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Recombinant disintegrin domain of ADAM15 inhibits the proliferation and migration of Bel-7402 cells

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ABSTRACT

ADAM15 (A Disintegrin And Metalloproteinase 15), a transmembrane protein containing seven domains, interacts with some integrins via its disintegrin domain and overexpresses in many solid tumors. In this study, the effect of the recombinant human disintegrin domain (rhddADAM15) on the proliferation and migration of Bel-7402 cells was evaluated *in vitro* and *in vivo* in zebrafish xenografts. rhddADAM15 (4 μM) severely inhibited the proliferation and migration of Bel-7402 cells, inducing a partial G_2/S arrest and morphological nucleus changes of apoptosis. Moreover, the activity of caspases 8, 9 and 3 in Bel-7402 cells was increased. In addition, the zebrafish was used as a model for apoptosis-induction and tumor-xenograft. rhddADAM15 (1 pM) inhibited the growth and metastasis of Bel-7402 cell xenografts in zebrafish and a lower concentration (0.1 pM) induced severe apoptosis in the somatic cells of zebrafish. In conclusion, our data identified rhddADAM15 as a potent inhibitor of tumor growth and metastasis, making it a promising tool for use in anticancer treatment.

1. Introduction

There are several ways of treating tumors, such as surgical resection, radiation therapy, chemical or biological drugs, and gene therapy. Biological and targeted methods have become the most active and promising areas of research in tumor therapy. However, metastasis remains the principal cause of treatment failure and poor prognosis in cancer patients. Metastasis is a multistage process involving proteolysis, cell motility and migration, new site proliferation, and neoangiogenesis [1]. As one of the major families of cell-surface receptors, integrins play an important role in tumor progression. A prerequisite for tumor invasion is adhesion to the extracellular matrix by integrins and their abnormal expression, which results in tumor growth and metastasis [2]. Integrins interact with different ligands to mediate physiological and pathological features [3]. It has been found that many integrins can recognize short linear amino-acid sequence of the ligands, such as RGD, DGEA, EIDV and GPRP. The RDG (Arg-Gly-Asp) motif is the most characteristic structure among these sequences [4].

ADAMs (A Disintegrin And Metalloproteinase), a family of transmembrane glycoproteins that contain seven functional domains, are expressed in many tissues and organs and have been implicated in a variety of physiological and pathological processes, such as sperm-egg binding, neuronal development, and myotube formation [5,6]. To date, nearly 40 ADAMs have been found, all of which potentially possess cell-adhesion and protease activity. In addition, the disintegrin domain located in the extracellular segment of ADAMs contains integrin-binding sequences that are thought to interact with integrins and may mediate cell–cell interactions [7].

ADAM15, a transmembrane ADAM, which is usually expressed on smooth-muscle, mesangial and endothelial cells, is over-expressed in many solid tumors such as breast, colorectal, and ovarian cancer [8–10]. It plays important roles in the degradation of extracellular matrix, cell adhesion, intracellular signal transduction and pathological changes in tumors. Because of the RGD motif in its disintegrin domain, ADAM15 is considered to interact with integrins multifariously [11]. Researchers showed that ADAM15 combines with integrins such as $\alpha_v\beta_3$ in an RGD-dependent or with $\alpha_9\beta_1$ in an RGD-independent manner [12,13]. Over-expression of ADAM15 in NIH3T3 cells enhances cell–cell interaction [14] while shRNA-mediated ADAM15 down-expression in PC-3 cells decreases migration and adhesion to specific extracellular matrix proteins [15]. Furthermore, the recombinant disintegrin of ADAM15 inhibits the growth and metastasis of MDA-MB-231 tumor *in vivo* as a therapeutic protein [16]. However, the role of the disintegrin domain of ADAM15 in human liver cancer Bel-7402 cells is still unknown. We have expressed the recombinant human disintegrin domain of ADAM15 (rhddADAM15) by *Escherichia coli* in our previous research [17,18].This study aimed at assessing the effect of rhddADAM15 on the proliferation and migration of Bel-7402 cells *in vitro*. In addition, the zebrafish model of apoptosis-induction and tumor-xenograft [19,20] was used to

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determine the effect of rhddADAM15 on the growth and metastasis of Bel-7402 cell xenografts *in vivo*.

2. Materials and methods

2.1. Cell culture

Human liver cancer Bel-7402 cells (from the cell bank of the Chinese Academy of Sciences) were grown in RPMI 1640 essential medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 IU/mL) and streptomycin (0.1 mg/mL). The cells were incubated at 37 °C in 5% CO_2.

2.2. Sulforhodamine B assay

Sulforhodamine B assay (SRB; Sigma) was used to assess cell proliferation. Six to seven thousand Bel-7402 cells/well were added to a 96-well plate and cultured for 24 h at 37 °C in 5% CO_2. The medium was then removed and replaced with 100 μl of medium containing rhddADAM15. After incubation for 24 h, the medium with rhddADAM15 was removed and the cells were fixed by adding 100 μl of cold 10% trichloroacetic acid and incubated for 60 min at 4 °C. Then the fixed cells were washed with water and stained with 100 μl of 0.4% (w/v) SRB dye at 37 °C. After 30 min, the unbound SRB was washed off with 1% acetic acid and the bound SRB was solubilized with 100 μl of 10 mM Tris base. The absorbance of the stained cells(Abs) in the wells was measured at 540 nm. The inhibition rate of cell proliferation was calculated using the formula: inhibition rate = $(Abs_{540\ nm,\ control} - Abs_{540\ nm,\ rhddADAM15})/(Abs_{540\ nm,\ control} - Abs_{540nm,\ blank}) \times 100\%$.

2.3. Wound-healing assay

Wound-healing assay was used to assess cell migration. Bel-7402 cells ($1–5 \times 10^5$) were seeded into a 24-well plate and incubated for 24 h. A "wound" was made by manually scraping the monolayer in the middle of each well with a pipette tip. The floating cells were washed off with PBS and the first set of images of each well was captured. The spots of captured images were marked at the bottom of the well externally. Fresh medium or the medium with rhddADAM15 was added and incubated for another 24 h. Then a second set of images of each well at the marked spots was captured. The area of the wound (A) was measured with ImageJ software (NIH, USA). The inhibition rate of cells migration was calculated using the following formulae:

$$\text{migration rate(MR)} = (A_{0h} - A_{24h})/A_{0h} \times 100\%$$
$$\text{inhibition rate} = \text{MR}_{\text{control}} - \text{MR}_{\text{rhddADAM15}}.$$

2.4. Chromatin fragmentation assay

DAPI staining was used to assess nucleus morphology and identify the Chromatin fragmentation when apoptosis occurred in Bel-7402 cells. Bel-7402 cells ($2–5 \times 10^4$) were seeded onto chamber slides in a 24-cell plate. After incubation for 24 h, the medium was removed and the cells were incubated with rhddADAM15 for 24 h. Then the cells were washed with PBS and fixed in cold 70% ethanol for 20 min, followed by two additional PBS washes. DAPI stain (Beyotime, Shanghai, China) was added to each well and allowed to incubate for 20 min in the dark, followed by two additional PBS washes. The slides were taken out carefully and the nuclei of the stained cells were observed randomly under a Nikon fluorescence microscope. At least 6 images were captured randomly in each group and the number of the apoptotic cells was counted. The nuclei of apoptotic cell display pyknosis, fragmentation, and a petal shape.

2.5. Assessment of caspase activity

Bel-7402cells ($3–5 \times 10^5$) in the logarithmic phase were seeded in a 6-well plate and cultured for 24 h. Then the medium was removed and replaced with fresh medium containing rhddADAM15. The cells were digested and the activity of caspases 8, 9 and 3 was determined by spectrophotometry using a caspase activity assay kit (C1115, Beyotime, China). The specific activity of caspase 8 or 9 or 3 was calculated using the formula: specific activity (U/mg) = total activity of the tested protein (U)/total amount of the tested protein (mg).

2.6. Cell-cycle analysis

Bel-7402 cells ($3–5 \times 10^5$) in the logarithmic phase were seeded into a 6-well plate and incubated for 24 h. Then the medium was removed and replaced with medium containing rhddADAM15 and incubated for 24 h. Then the cells were digested and washed with PBS and fixed in cold 70% ethanol for at least 2 h at 4 °C. The cells were further washed and incubated with RNase (Sigma) and propidium iodide (Beyotime, Shanghai, China) at 37 °C for 30 min. Cell cycle distribution was evaluated using BD FACSCalibur flow cytometer and the data were analyzed with ModFit software.

2.7. Apoptosis assay in zebrafish

Zebrafish (*albino*) were raised and maintained at Hunter Biotechnology Inc. The embryos of zebrafish were used in the experiment. Embryos were generated by natural pair-wise mating [21]. rhddADAM15, Cisplatin (Melonepharma, Dalian, China) used as the positive control and PBS as the negative control were microinjected into the embryos respectively. After 24 h of drug treatment, the embryos were stained with acridine orange and observed for apoptotic cells that would display yellow-green fluorescent spots under the fluorescence microscope. Embryos were treated with 0.4% tricaine and mounted in 1.5% methylcellulose before being imaged. Nikon NIS-Elements D 3.10 Advanced image processing software was used to capture and analyze the images. The fluorescence signal (S) from apoptotic cells was measured and the apoptotic rate was calculated using the formula: apoptotic rate (%) = $(S_{\text{rhddADAM15}}/S_{\text{control}} - 1) \times 100\%$. When the experiment was finished, zebrafish were sacrificed by excessive exposure to tricaine in compliance with the specifications of the American Veterinary Medical Association.

2.8. Xenografts in zebrafish

Zebrafish (*albino*) were used to determine the effects of rhddADAM15 on the growth and metastasis of xenografts of Bel-7402 cells. Zebrafish xenografts were generated by microinjection of approximately 800 Bel-7402 cells labeled with CM-Dil (Invitrogen) into the yolk sac of embryos that were 48 h postfertilization. Either rhddADAM15 or Batimastat (Biovision) as the positive control or PBS was microinjected into the yolk sac 24 h later. Before and 2 days after drug injection, anesthetized zebrafish were imaged. The fluorescence signal (S) and the area (A) of zebrafish xenografts of Bel-7402 cells were obtained, which are representative of the growth and metastasis ability of Bel-7402 cells respectively. The inhibition rate of growth and metastasis was calculated using the following formulae:

$$\text{inhibition rate of growth}(\%) = (1 - S_{\text{rhddADAM15}}/S_{\text{control}}) \times 100\%$$
$$\text{inhibition rate of metastasis}(\%) = (1 - A_{\text{rhddADAM15}}/A_{\text{control}}) \times 100\%$$

2.9. Data analysis

Results are presented as mean ± SD of triplicates from at least 3 independently performed experiments. Statistical differences were determined by Student's *t*-test. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of rhddADAM15 on the proliferation and migration of Bel-7402 cells in vitro and in zebrafish xenografts

The SRB assay showed that rhddADAM15 inhibited the proliferation of Bel-7402 cells *in vitro* in a dose-dependent manner (Fig. 1A), with an IC_{50} of 1.04 μM. The wound-healing assay showed that rhddADAM15 strongly inhibited cell migration (Fig. 1B and C). The cells almost stopped migrating at or over 4 μM rhddADAM15.

Then we determined the effect of rhddADAM15 on xenografts of Bel-7402 cells in zebrafish model. After 48 h xenotransplantation, Bel-7402 cells in the control group migrated away from the primary site, displaying an irregular boundary of the tumor xenograft (Fig. 2A, b), while in the rhddADAM15-treated groups the boundary of tumor xenograft was smooth. Compared with the control group, the xenografts of Bel-7402 cells had smaller metastasis area after treatment with Batimastat (Fig. 2B, b) or rhddADAM15 (Fig. 2B, c–d). In addition to local invasion, a substantial number of Bel-7402 cells migrated to distant parts of the zebrafish body in the control group, while the cells in the rhddADAM15-treated groups did not migrate far from the primary site. Moreover, the xenografts of Bel-7402 cells treated with Batimastat or rhddADAM15 had weaker fluorescence intensity than that in the control group, indicating that rhddADAM15 could kill Bel-7402 cells in zebrafish xenografts. Statistical results showed that compared with the control group, the fluorescence intensity and area of tumor xenograft was reduced by (65 ± 1.91)% and (48 ± 2.66)% respectively after treatment with 7.5 pM rhddADAM15.

3.2. Apoptosis-inducing effect of rhddADAM15 on Bel-7402 cells in vitro and in zebrafish

The above results showed that rhddADAM15 inhibited the proliferation and migration of Bel-7402 cells, but the mechanism is still unclear. DAPI staining was used to observe morphological nucleus changes of Bel-7402 cells (Fig. 3A). Bel-7402 cells treated with rhddADAM15 were smaller and the nuclei showed pyknosis, fragmentation, and a petal shape. The numbers of normal and apoptotic cells were counted and the proportion of apoptotic cells revealed an increased effect dose-dependently (Fig. 3B).

The tailbud of zebrafish was chosen for the observation of apoptosis induction [20]. Zebrafish treated with PBS had a few apoptotic cells, while Cisplatin treatment significantly increased the number of apoptotic cells. When treated with different concentrations of rhddADAM15, there was an increase in the apoptotic rate dose-dependently. At the maximum non-lethal dose, the apoptotic rate was (56 ± 4.14)% (Fig. 3C and D).

3.3. Effect of rhddADAM15 on caspase activity in Bel-7402 cells

Enzymatic activity was determined in Bel-7402 cells after 24 h of rhddADAM15 treatment (Fig. 3E). The activity of caspases 3, 8 and 9 was dose-dependent: caspase 9 activity doubled with 2 μM rhddADA15 and caspases 8 and 3 activity increased 1.5-fold with 4 μM rhddADA15 (Fig. 3E).

3.4. Effect of rhddADAM15 on the cell-cycle of BEL-7402 cells

Adherent Bel-7402 cells treated with rhddADAM15 were collected and the cell-cycle was analyzed. The number of cells in G_0/G_1 phase was decreased and there was an increase in G_2 and S

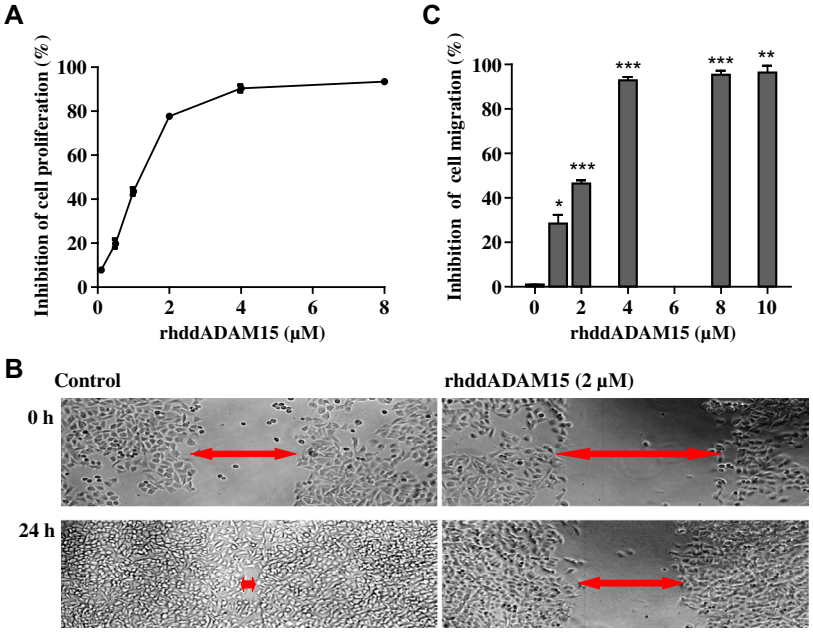

Fig. 1. Inhibitory effect of rhddADAM15 on the proliferation and migration of Bel-7402 cells. (A) Inhibitory effect of rhddADAM15 on the proliferation of Bel-7402 cells by SRB assay. rhddADAM15 inhibited the proliferation of Bel-7402 cells *in vitro* in a dose-dependent manner with an IC_{50} of 1.04 μM. (B) and (C) Inhibitory effect of rhddADAM15 on the migration of Bel-7402 cells by wound-healing assay. A wound was generated by scraping the monolayer of Bel-7402 cells. The images of Bel-7402 cells migrating into the wound before and 24 h after rhddADAM15 treatment were captured at the same spot and analyzed. ($n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* control).

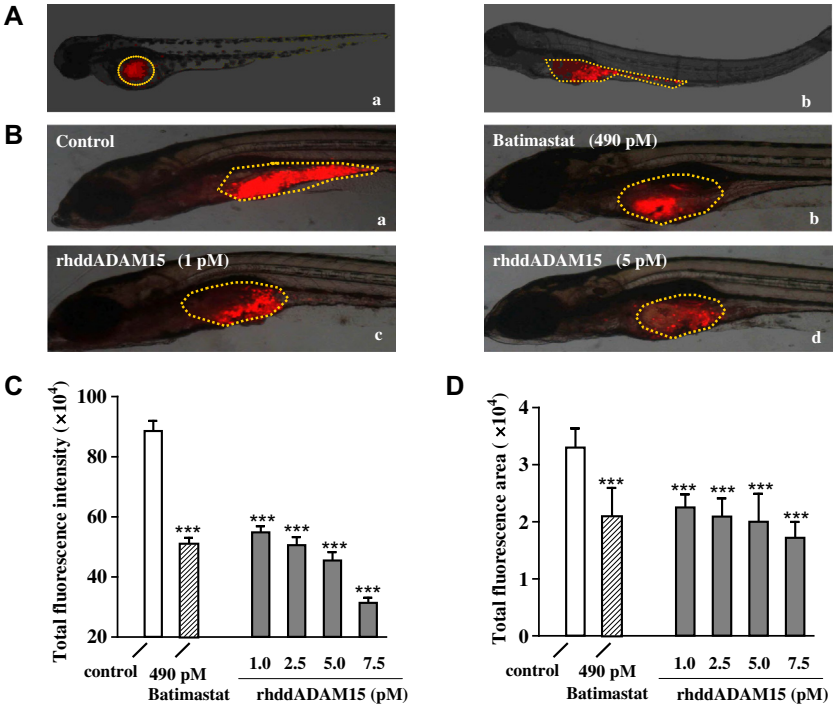

Fig. 2. Inhibitory effect of rhddADAM15 on the proliferation and metastasis of Bel-7402 cells in zebrafish xenografts. CM-Dil-labeled Bel-7402 cells (red) were microinjected into the yolk sac of zebrafish embryos and rhddADAM15 was microinjected after 24 h. The proliferation and metastasis of xenografts of Bel-7402 cells were imaged under a fluorescence microscope at day 2 post-injection. The labeled xenografts of Bel-7402 cells were marked by yellow dotted coil. (A) a, Primary Bel-7402 cells site (red) immediately after xenotransplantation. b, metastasis of xenografts of Bel-7402 cells at day 2 post-injection. (B) a, Negative control of which PBS was microinjected; b, positive control of which Batimastat, an inhibitor of cell metastasis and growth was microinjected; c–d, zebrafish treated with different concentrations of rhddADAM15; (C) fluorescence intensity of tumor xenograft, representing the number of Bel-7402 cells; (D) quantification of the fluorescent area of tumor xenograft, representing the metastasis of Bel-7402 cells ($n = 10$, $^{***}p < 0.001$ *vs* control).

phase, indicating that a proportion of the cells were blocked in G_2 and *S* phase (Table 1).

4. Discussion

Disintegrins, most of which contain an RGD motif, are small molecular proteins originally derived from snake venom (Viperidae, *Agkistrodon* species) and leech toxins [15]. As the main ligand of the integrins, disintegrins competitively inhibit integrin-ligand binding, blocking the physiological and pathological processes mediated by integrins. Therapeutic agents which inhibit cell migration and promoting apoptosis by blocking integrins have been tested in several laboratories [22]. The disintegrin domain of ADAM15 that contains an RGD motif binds to integrins, suggesting that the functions of ADAM15 and integrins may be mutually dependent [10]. However, the specific role of ADAM15 in tumor progression remains unclear. A considerable part of the research on the disintegrin domain of ADAM15 concerns its inhibitory effect on adhesion and migration in prostate cancer cells, ovarian cancer cells, and vascular endothelial cells. But there is no information about its effect on liver cancer cells. As the endogenous molecule of human origin, the disintegrin domain of ADAM15 might avoid immunogenic side effects for clinical use and arouses us great interest.

In this study, we found that as an external protein, rhddADAM15 had an inhibitory effect on the proliferation of liver cancer Bel-7402 cells *in vitro*. Then we determined the cell-cycle changes and the apoptotic induction of Bel-7402 cells. After rhddADAM15 (4 μM) treatment, the cells showed a partial G2/S block, indicating that part of the inhibitory effect of rhddADAM15 was through cells of *G*0/*G*1 phase. Cells require anchorage to the ECM to proliferate. Integrins that recognize several ECM proteins activate growth-promoting signaling pathways responsible for the anchorage requirement. And integrin signals are necessary for cells to traverse the cell-cycle [3]. Hence, rhddADAM15 may inhibit the functions of integrins in the regulation of the cell-cycle of Bel-7402 cells by binding to integrins. In addition, obvious morphological nucleus changes of apoptosis were found when Bel-7402 cells were treated with rhddADAM15. Studies have shown that there are three pathways in apoptosis: the death receptor pathway, the mitochondrial pathway, and the endoplasmic reticulum pathway. All of these pathways activate caspases, but apoptosis can occur without caspase participation [23]. Caspase 8 participates in the intracellular signaling cascade leading to apoptosis while caspase 9 has been linked to the mitochondrial death pathway. Caspase 3 is either partially or totally responsible for the proteolytic cleavage of many key proteins during apoptosis and interacts with caspase 8 and caspase 9. Our results showed that the activity of caspases 8, 9 and 3 was not increased greatly, suggesting that the apoptosis of Bel-7402 cells after rhddADAM15 treatment does not depend on the caspase pathway wholly.

The integrin-binding domain of rhddADAM15 is thought to be key for its inhibition in metastasis [12,13,24]. At present, the exact roles of the interaction of integrins and rhddADAM15 during tumor progression are unknown. Recently, Veronika Beck found that ADAM15 is a natural binding partner of integrin $\alpha_v\beta_3$ and decreases $\alpha_v\beta_3$-mediated ovarian cancer cell adhesion and motility in an RGD-dependent fashion [24]. In contrast, Lu demonstrated-that ddADAM-15 inhibits platelet-derived growth factor-induced airway smooth muscle cell migration, which is mediated predominantly by β_1-integrin but not by either β_3- or α_5-integrin subunits. Moreover, the study showed that the inhibitory activity was abolished when R64 was substituted for A64 in ddADAM-15 (RGD/

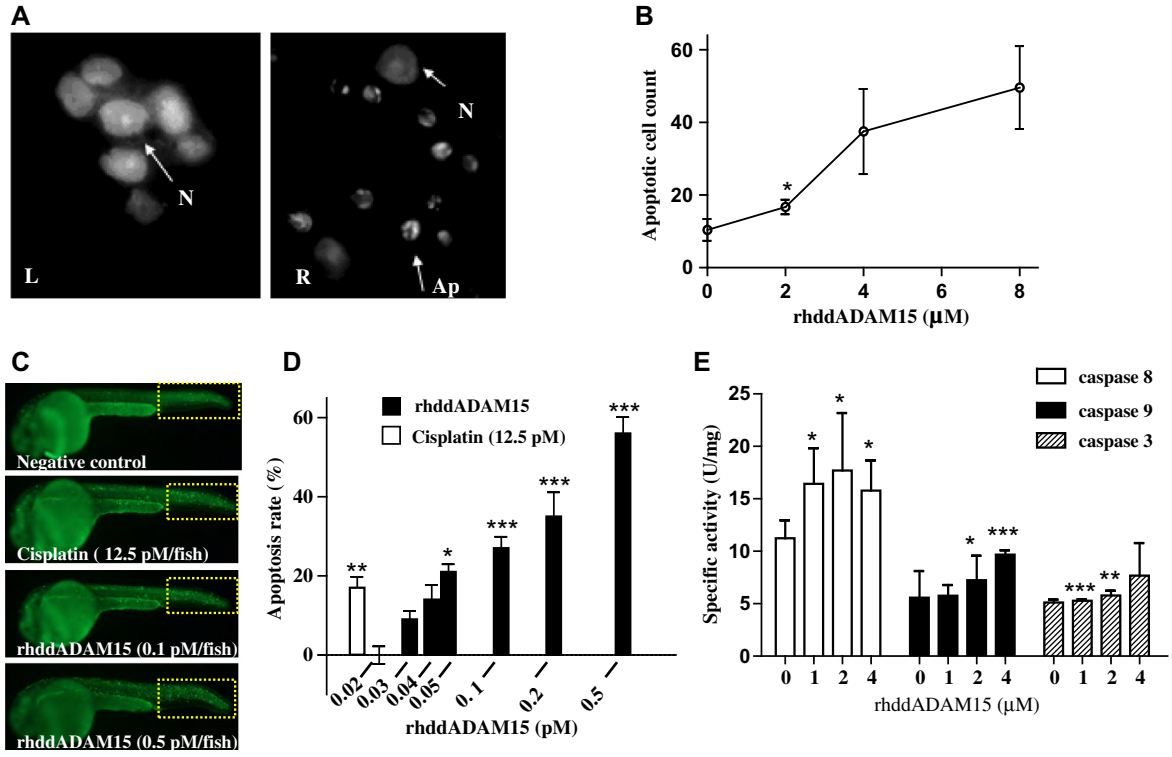

Fig. 3. rhddADAM15 induced apoptosis of Bel-7402 cells and the somatic cells of zebrafish and the assessment of caspases 8, 9 and 3 activity. (A) normal (N) and apoptotic Bel-7402 cells (Ap) stained with DAPI after treatment with rhddADAM15 (4 μM). Bel-7402 cells were fixed and induced by incubation for 24 h with PBS (L, left) or rhddADAM15 (R, right). (B) statistics of apoptotic cells. (C) the embryos were stained with acridine orange and observed for apoptotic cells displaying yellow-green fluorescent spots under the fluorescence microscope. After induction by rhddADAM15 or Cisplatin, the apoptotic cells marked by the yellow dotted box were found over the whole fish, especially on the head and the tailbud. The apoptotic cells in the tailbud were chosen to calculate the rate of apoptosis induction, (D) ($n = 10$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* control). (E) to assess activity of caspases 8, 9 and 3, Bel-7402 cells were incubated with different concentrations of rhddADAM15 for 24 h. The activity of caspases 8, 9 and 3 was determined by spectrophotometry using a caspase activity assay kit ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* control).

Table 1
Assessment of the DNA cycle in the Bel-7402 cells treated with rhddADAM15 by Flow cytometric.

rhddADAM15 (jμM)	G_0/G_1 phase (%)	*S* phase (%)	G_2 phase (%)
Control	65.29 ± 5.36	26.71 ± 5.37	8 ± 0
1	59.12 ± 4.31**	32.17 ± 4.04**	8.71 ± 0.81**
2	57.02 ± 5.74**	30.38 ± 3.28*	12.60 ± 3.99**
4	51.51 ± 12.74*	33.17 ± 5.86	15.36 ± 7.26*

Cells were treated with 1, 2, or 4 μM rhddADAM15 for 24 h. Cell cycle distribution was evaluated using BD FACSCalibur flow cytometer and the data were analyzed with ModFit software ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* control).

AGD), implying a crucial role for the RGD sequence [25]. Thus, rhddADAM15 may play its roles by binding to different integrins in different physiological and pathological conditions. In this study, we found that rhddADAM15 inhibited the migration of Bel-7402 cells *in vitro* and *in vivo*. Using flow cytometry and fluorescent antibody, we found that $\alpha_v\beta_3$ integrin was highly expressed on the Bel-7402 cell surface (data not shown). $\alpha_v\beta_3$ is an important integrin which can bind to rhddADAM15 [12]. We are still exploring the relationship between the inhibitory effect of rhddADAM15 on the proliferation and migration of Bel-7402 cells and the integrins.

Zebrafish is an important model for studies of evolutionary biology, toxicology and pharmacology and many other fields [26–28]. We further evaluated the effect of rhddADAM15 on apoptotic induction in the somatic cells of zebrafish and the growth and metastasis of Bel-7402 cells in zebrafish xenografts. Our results showed that rhddADAM15 strongly induced apoptosis in the somatic cells of zebrafish in a dose-dependent manner. Many of the same family of apoptosis genes of mammalian cell have been identified in the zebrafish DNA database, suggesting that most of the apoptotic pathways in zebrafish and higher vertebrates are highly conserved [29]. In addition, the growth and metastasis of zebrafish xenografts were also inhibited by rhddADAM15 at very low concentrations. These results implied that rhddADAM15 had higher biological activity *in vivo* and could be a potential anti-tumor drug for future development.

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