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# TIEG1 induces apoptosis through mitochondrial apoptotic pathway and promotes apoptosis induced by homoharringtonine and velcade

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**Abstract Overexpression of TGFβ inducible early gene (TIEG1) mimics TGFβ action and induces apoptosis. In this study, we found that TIEG1 was significantly up-regulated during apoptosis induced by homoharringtonine or velcade. Overexpression of TIEG1 could induce apoptosis in K562 cells and promote apoptosis induced by HHT or velcade. TIEG1-induced apoptosis was shown to involve Bax and Bim up-regulation, Bcl-2 and Bcl-XL down-regulation, release of cytochrome *c* from mitochondria into the cytosol, activation of caspase 3 and disruption of the mitochondrial membrane potential (ΔΨm). We concluded that TIEG1 is a key regulator which induces and promotes apoptosis through the mitochondrial apoptotic pathway.**



*Keywords:* TIEG1; Apoptosis; Homoharringtonine; Velcade; Mitochondrial pathway

## 1. Introduction

TGFβ inducible early gene (TIEG1), TIEG2 and TIEG3 constitute a new subfamily of transforming growth factor-β-inducible Sp1-like proteins [1,2]. TIEG1, TIEG2 and TIEG3 were immediately induced by TGFβ [3–5]. TIEG1 was also induced by nitric oxide [6], 15-hydroxy-eicosatetraenoic acid [7] and doxazosin [8]. We previously reported that TIEG1 mRNA was significantly induced by homoharringtonine (HHT) [9]. It was reported that overexpression of TIEG1 mimiced TGFβ action and induced apoptosis in human osteoblast cells, pancreatic carcinoma cells, epithelial and liver cancer cells [10–13]. However, so far, there have been no reports as to whether TIEG1 can induce apoptosis through mitochondrial apoptotic pathway.

In this study, for the first time, we found that human TIEG1 or mouse TIEG1 was significantly up-regulated during apoptosis in the human K562 leukemia cells or mouse B16 melanoma cells induced by HHT, human TIEG1 was also significantly up-regulated during apoptosis in the K562 cells induced by velcade. Overexpression of TIEG1 could induce apoptosis in K562 cells and promote apoptosis induced by HHT and velcade. TIEG1-induced apoptosis was shown to involve Bax and Bim up-regulation, Bcl-2 and Bcl-XL down-regulation, release of cytochrome *c* from mitochondria, activation of caspase 3 and destroying the mitochondrial membrane potential (ΔΨm).

We concluded that TIEG1 was up-regulated during apoptosis induced by HHT or velcade not only in human cells but also in mouse cells. TIEG1 is a key regulator which can induce and promote apoptosis through mitochondrial apoptotic pathway.

## 2. Materials and methods

### 2.1. Cell culture, transfection and plasmids

Human K562 leukemia cells and mouse B16 melanoma cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum in 95% air and 5% $CO_2$ at 37 °C. For the experiment, K562 cells were incubated with 10 nM velcade (was kindly provided by Dr. Hua Yan of Ruijin Hospital, Shanghai, China) or 10 μM HHT (Beijing Union Pharmaceutical Factory, Beijing, China) or RPMI 1640 medium alone for 24 h and 48 h, B16 cells were incubated with 36 μM HHT or RPMI 1640 medium alone for 24 h and 48 h.

TIEG1 overexpression vector with the amino-terminal FLAG epitope-tagged (pcDNA4/TO-TIEG) or its control empty vector (pcDNA4/TO) was transfected into k562 cells by using Lipofectamine 2000 (GIBCO BRL).

### 2.2. Detection of DNA fragmentation

The total cellular DNA was extracted from cells by the method described by Slin and Stafford with some slight modifications [14]. In brief, cells were washed and lysed overnight at 37 °C in lysis buffer containing Tris–HCl 10 mM (pH 8.0), edetic acid 10 mM, 0.4% sodium dodecylsulfate, and proteinase K 100 mg/l. Subsequently cells were treated with 0.5 μg/ml RNase A for 2 h. The genomic DNA was extracted by phenol–chloroformisoamyl alcohol extraction. Electrophoresis was performed on 2.0% agarose gel. The DNA was visualized by UV illumination.

### 2.3. Morphological observation of apoptotic cells

K562 cells or B16 cells after exposure to conditions indicated were centrifuged and washed with PBS, fixed with 4% paraformaldehyde and stained with Hoechst 33258 (5 μg/ml, Sigma) for 15 min, detect apoptosis by fluorescence microscopy.

### 2.4. RNA isolation

Total RNA from cells was extracted according to the original Chomczynski method with slight modifications [15]. Cells were

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*Abbreviations:* HHT, homoharringtonine; ΔΨm, mitochondrial membrane potential; TIEG1, TGFβ inducible early gene; PMSF, phenylmethylsulphonyl fluoride

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collected and homogenized in Solution D containing 1% β-mercaptoethanol. After centrifugation, supernatant was extracted with phenol:chloroform (1:1) twice and acidic phenol:chloroform (5:1) once. The RNA from aqueous phase was precipitated by cold isopropanol and dissolved in deionized $H_2O$. Messenger RNAs were purified using an Oligotex-dT mRNA Midi Kit (Qiagen, Inc., Carlsbad, CA).

*2.5. Detection and analysis of microarray*

The 14 218 microarray consists of 14 218 human full-length or partial complementary DNAs, the 8000 microarray consists of 8000 mouse full-length or partial complementary DNAs. All microarrays were provided by United Gene Holdings, Ltd. (1111 Zhongshan Bei Er Road, Shanghai, China). Microarrays were hybridized and scanned as previously described [9]. Genes were identified as differentially expressed if the ratio of Cy5/Cy3 was >2 or <0.5.

*2.6. Reverse transcription-PCR*

Total RNA was extracted from cells with TRIzol reagent (Invitrogen Life Technologies, Inc.). The reverse transcription reaction was performed using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies, Inc.). The newly synthesized cDNA was amplified by PCR. Human TIEG1 primer (5′-ACTGCGGAGGAAAGAATGGA-3′; 5′-CTGGGAGGAGTGCTGGGAAC-3′), mouse TIEG1 primer (5′-CGTCTAGTGTCTCAGTGCTC-3′; 5′-CTCTTTGAAAGGAGTGGC-3′). Human GAPDH primer (5′-GCCAAAAGGGTCATCATCTC-3′; 5′-GTAGAGGCAGGGATGATGTTC-3′) and mouse GAPDH primer (5′-ACAGCCGCATCTTCTTGTGCAGTG-3′; 5′-GGCCTTGACTGTGCCGTTGAATTT-3′) were used as an internal control. Amplification cycles were: 94 °C for 3 min, then 33 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1.5 min, followed by 72 °C for 10 min. Aliquots of PCR product were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining.

*2.7. Quantification of apoptosis*

Cells were stained with Hoechst 33258 and then observed under a fluorescence microscope. Apoptotic cells with condensed or fragmented nuclei were easily distinguished from normal cells with intact nuclei. Quantification of apoptosis was determined by counting the number of apoptotic cells: six randomly chosen fields of view were observed after exposure to the conditions indicated, with a minimum number of 500 cells scored in each condition.

*2.8. Preparation for cytosolic and mitochondrial fractions*

The cells were washed and resuspended in buffer A (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 mM HEPES–KOH, pH 7.4). After 30 min incubation on ice, cells were homogenized and centrifuged at $700 \times g$ for 10 min. The supernatant was collected, centrifuged at $10{,}000 \times g$ for 25 min, the supernatant was the cytosolic fraction and the pellets were resuspended in 50 μl of buffer A supplemented with 5 μl of buffer B (10% Triton X-100 and 10% DMSO in Buffer A), centrifuged at $5000 \times g$ and the resulting supernatant was the mitochondrial fraction.

*2.9. Western blot analysis*

To prepare the whole-cell extract, cells were washed, lysed in cold TNT buffer (20 mM Tris–HCl (pH 7.4), 200 mM NaCl, 1% Triton X-100, 1 mM PMSF, and 1% aprotinin) for 45 min. The lysates were centrifuged at $12{,}000 \times g$ for 40 min at 4 °C, the supernatant (50 μg of protein) were resolved by SDS–PAGE. Proteins were transferred to nitrocellulose. The membrane was incubated in blocking solution consisting of 5% powered milk in TBST (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20) for 1 h, then immunoblotted with TIEG1 antibody, Bcl-2 antibody, Bax antibody, Bcl-XL antibody (Santa Cruz Biotechnology, Inc.), cytochrome *c* antibody (BD Biosciences), COX-4 antibody (Molecular Probes), Bim antibody (Imgenex), cleaved caspase-3 antibody (Cell Signaling Technology, Inc.), anti-FLAG M2 antibody (Sigma), antitubulin antibody (Sigma–Aldrich). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Amersham Pharmacia Biotech).

*2.10. siRNA preparation and transfection*

TIEG1 siRNA is a pool of 3 target-specific 19 nt siRNAs designed to knock down TIEG1 expression. The siRNA was synthesized by Shanghai GeneChem (Zhangjiang, Shanghai). The cells in the exponential phase of growth were seeded in 6-well plates at a concentration of $5 \times 10^5$ cells/well. After incubation for 24 h, the cells were transfected with siRNA (100 nmol/l) and Lipofectamine 2000 (Invitrogen Life Technologies, USA) according to the manufacturer's protocol. Silencing was examined 48 h after transfection. The control cells were treated with non-targeting siRNA.

*2.11. Determination of $\Delta\Psi m$*

JC-1 staining was performed according to the manufacture's instructions (Molecular Probes). Briefly, cells were collected and washed with PBS. Cells were then incubated with 10 μg/ml of JC-1 in warm PBS for 15 min. After washing with PBS, the cells were analyzed using a flow cytometry (Becton Dickinson). Red and green fluorescence were expressed as percentages of total gated cells.

*2.12. Statistical analysis*

Data shown represent means ± standard deviation (S.D.). Statistical analyses for detection of significant differences between the control and experimental groups were carried out using Student's *t*-test.

## 3. Results

*3.1. Apoptosis in human K562 leukemia cells induced by HHT and velcade or in mouse B16 melanoma cells induced by HHT*

Apoptosis in human K562 leukemia cells or in mouse B16 melanoma cells induced by HHT or velcade was examined (Fig. 1A–C). DNA agarose gel electrophoresis showed that K562 cells and B16 cells presented typical DNA ladder patterns of apoptosis after treatment with 10 μM or 36 μM HHT for 24 h and 48 h. K562 cells presented a typical DNA ladder pattern of apoptosis after treatment with 10 nM velcade for 24 h and 48 h.

As shown with Hoechst-33258 staining, control K562 cells and B16 cells that were not submitted to HHT or velcade treatment did not exhibit chromatin condensation. By contrast, dense and thin crown of nuclear coloration, typical of chromatin condensation, could be observed in the HHT- or velcade-treated cells at 24 h or 48 h (Fig. 1E). We found that although several nuclei still displayed normal morphology, most of the cells exhibited very intense staining of condensed and fragmented chromatin. These evidences clearly showed that HHT could induce apoptosis in K562 cells and B16 cells, velcade could induce apoptosis in K562 cells.

*3.2. cDNA microarray analysis*

In order to identify apoptosis-responsive genes, RNA was isolated from K562 cells and B16 cells treated with HHT for 6 h, 24 h and subjected to microarray hybridization. Interestingly, TIEG1 was the most significant up-regulated gene during apoptosis both in K562 cells and in B16 cells, which was observed to be increased by 6–8 folds.

*3.3. TIEG1 increased in apoptotic K562 cells induced by HHT, velcade and in apoptotic B16 cells induced by HHT*

To confirm the results obtained from the microarray hybridization and to detect if TIEG1 was up-regulated by velcade, we performed Reverse Transcription-PCR and Western blot. As shown in Fig. 1F and G, TIEG1 mRNA and protein increased significantly in apoptotic K562 cells induced by

HHT, velcade and in apoptotic B16 cells induced by HHT. Expression patterns of TIEG1 correlated with the microarray results.

### 3.4. Overexpression of TIEG1 could induce apoptosis in K562 cells

To detect the overexpressed TIEG1 in K562 cells, we performed Reverse Transcription-PCR and Western blot. As shown in Fig. 3C and D, TIEG1 mRNA and protein increased significantly in K562 cells after transfection with pcDNA4/TO-TIEG1 for 24 h and 48 h. DNA agarose gel electrophoresis showed that K562 cells presented typical DNA ladder patterns of apoptosis after transfection with pcDNA4/TO-TIEG1 for 24 h and 48 h (Fig. 1D). As shown with Hoechst-33258 staining, control K562 cells did not exhibit chromatin condensation. By contrast, dense and thin crown of nuclear coloration, typical of chromatin condensation, could be observed in the cells transfected with pcDNA4/TO-TIEG1 for 24 h and 48 h (Fig. 1E).

### 3.5. TIEG1 promoted apoptosis induced by HHT and velcade, inhibition of TIEG1 by siRNA attenuated HHT- and velcade-induced apoptosis

To further examine the role of TIEG1 in apoptosis induced by HHT and velcade, K562 cells were transfected with pcDNA4/TO-TIEG1 (Fig. 3C and D) or siRNA targeting TIEG1 (Fig. 3A and B) for 8 h, then treated with 10 μM HHT or 10 nM velcade for 24 h and 48 h. Percentages of apoptotic cells were assessed by counting the cells stained with Hoechst 33258. As shown in Fig. 2A and B, the overexpression of

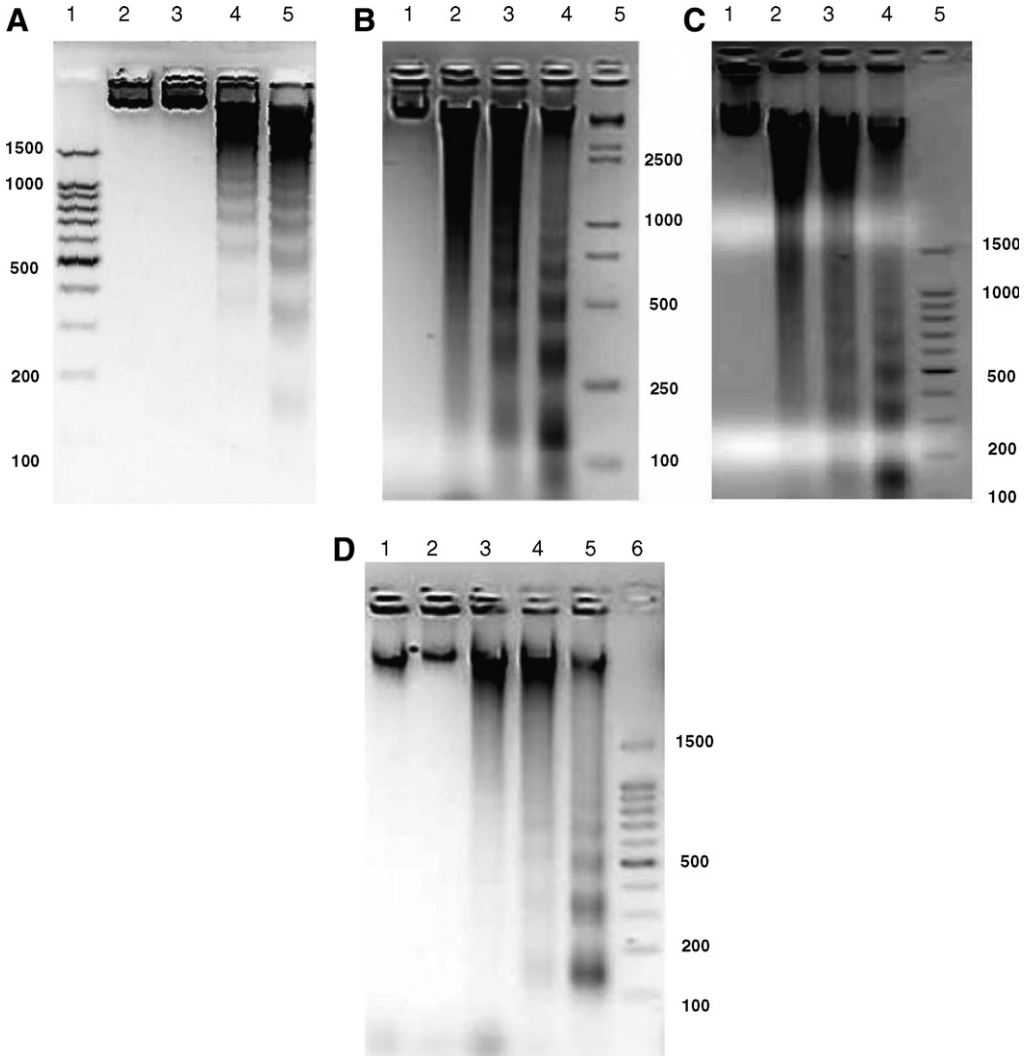


Fig. 1. Patterns of DNA fragmentation during apoptosis in K562 cells induced by HHT (A), velcade (B), overexpressed TIEG1 (D), in B16 cells induced by HHT (C). (A) 1: 100 bp DNA Ladder Marker; 2: untreated cells; 3: HHT 10 μM, 3 h; 4: HHT 10 μM, 24 h; 5: HHT 10 μM, 48 h. (B) 1: untreated cells; 2: velcade 10 nM, 6 h; 3: velcade 10 nM, 24 h; 4: velcade 10 nM, 48 h; 5: Lambda DNA/Eco130I (StyI) marker (bp). (C) 1: untreated cells; 2: HHT 36 μM, 6 h; 3: HHT 36 μM, 24 h; 4: HHT 36 μM, 48 h; 5: 100 bp DNA Ladder Marker. (D) 1: untreated cells; 2: K562 cells transfected with control empty vector pcDNA4/TO 48 h; 3: K562 cells transfected with pcDNA4/TO-TIEG1, 6 h; 4: K562 cells transfected with pcDNA4/TO-TIEG1, 24 h; 5: K562 cells transfected with pcDNA4/TO-TIEG1, 48 h; 6: Lambda DNA/Eco130I (StyI) marker (bp). (E) Photomicrograph of apoptotic changes in B16 cells induced by HHT for 24 h, 48 h, in K562 cells induced by HHT, velcade or overexpressed TIEG1 for 24 h, 48 h, K562 cells transfected with TIEG1 expression vector pcDNA4/TO-TIEG1 or its control vector pcDNA4/TO, transfected or untransfected K562 cells treated with HHT or velcade for 24 h, 48 h. (F) TIEG1 mRNA and protein (G) increased in apoptotic K562 cells induced by HHT (a), Velcade (b) and apoptotic B16 cells induced by HHT (c). (a) 1: untreated K562 cells; 2: HHT 10 μM, 24 h; 3: HHT 10 μM, 48 h; (b) 1: untreated K562 cells; 2: velcade 10 nM, 24 h; 3: velcade 10 nM, 48 h; (c) 1: untreated B16 cells; 2: HHT 36 μM, 24 h; 3: HHT 36 μM, 48 h.

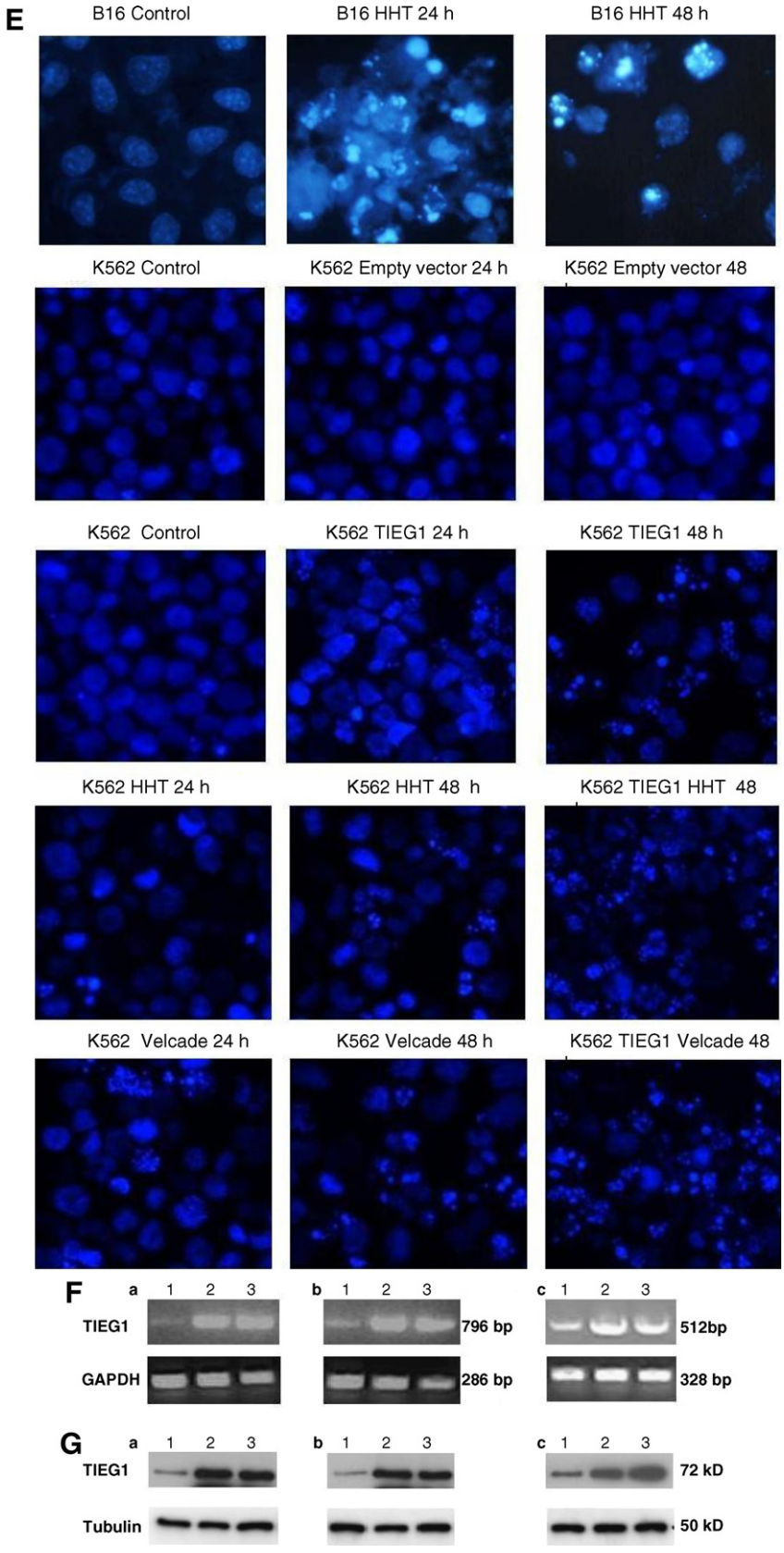


Fig. 1 *(continued)*

TIEG1 significantly sensitized K562 cells to HHT- or velcade-induced apoptosis, the inhibition of TIEG1 significantly decreased sensitivity of K562 cells to HHT- or velcade-induced apoptosis.

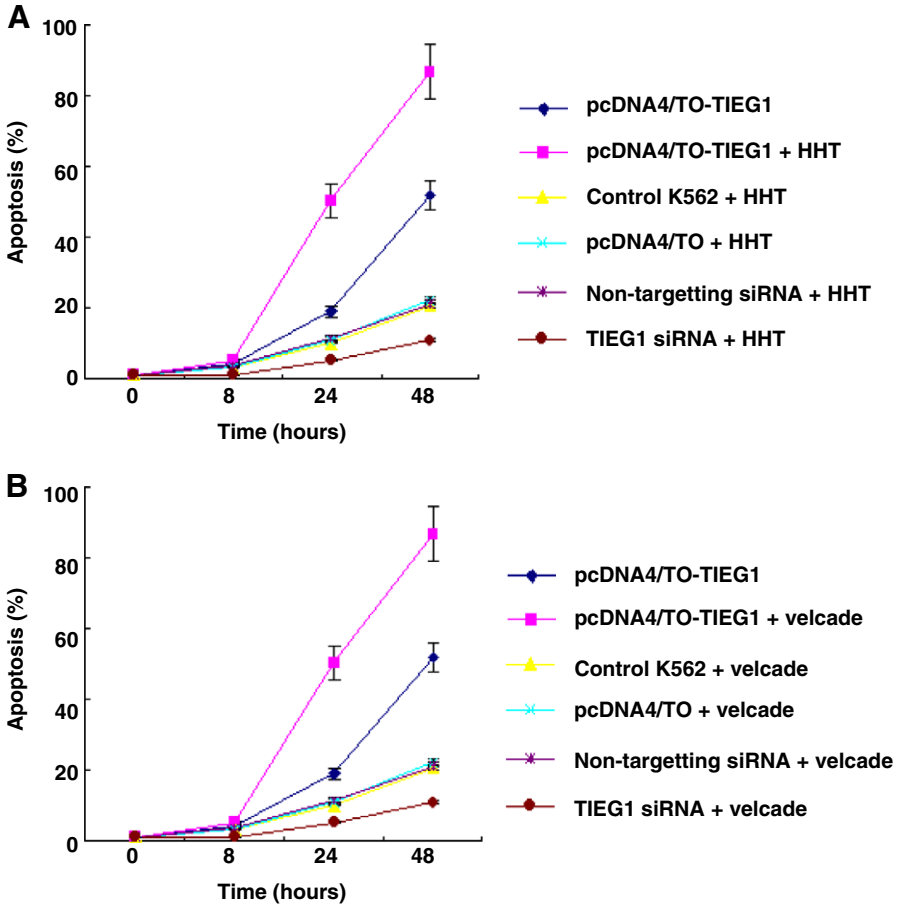


Fig. 2. Overexpression of TIEG1 promoted HHT- and velcade-induced apoptosis, inhibition of TIEG1 by siRNA attenuated HHT- and velcade-induced apoptosis. K562 cells were transfected with pcDNA4/TO-TIEG1 or siRNA targeting TIEG1 (A, B), after 8 h, transfected or untransfected k562 cells were treated by HHT or velcade. Apoptotic cells were measured by counting the cells after staining with Hoechst 33258. Different treated cells was compared with parental K562 cells ($^*P < 0.01$). (A) K562 cells transfected with pcDNA4/TO-TIEG1 or TIEG1-siRNA treated by HHT. (B) K562 cells transfected with pcDNA4/TO-TIEG1 or TIEG1-siRNA treated by velcade.

### *3.6. Effect of TIEG1 on the expression levels of the Bcl-2 family proteins, caspase 3 and cytochrome c*

In order to determine whether Bcl-2 family proteins were involved in the apoptotic process induced by TIEG1, Bcl-2 family pro-apoptotic proteins, including Bim, Bax and anti-apoptotic proteins, including Bcl-2 and Bcl-XL, were detected by Western blot. As shown in Fig. 3E, TIEG1 could increase the expression of Bax and Bim proteis and inhibit the expression of Bcl-2 and Bcl-Xl proteins.

As shown in Fig. 3F, the cleaved caspase 3 was activated after transfection with pcDNA4/TO-TIEG1 for 24 h and 48 h. As shown in Fig. 3G, the cytosolic and mitochondrial fractions of TIEG1-overexpressed cells were detected by immunoblotting with cytochrome *c* antibody. Release of cytochrome *c* from mitochondria into cytosol increased significantly in pcDNA4/TO-TIEG1 cells at 48 h compared with pcDNA4/TO cells.

### *3.7. Effect of TIEG1 on ΔΨm*

To study the effect of TIEG1 on mitochondrial apoptotic events, we examined changes of ΔΨm using JC-1 staining in K562 cells transfected with pcDNA4/TO-TIEG1 for 8 h, 24 h and 48 h. Fig. 4 showed that overexpression of TIEG1 resulted in significant reduction in ΔΨm.

## 4. Discussion

In this study, our experiments showed that HHT could induce apoptosis in K562 cells and B16 cells, velcade could induce apoptosis in K562 cells. During these apoptosis, TIEG1 mRNA and protein increased significantly. To further elucidade the contribution of TIEG1 in mediating apoptosis induced by HHT and velcade, we overexpressed TIEG1 in K562 cells and found that overexpression of TIEG1 could induce apoptosis. We also found that overexpression or inhibition of TIEG1 significantly promoted or attenuated HHT- or velcade-induced apoptosis. From these data, we identified apoptosis induced by HHT or velcade was TIEG1-dependent and TIEG1 was a key factor in apoptosis.

Apoptosis can be initiated via two alternative signal pathways: the death receptor-mediated "extrinsic apoptotic pathway" and the mitochondrion-mediated "intrinsic apoptotic pathway [16,17]." Mitochondria plays a critical role in the regulation of various apoptotic processes including drug-induced apoptosis [18]. The mitochondrial death pathway is controlled by the members of the Bcl-2 family. The Bcl-2 family consists of pro-apoptotic and anti-apoptotic members [19]. During apoptosis, Bcl-2 family pro-apoptotic proteins, including Bim, Bax and Bid, can translocate to the outer

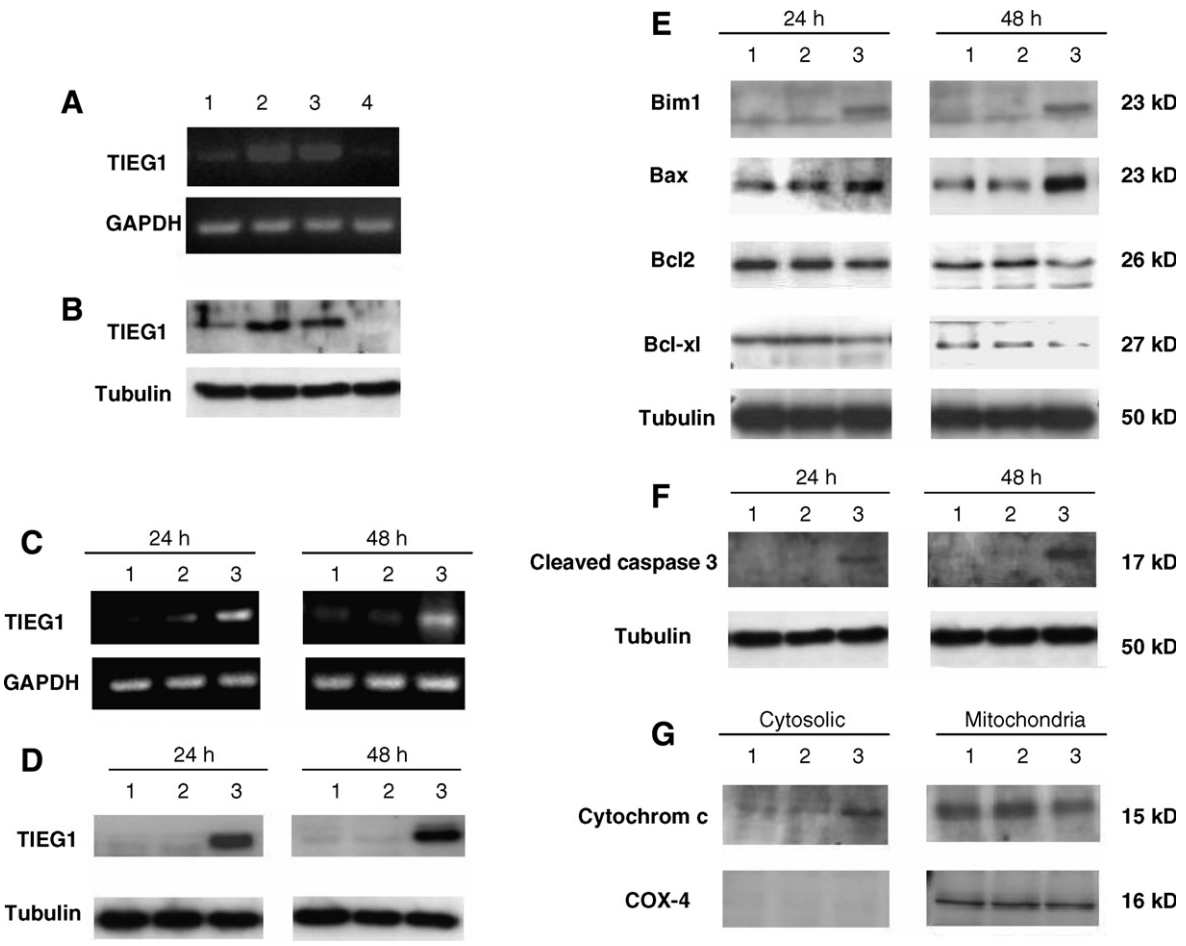


Fig. 3. Effect of TIEG1 on the expression levels of the Bcl-2 family proteins, caspase 3 and cytochrome *c*. (A) RT-PCR for TIEG1 mRNA in control K562 cells or TIEG1-siRNA K562 cells. (B) Western blot assay for TIEG1 protein in control K562 cells or TIEG1-siRNA K562 cells. (C) RT-PCR for TIEG1 mRNA in control K562 cells or TIEG1-overexpressed K562 cells. (D) Western blot assay for TIEG1 protein in control K562 cells or TIEG1-overexpressed K562 cells. (E) Effect of overexpressed TIEG1 on the expression of Bcl-2 family proteins. (F) Effect of overexpressed TIEG1 on the expression of cleaved caspase 3. (G) Effect of overexpressed TIEG1 on the expression of cytochrome *c*. From (A) to (B), 1: K562 cells transfected with pcDNA4/TO; 2: K562 cells transfected with pcDNA4/TO-TIEG1; 3: K562 cells co-transfected with pcDNA4/TO-TIEG1 and non-targeting siRNA; 4: K562 cells co-transfected with pcDNA4/TO-TIEG1 and siRNA targeting TIEG1. From (C) to (G), 1: Control K562 cells; 2: K562 cells transfected with pcDNA4/TO; 3: K562 cells transfected with pcDNA4/TO-TIEG1.

membrane of mitochondria, promote the release of pro-apoptotic factors and induce apoptosis. Bcl-2 family anti-apoptotic proteins, including Bcl-2 and Bcl-XL, sequestered in mitochondria, inhibit the release of pro-apoptotic factors and prevent apoptosis. When interacting with activated pro-apoptotic proteins, the anti-apoptotic proteins lose inhibiting ability of pro-apoptotic factors' release, and again promote apoptosis. Alteration in the levels of anti- and pro-apoptotic Bcl-2 family proteins influences apoptosis [20], and causes the loss of mitochondrial membrane potential ($\Delta\Psi$m), release of cytochrome *c* from the mitochondrial intermembrane space toward the cytosol and proteolytic activation of caspase 9 and 3 [21].

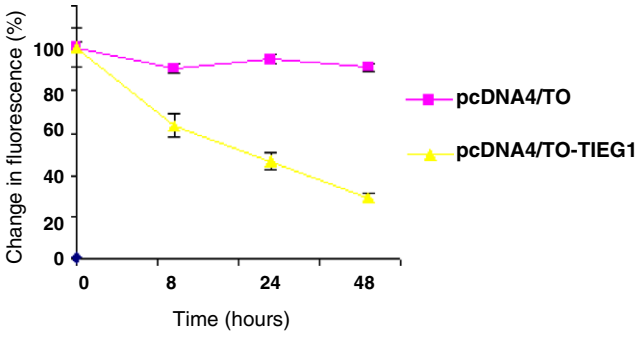


Fig. 4. Loss of $\Delta\Psi_m$ in K562 cells transfected with TIEG1. K562 cells were transfected with pcDNA4/TO-TIEG1 or the control empty vector pcDNA4/TO; the $\Delta\Psi_m$ was measured as described in Section 2. Results in transfected cells are expressed as a percent of parental K562 cells ($^*P < 0.01$).

In this experiment, overexpressed TIEG1 increased the level of the pro-apoptotic proteins Bax and Bim, decreased the level of the anti-apoptotic proteins Bcl-2 and Bcl-XL. This result indicated that TIEG1 induced apoptosis in K562 cells by modulating Bcl-2 family proteins activity. Results also demonstrated that cytochrome *c* was released from mitochondria into cytosol in TIEG1 overexpressed cells, indicating that caspases are partially responsible and this effect was accompanied by the increase of cleaved caspase 3 and destroying the $\Delta\Psi$m. These data indicated that TIEG1 induced apoptosis via mitochondrial pathway.

In conclusion, we demonstrated that TIEG1 was significantly up-regulated during apoptosis induced by HHT or velcade. Overexpression of TIEG1 could induce apoptosis in K562 cells and in return, promoted apoptosis induced by HHT or velcade. TIEG1-induced mitochondrial dysfunction resulted in the reduction of $\Delta\Psi m$ accompanied by the release of cytochrome *c* and caspase 3 activation.

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