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Diallyl disulfide inhibits proliferation, epithelial–mesenchymal transition, and invasion through RORα-mediated downregulation of Wnt1/β-catenin pathway in gastric cancer cells

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Abstract

Overactivation of Wnt/β-catenin pathway due to dysfunction of retinoid-related orphan receptor α (RORα) is related to cancer development and progression. Diallyl disulfide (DADS), an active component of garlic, has been reported in our previous study for upregulation of RORα expression in gastric cancer (GC) cells. It remains to be elucidated the role and mechanism of RORα in DADS against GC. This study revealed that DADS treatment resulted in reduced expression levels of Wnt1, β-catenin, TCF-4, intranuclear β-catenin and p-β-catenin in GC cells, concomitant with the compromised expression of β-catenin target genes (Axin, c-Jun, and c-Myc). RORα overexpression augmented DADS-induced downregulation of Wnt1/β-catenin pathway, G2/M phase arrest, and cell growth inhibition in vitro and in vivo. Contrarily, knockdown of RORα attenuated these effects of DADS. Interestingly, DADS induced an increase in the binding of RORα to β-catenin, which may lead to reduction of β-catenin phosphorylation and nuclear translocation. This interplay modulated by DADS may affect β-catenin target gene expression for that the opposite results were observed in DADS-treated RORα knockdown and overexpression cells. DADS caused a decrease in vimentin, snail and MMP-9, as well as an increase in E-cadherin and TIMP3 expression, which restricted epithelial–mesenchymal transition (EMT), migration, and invasion. The aforementioned effects of DADS were weakened simultaneously when the suppression of DADS on the Wnt1/β-catenin pathway was resisted by knockdown of RORα. In contrast, overexpression of RORα enhanced the effects of DADS. Therefore, RORα-mediated downregulation of Wnt1/β-catenin pathway could undertake an important role in anticancer activity of DADS against GC cell proliferation, EMT, migration, and invasion.

Keywords: Diallyl disulfide, Gastric cancer cell epithelial–mesenchymal transition, Proliferation, RORα, Wnt/β-catenin pathway

Abbreviations: DADS, diallyl disulfide; EMT, epithelial–mesenchymal transition; GC, gastric cancer; MMP-9, matrix metalloproteinase-9; RORα, retinoid-related orphan receptor α; RT-PCR, reverse transcription-polymerase chain reaction; TCF-4, T cell factor-4; TIMP3, tissue inhibitor of metalloproteinase 3.

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1. Introduction

Gastric cancer (GC) remains one of the most frequent cancers accounting for 8.2% of global cancer deaths in 2018 [1], and it was the second leading cause of cancer mortality in China in 2015 [2]. Early surgery is an effective treatment, but the majority of diagnosed cases are at an advanced stage, with a 5-year survival rate less than 10% [3]. Thus, it is still urgent to explore the potential molecular targets for prevention and therapy of GC.

Diallyl disulfide (DADS), a sulfur compound extracted from garlic, shows inhibitory activities against numerous tumors through various mechanisms [4]. We previously demonstrated that DADS restrained invasion and migration of GC cells, and obtained DADS-induced differential protein expression profile which presented an increase of retinoid-related orphan receptor α (RORα) expression in GC cells via proteomic approaches [5], however, it remains to be determined whether upregulation of RORα by DADS is implicated in its anti-GC activity.

RORα, one of the orphan nuclear receptors, emerges as a critical role in the signal integration and coordinating gene expression through interplay with co-regulators [6]. The mounting evidence indicates that RORα downregulation and hypo-activation are implicated in tumorigenesis and progression, and it could identified as a potential therapeutic target for many solid tumors [6]. Low expression of RORα is associated with aggressive cell phenotypes and unfavorable prognosis in breast cancer and hepatocellular carcinoma, while restoring its expression can inhibit cell proliferation, invasion, and migration, which is associated with its modulating expression and/or function of oncogenes and tumor suppressor genes (β-catenin, SEMA3F, p53, etc.) [7–9]. Interestingly, RORα phosphorylated by the Wnt5a/PKCα pathway has been reported to exert its transrepression activity via binding β-catenin competitively on the promoter with co-activator in colon cancer cells [10]. RORα binding to β-catenin affects the transcripational activity of β-catenin, but it is unclear whether this interaction also limits β-catenin nuclear translocation. Therefore, it is necessary to further elucidate the mechanism by which RORα regulates β-catenin signaling pathway.

DADS inhibits growth, epithelial–mesenchymal transition (EMT) of breast cancer cells through inactivating β-catenin pathway [11]. Our studies have shown that DADS represses proliferation and induces apoptosis through restraining Wnt1/β-catenin pathway in GC cells via miR-200b and miR-22 [12], and prevents upregulation of β-catenin via negatively regulating TGF-β1/Rac1 pathway, prohibiting EMT and tumor growth [13]. Thus, it is worth determining whether RORα participates in the anti-GC effects of DADS through Wnt1/β-catenin pathway.

In this study, the role of RORα was identified in the inhibition of cell proliferation, EMT, migration, and invasion by DADS. Furthermore, we investigated the underlying mechanism by which RORα was involved in DADS antagonizing Wnt1/β-catenin pathway and β-catenin target gene expression in GC cells.

2. Materials and methods

2.1. Reagents and antibodies

DADS, purchased from Fluka Co. (Milwaukee, Wisconsin, USA), was dissolved in Tween-80 and stored at −20 °C after a 100-fold dilution with saline. The primary antibodies against RORα (ab60134), MMP-9 (ab38898), TIMP3 (ab39184), vimentin (ab92547), E-cadherin (ab40772), snail (ab53519), Ki-67 (ab66155), and CD34 (ab81289) were provided by Abcam (Cambridge, MA, UK). The primary antibodies against Wnt-1 (sc-514531), β-catenin (sc-1496), Axin (sc-14029), c-Jun (sc-44), c-Myc (sc-40), TCF-4 (sc-271287), β-actin (sc-8432) and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibody against p-β-catenin was purchased from Cell signaling technology (Danvers, MA, USA). pGL3-c-Myc promoter luciferase reporter plasmid was obtained from Guangzhou Cyagen Biosciences Inc.

2.2. Cell culture and cell line establishment

Human GC cell lines (MGC803 and SGC7901) were obtained from the Cancer Research Institute, Central South University in China. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Vienna, Austria), 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C under a humidified atmosphere of containing 5% CO2. The pcDNA™6.2-GW/EmGFPMiR RORα-microRNA (miR)-expressing and pcDNA3.1 RORα-expressing plasmids were constructed by Invitrogen Corporation. Sequences of DNA oligomers inserted into pcDNA™6.2-GW/EmGFPMiR were listed as follow: F: 5’-TGCTGTTGATGCGACACAAATGCGAATTTGGCCACTGACTGACTGCGAATTGTCATCAA-3’,
2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from SGC7901 cells using Trizol reagent (Invitrogen). Reverse transcription was carried out using the RT-PCR system (Promega) following the manufacturer's instructions. The mRNA levels were normalized to β-actin. The RORα, c-Jun: R: 5′-CAGGATGGCAGAGGGTTCATG-3′; GAGGGTTCATG-3′, and the expression changes were calculated through relative quantification to the control group.

2.4. Western blot and Co-immunoprecipitation

For total protein extraction, the cell lysates were harvested in RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1m M NaF, 1m M Na3VO4, and complete protease inhibitor cocktail). Nuclear extracts were prepared according to the manufacturer's instructions using NE-PER® Kit (Pierce, Rockford, IL). Co-immunoprecipitation was conducted using Protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The pellets were washed three times using RIPA buffer. The immunoprecipitated proteins were analyzed by Western blot.

Proteins were resolved on 10% SDS–PAGE gel and transferred onto PVDF membrane. The blots were blocked in 5% skim milk in Tris buffered saline (TBS) containing 0.1% Tween 20 for 2 h at room temperature, and then incubated with the primary antibody at 4 °C overnight. After washed with TBS-T, the membranes were incubated with HRP-conjugated secondary antibody. The membranes were developed by an enhanced chemiluminescence plus (ECL Plus) kit (Amersham Biosciences, Buckinghamshire, England). The target protein amounts were normalized to β-actin and the expression changes were calculated through relative quantification to the control group.

2.5. Cell proliferation and cell cycle analysis

Cell proliferation was evaluated by MTS and EdU assays. MTS assay was performed using a CellTiter 96 AQ One Solution Cell Proliferation Assay kit (MTS, Promega, Madison, USA) according to the manufacturer's instructions. Cells were cultured in 96-well plates at 1 × 104 cells per well. Transfected and untransfected cells were treated with 30 mg/L DADS for 24 h or left untreated, and the absorbance was recorded at 490 nm using an ELISA plate reader. Each assay was replicated 5 times.

EdU assay was conducted with an EdU kit (RiboBio, Guangzhou, China). The untreated and treated cells were incubated with the diluted EdU medium for 2 h, and then washed in PBS and subjected to DAPI staining. Finally, the images were taken and analyzed using an inverted microscope (Olympus, Tokyo, Japan). All the assays were repeated three times.

For cell cycle analysis, cells were fixed in ice-cold 70% ethanol and stained with propidium iodide (PI). The cell cycle profiles were assayed using Elite ESP flow cytometry at 488 nm and the data were analyzed using CELL Quest software (BD Biosciences, San Jose, CA, United States).

2.6. Luciferase reporter assay

Briefly, 3 × 104/well cells were plated in 24-well plates. Then, c-Myc-pGL-3 plasmids were transfected into cells using Lipofectamine 2000. After
cells were treated or left untreated with DADS for 24 h, luciferase activities were analyzed with a liquid scintillator. The reporter activity was normalized to the control renilla luciferase activity.

2.7. Cell migration and invasion assays

Cell migration and invasion assays were performed using Transwell® plates (Corning, Corning, NY) as previously described [13]. In brief, SGC7901 cells were seeded onto the Matrigel-uncoated filters (8-μm pore size), then were treated or left untreated with DADS for 24 h. The cells that had migrated the lower surface of the filter were fixed and stained with hematoxylin. The migrated cells were counted in four microscopic fields per well, and the extent of migration was expressed as an average number of cells per microscopic field. Migration rates are expressed as the ratio of the treated group value to the control group value. Invasion assays were done using the same procedure as for migration assay, except using the Matrigel-coated filters.

2.8. Animal models of tumor

Transfected and untransfected SGC7901 cells were injected into the subcutis of the right axillary of male athymic BALB/c nude mice. Intraperitoneal injection of saline or DADS (100 mg/kg) was performed every 2 days until the end of the experiment. Tumor volume (mm³) was calculated using a standard formula (width² × length × 0.5) every 7 days. Average tumor volumes are displayed (n = 5 for each group) from the 7th day and continued until the 70th day. The weight of the xenograft was measured on the 70th day. All experiments were performed according to the guidelines for animal use of the Ethics Committee of University of South China.

2.9. Immunohistochemistry

The sections were prepared from the transplanted tumor tissues. After dewaxing the slide in xylene and hydration in a graded alcohol solution, antigen repair was performed by heat treatment in 10 mM sodium citrate buffer (pH 8.0). The slides were placed in 3% H₂O₂ solution to quench endogenous peroxidase activity, and then blocked with normal goat serum. The slides were incubated with primary antibodies (dilution 1:100) at 4 °C overnight. Positive signals were developed with peroxidase-conjugated secondary antibodies and 0.5% diaminobenzidine/ H₂O₂ followed by counterstaining with Mayer's hematoxylin. The slides that were treated with normal goat serum were used as negative controls.

2.10. Statistical analysis

All results are presented as the mean ± SD of three independent experiments. Student’s t tests and one-way ANOVA were used to analyze expression differences among groups. Statistical analysis was conducted using SPSS17.0 software. P-values < 0.05 were considered to be statistically significant.

3. Results

3.1. DADS suppress GC cell proliferation in vitro through upregulation of RORα

Since 30 mg/L DADS have been proved to upregulate the expression of RORα in proteomics experiments [5], we first confirmed that the effects of DADS on RORα expression in MGC803 and SGC7901 cells after treatment with 30 mg/L DADS for 8, 12, 24 h (Fig. 1A). The protein levels of RORα increased in a time-dependent manner in SGC7901 cells, and the similar results were observed in MGC803 cells, which was in line with our previous data [5]. The established RORα knockdown and overexpression SGC7901 cell lines were identified by RT-PCR and Western blot, respectively (Fig. 1B and C). DADS-induced upregulation of RORα was abolished by knockdown of RORα (Fig. 1B). Conversely, this effect of DADS was augmented in cells transfected with the RORα-expressing plasmid (Fig. 1C). Then, we explored the effect of RORα on DADS inhibiting GC cell proliferation. Flow cytometry analysis indicated an increase in the percentage of G2/M phase cells after DADS treatment compared with the untreated empty vector group. Whereas, the proportion of cells arrested in G2/M phase reduced in the DADS-treated RORα knockdown cells (Fig. 1D). The MTS and EdU assays showed that the inhibitory effect of DADS on cell proliferation was attenuated by knockdown of RORα (Fig. 1E and F).

In the overexpressed RORα cells upon DADS treatment, the percentage of G2/M phase cells decreased in contrast to the cells treated with DADS alone (Fig. 1G). Likewise, DADS exerted a more potent effect on cell proliferation in RORα overexpression cells (Fig. 1H and I).

3.2. Effect of RORα knockdown on DADS-induced downregulation of Wnt/β-catenin pathway

The mRNA and protein levels of Wnt1 and β-catenin were downregulated after SGC7901 cells exposure to 30 mg/L DADS for 24 h. Knockdown of RORα partially offset the effect of DADS on the
expression of Wnt1 and β-catenin (Fig. 2A and B). DADS decreased nuclear β-catenin and p-β-catenin levels, suggesting that it may have a suppressive effect on β-catenin activation and nuclear accumulation. β-Catenin and p-β-catenin levels increased in the RORα KD + DADS group by comparison with the DADS group (Fig. 2C). To determine whether DADS prohibits β-catenin from entering the nucleus through inducing the binding of RORα to β-catenin, besides downregulating the total β-catenin expression, the interaction between RORα and β-catenin was assessed in cells incubated with DADS for 12 h. As shown in Fig. 2D, the interaction of them was increased after DADS treatment. However, knockdown of RORα caused a reduction in DADS-induced the interaction of them.

DADS decreased TCF-4 and β-catenin target gene expression (Axin, c-jun, and c-myc). Compared with the DADS group, the expression of these genes rebounded in RORα KD + DADS group (Fig. 2E and F). And, knockdown of RORα antagonized the repressive effect of DADS on the c-myc promoter activity (Fig. 2G).

3.3. Effect of RORα overexpression on DADS-induced downregulation of Wnt/β-catenin pathway

The mRNA and protein levels of Wnt1 and β-catenin were lower in the RORα + DADS group than those in the DADS group (Fig. 3A and B). Compared with the cells exposed to DADS alone, the intranuclear β-catenin and p-β-catenin levels were further decreased in RORα overexpressed cells upon DADS treatment (Fig. 3C). In contrast with the DADS group, the increased interaction between RORα and β-catenin occurred in the RORα + DADS group (Fig. 3D). It meant that upregulation of RORα by DADS may promote its binding to β-catenin, hindering β-catenin phosphorylation and nuclear localization.

RORα overexpression augmented the inhibition of DADS on TCF-4 and β-catenin target gene expression (Fig. 3E and F). Meanwhile, DADS exhibited a stronger repressive effect on the activity of c-myc promoter in cells overexpressing RORα (Fig. 3G). These data described as above suggest that RORα could be an active participant in DADS-induced downregulation of Wnt/β-catenin pathway.

3.4. RORα contributes to the inhibitory effects of DADS on cell migration and invasion

To further ascertain whether RORα-mediated downregulation of Wnt/β-catenin pathway is related to DADS suppressing cell motility and invasiveness, the effects of DADS on migration and invasion were assessed in the RORα knockdown and overexpression cells. After SGC7901 cells were treated with 30 mg/L DADS for 24 h, both the migration and the invasion rates were significantly reduced compared to the empty vector groups. The effects of DADS on cell migration and invasion were weakened in the RORα KD + DADS group (Fig. 4A and B), while they were strengthened in the RORα + DADS group (Fig. 4C and D). Knockdown of RORα resisted DADS from downregulating MMP-9 and upregulating TIMP3 expression (Fig. 4E and F). Whereas, RORα overexpression enhanced the effects of DADS on cells (Fig. 4G and H). These results suggest that the inhibition of DADS on cell migration and invasion may result from recovering the suppressive role of RORα in regulating Wnt/β-catenin pathway.

3.5. RORα augments the inhibitory effect of DADS on EMT

Next, cell morphology changes were evaluated to explore the influence of RORα on EMT in DADS-treated cells. As shown in Fig. 5A and B, most cells presented a fibroblast-like shape and heteromorphism with almost no cell connections in the empty vector group. While in the DADS group, many cells showed an epithelial-like morphology and grew in clusters with cell connections. These changes in cell morphology were more distinctly observed in the RORα + DADS group (Fig. 5B). However, there were cells of different sizes, an increase of fibroblast-like cells, and a reduction in cell connections demonstrated in the RORα KD + DADS group (Fig. 5A).

Consistent with these morphological changes, the decrease of vimentin and snail and the increase of...
E-cadherin were verified in the DADS group compared with the vector group (Fig. 5C, D, E and F). These effects of DADS on the expression of EMT markers were neutralized by knockdown of RORα (Fig. 5C and D). By contrast, overexpression of RORα strengthened the effects of DADS (Fig. 5E and F). The data indicate that upregulation of RORα by DADS may be beneficial to negatively regulating the Wnt/β-catenin pathway, inhibiting EMT, subsequent cell motility and invasiveness.
3.6. RORα is implicated in the DADS anti-tumor effects in vivo

To determine the association between the anti-proliferative effect of DADS and RORα in vivo, the xenograft growth was examined in nude mice transplanted with RORα overexpression and knockdown SGC7901 cells. Compared to the control group, the DADS group demonstrated a decrease in tumor volume. Moreover, mice treated with RORα + DADS exhibited a greater degree of decline in tumor volume (Fig. 6A). Inversely, an increased tumor volume was observed in the RORα-miR + DADS group in contrast to the DADS group (Fig. 6A). Furthermore, the same changes in weight were obtained from the xenograft tumors removed.
at 70 days (Fig. 6B and C). The data indicate that, in vivo, ROR\(\alpha\) could play a similar role in DADS inhibiting GC cell growth to that in vitro.

In addition, we detected the protein expression of the markers involved in cell proliferation and EMT in transplanted tumor tissues. Immunohistochemistry results illustrated that DADS upregulated E-cadherin, and downregulated CD34, Ki-67, snail, and vimentin protein levels (Fig. 6D). In contrast with the DADS group, these effects of DADS were attenuated in the ROR\(\alpha\) KD group, while potentiated in the ROR\(\alpha\)-overexpressing group (Fig. 6D).
4. Discussion

DADS possesses the property of multi-target activity, which renders it to interfere with various signaling pathways [14]. It suppresses tumor growth by inducing cell cycle arrest at the G0/G1 or G2/M phase, and apoptosis [4]. DADS induces G2/M arrest via PI3K/Akt/mTOR pathway in human osteosarcoma cells [15]. We have revealed that DADS arrested GC cells at G2/M phase by selectively activating Chk1, downregulating CDC25C and cyclin B1 [16,17]. In addition, anti-cancer activity of DADS is conferred by increasing the expression and activity of tumor suppressors [4,14]. RORα has been recognized as a tumor suppressor in many tumors [6]. Reduced RORα expression has been shown to positively correlate with clinical progression, metastasis [18,19], and shorter overall survival in GC [19], whereas, GC patients with higher RORα expression levels have a better prognosis [19], and AMP-activated protein kinase activator could bring about GC cell apoptosis by increasing RORα expression and activity [18], prompting that RORα may be a potential target for prevention and therapy in GC. In this study, our results showed that DADS induced G2/M phase arrest, accompanied by increased RORα expression in GC cells. RORα knockdown partially counteracted DADS-induced G2/M arrest in vitro and inhibition of cell growth in vitro and in vivo, while the effects of DADS were reinforced in RORα overexpression cells. Thereby, upregulation of RORα was conducive to the inhibitory effect of DADS on GC cell proliferation.

RORα acts as a pivotal transcription regulator through binding to ROR-responsive elements, and regulates tumor suppressor expression and/or function. It promotes the expression of SEMA3F, a suppressive microenvironmental factor, attenuating breast cancer cell invasiveness [8]. RORα conduces to p53 stabilization, which enhances the expression of p53 target genes involved in apoptosis [20]. Additionally, RORα positively regulates p53 expression and activity, and represses proliferation of oral squamous cell carcinoma cells [21]. On the other hand, RORα controls the expression of...
oncogenes in tumor cells. The function of RORz repressing the expression of β-catenin target genes (such as cyclin D1, Axin, and LEF1) might be attributed to the competition with co-activators for β-catenin binding, and the recruitment of histone lysine methyltransferases in colon cancer cells [10]. The decreased RORz level is correlated with the increased expression of Wnt/β-catenin target genes in prostate cancer, and restoration of RORz expression depresses hyperactivation of proliferative genes (cyclin D1, c-Jun, and c-Myc) [22]. Our studies indicated that DADS upregulated RORz while reducing Wnt1, β-catenin and TCF-4 expression in GC cells. And knockdown of RORz weakened the DADS-induced decrease of Wnt1, β-catenin, TCF-4, Axin, c-Jun, and c-Myc expression. Thence, DADS could downregulate β-catenin target gene expression through RORz negatively regulating Wnt1/β-catenin pathway.

Aberrant activation of Wnt/β-catenin pathway accounts for EMT, subsequent invasion and migration in that the metastatic phenotypes of cancer cells can be reversed by inactivating this pathway [23,24]. RORz overexpression causes downregulation of β-catenin and N-cadherin in hepatoma cells, inhibiting invasion and migration [9]. Wnt/β-catenin pathway overactivation arising from knockdown of RORz potentiates proliferation, EMT and metastatic capacity of hepatoma cells in vitro and in vivo [25]. We provided evidence that DADS showed stronger effects in decreasing vimentin, snail, and MMP-9, and increasing of E-cadherin and TIMP3 expression in RORz overexpressed GC cells, and DADS-induced inhibition of EMT, migration, and invasion was in accordance with the decrease of Wnt1, β-catenin, TCF-4, intranuclear β-catenin and p-β-catenin. Nevertheless, these effects of DADS were partially eliminated after knockdown of RORz. The data suggest that upregulation of RORz may play an important role in DADS inhibiting EMT regulated by Wnt/β-catenin pathway in GC cells.

The excessive activation of Wnt/β-catenin pathway benefits from the participation of other signaling pathways, which converge on β-catenin and augment its activation, nuclear localization, and transcriptional activity. Rac1/PAK1 phosphorylates β-catenin to enhance its stability and activity [26]. Rac1-mediated activation of β-catenin facilitates it entry into the nucleus, and the formation of nuclear β-catenin-TCF-LEF complex in colon cancer cells [27,28]. KRT19 regulates β-catenin nuclear translocation dependent on its interaction with Rac1/β-catenin complex in breast cancer cells [29]. We evidenced that DADS down-regulates Rac1/LIMK1 in GC cells, suppressing EMT, invasion, and proliferation [30]. Furthermore, DADS and Rac1 inhibitor rescue TGF-β1-induced increment of total β-catenin level [13]. Herein, the reduced total β-catenin, intranuclear β-catenin and p-β-catenin levels were observed in DADS-treated GC cells. Owing to inhibition of Rac1 pathway by DADS, we supposed that the decrease of β-catenin entering nucleus may result from the reduction of Rac1-mediated β-catenin expression and phosphorylation activation.

DADS reverses EMT in vitro, and inhibits cell growth in vivo by downregulating active β-catenin, snail, and MMP-9 in breast cancer cells [11]. A recent study proffered that DADS hindered fibronectin-induced EMT via suppressing nuclear translocation of β-catenin, TCF activity, and snail expression in lung cancer cells [31]. Our previous study showed that the total β-catenin in GC cells was downregulated by DADS at 24 h [13], and it is noteworthy that the increased interaction between RORz and β-catenin was observed at 12 h in DADS-treated cells in this study. These data allowed us to consider that the reduction of β-catenin and p-β-catenin levels in nucleus was not entirely due to DADS downregulating the total β-catenin expression, and DADS-induced interaction of RORz with β-catenin may also affect β-catenin activation and nuclear translocation. Therefore, the interplay between RORz and β-catenin in the cytoplasm may be another mechanism interpreting RORz-manipulated downregulation of β-catenin function, despite it has been shown that RORz exerts its trans-repression activity by binding β-catenin competitively with co-activator in colon cancer cells [10].

In summary, we provided the first evidence that RORz-mediated downregulation of Wnt/β-catenin pathway contributed to the inhibitory effects of DADS on GC cell proliferation, EMT, invasion, and migration. Upreregulation of RORz by DADS may be, at least in part, responsible for deceasing Wnt1, β-catenin, TCF-4, and β-catenin target gene
expression, and impeding β-catenin activation and nuclear translocation through promoting RORζ binding to β-catenin. DADS downregulating Wnt/β-catenin pathway via RORζ could provide a new strategy for prevention and adjuvant therapy of GC.

Conflict of interest

The authors declare no conflict of interest.

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References

