The saponin monomer of dwarf lilyturf tuber, DT-13, inhibits angiogenesis under hypoxia and normoxia via multi-targeting activity

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Received October 31, 2012; Accepted November 26, 2012

DOI: 10.3892/or.2013.2272

Abstract. The saponin monomer of dwarf lilyturf tuber, DT-13, exhibits anticancer activity by reducing human breast cancer cell adhesion and migration under hypoxia. To further investigate the anticancer activity of DT-13, we investigated whether DT-13 exhibits anti-angiogenic activity. DT-13 showed no effect on human umbilical vein endothelial cell proliferation but inhibited tube formation and migration under normoxia and hypoxia. Moreover, DT-13 significantly reduced density of vessels in vivo observed from a chicken chorioallantoic membrane model. Western blotting results showed that DT-13 suppressed the increased level of hypoxia-inducible factor 1α , p-extracellular signal-regulated kinase 1/2 and p-Akt induced by hypoxia. Enzyme-linked immunosorbent assay revealed that vascular endothelial growth factor excretion was suppressed by DT-13. DT-13 inhibited migration and tube formation induced by vascular endothelial growth factor under normoxia and hypoxia. In addition, DT-13 reduced the level of p-vascular endothelial growth factor receptor 2 and p-Akt induced by vascular endothelial growth factor. Our data suggest that DT-13 inhibits angiogenesis under normoxia and hypoxia and also inhibits angiogenesis induced by vascular endothelial growth factor via targeting at multi elements.

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Introduction

Aside from tumor cell proliferation, expansion and metastasis, angiogenesis is one of the essential steps in tumor progression. When the diameter is over 0.5 mm, the tumor stimulates blood vessel growth to cover the need for nutrition and oxygen, known as the process of angiogenesis (1). The correlation between angiogenesis and tumor metastasis has been described in two points indicating that angiogenesis plays a key role in tumor metastasis: first, the increased vascularity of the primary tumor always leads to increased number of metastatic colonies; second, increased vascular density is indicative of increased metastasis colony size (2). Thus, antiangiogenesis is one of the crucial targets of tumor therapy and anti-angiogenic therapy has been used to inhibit cancer growth and metastasis in the clinic (3). For instance, bevacizumab, the first anti-angiogenic drug approved by the FDA, is a monoclonal antibody against vascular endothelial growth factor (VEGF)-A, inhibiting tumor growth and metastasis (4,5) via anti-angiogenesis (6). Various factors induce angiogenesis, including hypoxia. In tumor, due to the accelerated proliferation of tumor resulting in an abnormal and chaotic blood supply, blood vessels cannot provide oxygen adequately or consistently to the whole tumor. Moreover, the temporal changes in blood flow cause endothelium to suffer hypoxia in certain regions (7).

Dwarf lilyturf tuber, a traditional Chinese medicine, is generally used for cardiovascular disease treatment (8). It has been proved that the saponin monomer of dwarf lilyturf tuber, DT-13, exerts anticancer activity (9,10). It has also been reported that some saponin, such as ginseng saponin, could inhibit angiogenesis (11,12). Previous studies on cancer cell lines indicated that DT-13 inhibits MDA-MB-435 cell migration and adhesion. DT-13 downregulates early growth response gene-1 expression resulting in the reduced excretion of tissue factor under hypoxia (13). In addition, DT-13 decreases the excretion and expression of matrix metallopeptidase 2/9 (MMP-2/9) and reduces the phosphorylation of p38

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Key words: DT-13, dwarf lilyturf tuber, hypoxia, normoxia, angiogenesis, vascular endothelial growth factor, human umbilical vein endothelial cells

(14). However, the effect of DT-13 on angiogenesis remains unclear. In the present study, we investigated if DT-13 impacts angiogenesis under normoxia or hypoxia and we examined the potential mechanisms.

Materials and methods

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated as previously described (15). HUVECs were cultured in complete medium consisting of M199 medium (Gibco), 20% FBS, 50 ng/ml of endothelial cell growth supplement (ECGS, Sigma) and 100 ng/ml heparin. Endothelial cells from third to sixth passages were used for experiments. The cells were incubated in a humidified atmosphere of 95% air, 5% CO₂ at 37°C. For hypoxic treatment, we placed 70-80% confluent cells in a tissue culture incubator in an atmosphere of 1% O₂ and 5% CO₂ at 37°C (16).

HUVEC proliferation assay. HUVECs were harvested, washed with phosphate buffer solution (PBS), re-suspended in complete medium and plated in 96-well plates at the density of 5x10³ cells/well, followed by a 12-h incubation. Cells were then treated with 10⁻⁸, 10⁻⁷ and 10⁻⁶ M DT-13 (provided by Professor Bo-Yang Yu) (Fig. 1) for 24 and 48 h under normoxia. The cell viability was evaluated by the Cell Counting kit (CCK-8, Dojindo Laboratories), or the medium was changed to ECGS-free medium (M199 medium with 1% FBS without ECGS). HUVECs were pretreated with 10⁻⁸, 10⁻⁷ and 10⁻⁶ M DT-13 for 1 h, and were then treated with 10 ng/ml VEGF for 48 h. Following treatment, the cell viability was evaluated by the Cell Counting kit.

Cell migration assay. The cell migration assay was performed by using a Transwell chamber migration system with polycarbonate membranes (8.0 μ M pore size), as previously described (17). Briefly, HUVECs were harvested, re-suspended in serum-free culture medium and (2x10⁴ cells/well) seeded with various concentrations of DT-13 into the upper chamber. Complete culture medium was added into the lower chamber. The cell migration system was incubated at 37°C for 8 h, or, HUVECs were harvested, re-suspended with ECGS-free medium and plated (2x10⁴ cell/well) in the presence of various concentrations of DT-13 into the upper chamber. VEGF (10 ng/ml) in ECGS-free medium was added into the lower chamber. The cell migration system was incubated at 37°C for 4 h. Following treatment, cells were stained and counted in three randomly selected fields.

Tube formation assay. Tube formation assay was performed as previously described (18). Briefly, 96-well plates were coated with Matrigel (50 μ l/well), and incubated at 37°C for 1 h to solidify Matrigel. HUVECs (1.5x10⁴ cells/well) were incubated with various concentrations of DT-13 at 37°C. After 6 h, tubular structures were quantified by manual counting of the number of tubes in three randomly selected fields. When HUVECs were treated with 10 ng/ml of VEGF, the growth factors reduced Matrigel and ECGS-free medium was preferred, and HUVECs were incubated under normoxia. If not, basement Matrigel and complete medium was preferred, and HUVECs were incubated under hypoxia and normoxia.



Figure 1. Chemical structure of the saponin monomer of dwarf lilyturf tuber (DT-13).

Chicken embryo chorioallantoic membrane (CAM) assay. A small window was created on the broad side of the 5-dayold egg. The filter paper with different doses of DT-13 (final concentration 0.001, 0.01 and 0.1 nmol/egg) was dropped on the window. The eggs were incubated at 37° C for another 2 days. We then observed the density and length of vessels toward the CAM face and then the CAMs were photographed. Eight eggs were used per group, and the newly formed vessels were counted.

Western blot analysis. Cellular protein extraction and western blot analysis were performed as previously described (16). Briefly, 40 μ g total protein was fractionated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes under semi-dry conditions. Membranes were probed with rabbit anti-human p-extracellular signal-regulated kinase (ERK) 1/2 (CST), ERK1/2 (CST), p-AKT (CST), AKT (CST), HIF-1 α (Santa Cruz), and murine anti-human β -actin (Sigma). Horseradish peroxidase (HRP) linked anti-mouse immunoglobulin G (Sigma) and anti-rabbit immunoglobulin G (CST) were used as the secondary antibodies. Protein bands were visualized by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Enzyme-linked immunosorbent assay (ELISA). HUVECs were plated into 60-mm dishes with completed medium overnight. HUVECs were changed medium to serum and growth factor supplement-free medium. After 2 h, cells were treated with various concentrations of DT-13 for 12 h under hypoxia or normoxia. After treatment, the medium was harvested to detect the concentration of VEGF by ELISA. The level of VEGF in HUVEC medium was measured with a VEGF ELISA kit (4A Biotech) according to the manufacturer's protocol.

Statistical analysis. The data represent at least three independent experiments and are expressed as the means \pm SEM. For statistical analysis, the Student's t-test was used as appropriate.

Results

Effect of DT-13 on HUVEC proliferation. To identify the cytotoxicity of DT-13, we treated HUVECs with 0.01, 0.1 and 1 μ M DT-13 for 24 and 48 h. DT-13 at 1 μ M inhibited cell proliferation to 71 and 86% of control at 24 and 48 h of treatment, respectively (Fig. 2A and B). However, 0.01 μ M of DT-13 slightly improved HUVEC proliferation after 48 h of



Figure 2. DT-13 inhibits angiogenesis under normoxia and hypoxia *in vitro*. (A) The effect of DT-13 on HUVEC proliferation for 24 h under normoxia. HUVECs were treated with DT-13 (0.01, 0.1 and 1 μ M) or PBS for 24 h under normoxia. Following treatment, the number of cells was counted by CCK-8. ^{**}p<0.01, statistical significance relative to vehicle control. (B) The effect of DT-13 on HUVEC proliferation for 48 h under normoxia. HUVECs were treated with DT-13 (0.01, 0.1 and 1 μ M) or PBS for 48 h under normoxia. Following treatment, the number of cells was counted by CCK-8. ^{*}p<0.05, statistical significance relative to vehicle control. (C and E) The effect of DT-13 on tube formation under normoxia. HUVECs were plated into 96-well plates pre-coated by Matrigel and treated with DT-13 (0.01, 0.1 and 1 μ M) or PBS. HUVECS were incubated under normoxia for 6 h. After treatment, the tubes were captured and counted. ^{**}p<0.01, statistical significance relative to vehicle control. (D and F) The effect of DT-13 on tube formation under normoxia for 6 h. After treatment, the tubes were captured and treated with DT-13 (0.01, 0.1 and 1 μ M) or PBS. HUVECS were incubated under normoxia for 6 h. After treatment, the tubes were captured and treated with DT-13 (0.01, 0.1 and 1 μ M) or PBS. HUVECs were incubated under hypoxia for 6 h. After treatment, the tubes were captured and counted. ^{**}p<0.01, statistical significance relative to vehicle control. (D and F) The effect of DT-13 on tube formation under hypoxia for 6 h. After treatment, the tubes were captured and counted. ^{**}p<0.01, statistical significance relative to vehicle medium with DT-13 (0.01, 0.1 and 1 μ M) or PBS were plated into 96-well plates pre-coated by Matrigel and treated with DT-13 (0.01, 0.1 and 1 μ M) or PBS were plated into the inner chamber of h. After treatment, the tubes were captured and counted. ^{**}p<0.01, statistical significance relative to vehicle control. (G) The effect of DT-13 on HUVECs in serum-free medium with DT-13 (0.01, 0.1

treatment. These results indicate that DT-13 inhibits HUVEC proliferation at high concentrations but promotes proliferation at low concentrations.

DT-13 inhibits tube formation and HUVEC migration under hypoxia and normoxia. To further investigate the effect of DT-13 on angiogenesis, we performed tube formation and migration assays under normoxic and hypoxic conditions, since DT-13 displayed marked anticancer activity under hypoxia (13). In the tube formation study, HUVECs were treated with 0.01, 0.1 and 1 μ M DT-13 and incubated under normoxia or hypoxia for 6 h. Under normoxia, different concentrations of DT-13 showed similar inhibitory effects on tube formation by 20% compared with control (21, 20 and 21% by 0.01, 0.1 and 1 μ M of DT-13 respectively) (Fig. 2C and E). Under hypoxia, the inhibitory activity was 25, 19 and 29% compared with control, respectively (Fig. 2D and F).

In the migration assay, we planted HUVECs into the upper chamber of Transwell with serum-free medium in the presence or absence of DT-13, and added complete medium into the lower chamber. Under normoxia, DT-13 reduced the number of migrated cells with the inhibitory rate of 20, 40 and 48% at the concentration of 0.01, 0.1 and 1 μ M, respectively (Fig. 2G). Under hypoxia, DT-13 performed stronger activity on inhibiting migration; different concentrations of DT-13 showed similar inhibitory effects on migration by 40% compared with control (36, 43 and 36% by 0.01, 0.1 and 1 μ M of DT-13 respectively) (Fig. 2G). These results indicate that DT-13 inhibits tube formation and migration under normoxia and this effect is stronger under hypoxia.

DT-13 inhibits angiogenesis in vivo. To confirm the antiangiogenesis activity of DT-13 *in vivo*, we used a CAM model. Five-day-old eggs were treated with PBS or DT-13 (0.1, 0.01 and 0.001 nmol/egg) for 48 h under normoxia. The normal branching pattern of blood vessels formed after 2 days of incubation. As shown in Fig. 3, DT-13 significantly reduced the relative length of vessels. Different concentrations of DT-13 showed similar inhibitory rates approximately 35% compared with PBS. These data suggest that DT-13 inhibits angiogenesis *in vivo* with a large spectrum of dose.

DT-13 downregulates the level of HIF-1 α , p-ERK, and p-Akt under hypoxia. Hypoxia-inducible factor 1 α (HIF-1 α) accumulates under hypoxia and is a crucial factor for angiogenesis (19). Thus, we investigated if DT-13 affects the level of HIF-1 α . Hypoxia strongly upregulated HIF-1 α expression compared with normoxia (Fig. 4A). However, DT-13 decreased the amount of HIF-1 α both under normoxia and hypoxia. Under hypoxia, DT-13 at the concentrations of 0.01 and 1 μ M significantly downregulated increased HIF-1 α induced by hypoxia. Moreover, DT-13 significantly downregulated ERK1/2 and Akt phosphorylation in a dose-dependent manner under hypoxia (Fig. 4A). Under normoxia, DT-13 only slightly reduced the level of p-Akt at 1 μ M (Fig. 4A). These data suggest that DT-13 exerts anti-angiogenic effects under hypoxia by inhibiting ERK1/2 and Akt phosphorylation.

DT-13 inhibits VEGF excretion under hypoxia. We also detected whether DT-13 impacts VEGF secretion under normoxia and

 $\mathbf{Figure 3. The effect of DT-13 on new blood vessel formation in CAM models.}$

Figure 3. The effect of DT-13 on new blood vessel formation in CAM models. Filter paper with DT-13 (0.001, 0.01 and 0.1 nmol/egg) or PBS was plated on chicken embryo chorioallantoic membranes of 5-day eggs, then incubated at 37°C for 2 days. ****p<0.001, statistical significance relative to vehicle control.

hypoxia with the ELISA assay. The medium of HUVECs was changed to serum-free medium and incubated with or without DT-13 under normoxia or hypoxia for 12 h. The conditioned medium was collected and examined. As shown in Fig. 4B, under normoxia DT-13 decreased VEGF secretion in a dosedependent manner with the inhibition rate of 11, 15 and 40% by 0.01, 0.1 and 1 μ M of DT-13. Under hypoxia, DT-13 always showed similar inhibitory rates by 40% compared with control (37, 42 and 38% by 0.01, 0.1 and 1 μ M of DT-13 respectively) (Fig. 4B).

DT-13 inhibits angiogenesis induced by VEGF. As shown above, DT-13 exhibited anti-angiogenic ability and inhibited VEGF excretion under normoxia and hypoxia, thus we investigated whether DT-13 inhibits angiogenesis induced by VEGF. Initially, DT-13 did not inhibit cell proliferation at the presence of VEGF within 48 h (Fig. 5A). However, DT-13 exhibited anti-angiogenic effects observed from cell migration and tube formation assays. DT-13 reduced the number of migrated cells induced by VEGF with the inhibitory rate of 61.5, 40.1 and 38.9% at the concentration of 0.01, 0.1 and 1 μ M respectively (Fig. 5B), and decreased tube formation to 63.4, 49.3 and 35% of control at the concentration of 0.01, 0.1 and 1 μ M (Fig. 5C).







Figure 4. (A) The effect of DT-13 on ERK1/2 and Akt activation and the level of HIF-1 α under normoxia and hypoxia. Western blot analysis demonstrated that DT-13 inhibits phosphorylation of ERK1/2, Akt and HIF-1 α . Total cell lysate was mixed with SDS loading buffer and separated by SDS-PAGE, blotted onto nitrocellulose membranes. Membranes were immunoblotted with the respective primary antibodies: p-ERK1/2, ERK1/2, p-Akt, Akt, HIF-1 α and β -actin. *p<0.05; **p<0.01, statistical significance relative to vehicle control under hypoxia. ##p<0.01, statistical significance relative to vehicle control under normoxia and hypoxia. HUVECs were plate into 60-mm dishes with completed medium overnight. HUVECs were changed medium to serum and growth factor supplement-free medium. After 2 h, cells were treated with various concentrations of DT-13 for 12 h under hypoxia. ##p<0.01, statistical significance relative to vehicle control under normoxia. ##p<0.01, statistical significance relative to vehicle control under normoxia. ##p<0.01, statistical significance relative to vehicle control under normoxia. After treatment, the medium was harvested to detect the concentration of VEGF by ELISA. **p<0.01, statistical significance relative to vehicle control under normoxia. ##p<0.01, statistical significance relative to vehicle control under normoxia.

The results indicate that DT-13 attenuates the effect of VEGF on angiogenesis by inhibiting HUVEC migration and tube formation, but does not affect HUVEC proliferation induced by VEGF.

DT-13 exhibits anti-angiogenic activity by inhibiting the VEGF pathway. As shown above, angiogenesis induced by VEGF is weakened by DT-13. Therefore, we evaluated if DT-13 affects the downstream of VEGF pathway. Firstly, we detected the phosphorylation of VEGFR2, the receptor of VEGF. We pretreated HUVECs with or without DT-13 for 1 h and then treated with 10 ng/ml VEGF for 15 min. VEGF significantly promoted the phosphorylation of VEGFR2 compared with PBS, while DT-13 decreased the level of p-VEGFR2 induced by VEGF, with a significant difference at the concentration of 1 μ M (Fig. 6). Furthermore, we examined the phosphorylation of Akt and ERK1/2 after VEGF and DT-13 treatment. VEGF increased the level of p-Akt, which was markedly attenuated by 1 μ M DT-13 (Fig. 6). However, increased p-ERK1/2 was slightly decreased by DT-13 without statistical difference (Fig. 6). These results suggest that under hypoxia, DT-13 inhibits angiogenesis by inhibiting the VEGF pathway.

Discussion

In this study, we demonstrated that DT-13 exhibits antiangiogenic activity under hypoxia and normoxia, and inhibits angiogenesis induced by VEGF under normoxia *in vitro*. DT-13

(µM)

1 (μM)



Figure 5. DT-13 inhibits angiogenesis stimulated by VEGF *in vitro*. (A) The effect of DT-13 on HUVEC proliferation stimulated by VEGF for 48 h under normoxia. HUVECs were pre-treated with DT-13 (0.01, 0.1 and 1 μ M) or PBS for 1 h, then treated with 10 ng/ml VEGF for 48 h. After treatment, the number of cells was counted by CCK-8. (B) The effect of DT-13 on HUVEC migration stimulated by VEGF. HUVECs in 1% FBS and ECGS-free medium with DT-13 (0.01, 0.1 and 1 μ M) or PBS were plated into the inner chamber of Transwell and 1% FBS and ECGS-free medium plus 10 ng/ml VEGF was added into the outer chamber. The Transwell system was incubated for 4 h. After treatment, the cells on the outer side membrane were fixed and stained by crystal violet. These cells were captured and counted. ***p<0.001, statistical significance relative to the VEGF plus group. ###p<0.001, statistical significance relative to the VEGF. HUVECs in 1% FBS and ECGS-free medium were plated into 96-well plates pre-coated by growth factor reduced Matrigel and treated with VEGF (10 ng/ml), DT-13 (0.01, 0.1 and 1 μ M) or PBS. HUVECS were incubated for 6 h. After treatment, the tubes were captured and counted. ***p<0.01; ***p<0.001, statistical significance relative to the VEGF plus group. ##p<0.01, statistical significance for 6 h. After treatment, the tubes were captured and counted. ***p<0.01; ***p<0.001, statistical significance relative to the VEGF plus group. ##p<0.01, statistical significance relative to the VEGF plus group. ##p<0.01, statistical significance relative to the VEGF plus group. ##p<0.01, statistical significance relative to the VEGF plus group. ##p<0.01, statistical significance relative to the VEGF plus group. ##p<0.01, statistical significance relative to the VEGF plus group. ##p<0.01, statistical significance relative to the VEGF plus group. ##p<0.01, statistical significance relative to the VEGF plus group. ##p<0.01, statistical significance relative to the VEGF plus group. ##p<0.01, statistical significance relative to the V

administration resulted in a significant reduction of HUVEC proliferation, migration and tube formation under hypoxia and normoxia. Further evaluation with CAM likely results in reduced relative vessel length in DT-13-treated eggs. To address the potential mechanism of the anti-angiogenic effects of DT-13, we detected the elements involved in angiogenesis, such as HIF-1 α ,

p-Akt, and ERK1/2 which showed significantly decreased levels after DT-13 treatment under hypoxia but not under normoxia. Additionally, DT-13 reduced VEGF secretion and VEGF induced p-VEGFR2 and p-Akt. These findings indicate a role for DT-13 in the inhibition of angiogenesis under hypoxia and normoxia or even of angiogenesis induced by VEGF.



Figure 6. The effect of DT-13 on VEGF-stimulated activation of VEGFR-2 and related signaling pathways. Western blot analysis demonstrated that DT-13 inhibits phosphorylation of VEGFR2, ERK1/2 and Akt. Total cell lysate was mixed with SDS loading buffer and separated by SDS-PAGE, blotted onto nitrocellulose membranes. Membranes were immunoblotted with the respective primary antibodies: p-ERK1/2, ERK1/2, p-Akt, Akt, p-VEGFR2 and β -actin. *p<0.05; **p<0.01, statistical significance relative to the VEGF plus group. #p<0.05; ##p<0.01, statistical significance relative to the VEGF plus group.

At present, there are three types of anti-angiogenesis drugs: antibody, synthetic small molecules, and natural products (20). Although the effects of the anti-VEGF-A antibody, bevacizumab, have been confirmed in clinical anti-angiogenic therapy combined with a cytotoxic agent (21), there are still adverse effects in the cardiovascular and renal systems (22,23). Multiple-target small molecules and natural products performed anti-angiogenic activity, such as sorafenib and curcumin (20). Previous studies on cancer cells suggested that DT-13 exhibits anticancer activity via inhibiting cancer cell migration and adhesion in vitro (13,14). Our data showed that DT-13 displays anti-angiogenic activity by inhibiting tube formation and HUVEC migration. Endothelial cells in tumor are under two main oxygen conditions, normoxia and hypoxia. Endothelial cells are under hypoxia during the pathological state, such as atherosclerosis and cancer (19,24-26). Hypoxic conditions stimulate the expression of pro-angiogenic protein, such as VEGF and its receptor VEGFR2, in endothelial cells (27-29). VEGFR-2 plays a crucial role in angiogenesis and activation of VEGFR-2 promotes endothelial cell growth, migration and tube formation (30). In VEGFR-2-deficient mice, failed formation of blood islands and blood vessels led to embryonic lethality (31), which indicates that VEGFR-2 is essential in angiogenesis. Stimulation of VEGFR-2 phosphorylates downstreams, such as ERK1/2 (32) and Akt (33). DT-13 reduces the level of p-Akt and ERK1/2 under hypoxia and p-VEGFR-2 and p-Akt induced by

VEGF under normoxia, but displays little effect on p-ERK1/2 induced by VEGF. DT-13 displays higher activity of inhibition on p-Akt and p-ERK1/2 under hypoxia than under normoxia. Furthermore, DT-13 reduces the increased HIF-1 α induced by hypoxia, which may also reduce angiogenesis considering the role of HIF-1 α in angiogenesis (19). Thus, DT-13 could be a new multi-targeting anticancer agent by inhibiting angiogenesis, tumor growth and metastasis.

In conclusion, we provided evidence for the first time that DT-13 exhibits anti-angiogenic activity. Further animal studies are required to confirm the effect of DT-13 on angiogenesis and to explore the side-effects of DT-13.

Acknowledgements

This study was financially supported by the National Natural Science Fund Nos. 81102853 and 81071841, and 2011' Program for Excellent Scientific and Technological Innovation Team of Jiangsu Higher Education.

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