1 is mainly involved in tumor cell invasion and migration. LIMK1 regulates ADF/cofilin to reorganize the actin cytoskeleton, thus promoting tumor cell migration and invasion (22,23). The ectopic expression of LIMK1 activates the metastatic ability of benign prostate cells. The

downregulation of LIMK1 by suppressors or RNAi interference suppresses tumor cell migration and invasion (23).

DADS is a fat-soluble, active ingredient in garlic that suppresses the growth of various tumors. Based on epidemiological study, the incidence and mortality rates of colorectal cancer patients are lower with the consumption of garlic (24). Our previous studies showed the significant suppressive effect of DADS on human colorectal cancer cell proliferation, blocking the cells in the G2/M phase (14,15). Proteomics studies demonstrated that DADS significantly downregulated LIMK1 expression and its downstream target, ADF/cofilin (16). In this study, the immunocytochemistry results showed the high expression of LIMK1 in SW480 human colorectal cancer cells, and the significant suppressive effect of DADS on LIMK1 gene expression at the mRNA and protein levels. Further analyses showed that following DADS treatment or LIMK1 gene silencing, the migration and invasion ability of the SW480 cells was compromised. When LIMK1 gene silencing and DADS treatment were applied simultaneously, the suppressive effect was more profound. Therefore, LIMK1 expression positively correlates with the migration and invasion ability of SW480 cells, and DADS suppresses SW480 cell invasion and migration, which may be associated with the downregualtion of LIMK1 gene expression.

LIMK1 is regulated by various upstream signaling pathways, and the Rho GTPase family is mainly involved in migration and invasion (25). The Rho GTPase family, including Rho, Rac and Cdc42, is activated by different transmembrane receptors, and signals the downstream effector proteins, ROCK1 and PAK1 (26). The ROCK1 gene encodes a protein serine/threonine kinase that is activated when bound to the GTP-bound form of Rho. Rac indirectly activates LIMK1 through PAK1. ROCK1 and PAK1 connect to the active GTPase and their conformational changes lead to the phosphorylation of LIMK1 at threonine 508. Thereafter the third serine on cofilin 1 is phosphorylated, eventually leading to changes in actin dynamics and resulting in the formation of the Rho-of Rac1-ROCK1/PAK1-LIMK1-ADF/cofilin signaling pathway (27-30). ADF/cofilin is an ADF, and the ADF/cofilin family, including destrin, cofilin 1 and cofilin 2 (31), is the key regulator of the actin cytoskeleton that stimulates actin depolymerization and separation. Its activity is associated with the malignancy and invasion ability of cancer cells (32,33). LIMK regulates the phosphorylation and dephosphorylation balance of ADF/cofilin, and affects the actin cytoskeletal structure (31). The phosphorylation and activation of LIMK1 in turn phosphorylates ADF/cofilin, promoting tumor cell migration, and the silencing of ADF or cofilin 1 by siRNA significantly reduces tumor cell migration (34).

Our previous study using suppression subtractive hybridization demonstrated that DADS significantly downregulated Rac1 gene expression in colorectal cancer cells (18), suggesting that the downregulation of LIMK1 protein expression by DADS may be associated with downregulation of the Rho GTPase pathway. In addition, in our previous proteomics study, we found that DADS significantly downregulated LIMK1 expression and its downstream target molecules of ADF/cofilin (16). Therefore, in this study, we examined the effect of DADS on the Rac1-ROCK1/PAK1-LIMK1-ADF/cofilin signaling pathway. The results showed that the DADS suppression of Rac1, ROCK1, PAK1, LIMK1, destrin mRNA and protein expression, and the protein phosphorylation of LIMK1 and cofilin 1 was time-dependent. Therefore, these data indicate that DADS negatively regulates the Rac1-ROCK1/PAK1-LIMK1-ADF/cofilin signaling pathway, and that LIMK1 may play a key role in the migration and invasion ability of cancer cells.

In conclusion, DADS suppresses the migration and invasion of human colorectal cancer cells by negatively regulating the Rac1-ROCK1/PAK1-LIMK1-ADF/cofilin signaling pathway.

Acknowledgements

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