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ARTICLE

First Evidence that Sika Deer (*Cervus nippon*) Velvet Antler Extract Suppresses Migration of Human Prostate Cancer Cells

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Abstract

Deer velvet antler (DVA) is one of the most popular medicines in China. Numerous studies have demonstrated that velvet antler possess biological effects. However, data regarding its anti-migration activity on prostate cancer is scarce. In this study, we investigated the inhibitory effect of top DVA (T-DVA) on the expression of prostate-specific antigen (PSA) and migration-related genes in the human prostate cancer cell, LNCaP. The T-DVA down-regulated the expression of PSA. In addition, the Radius^{TM} assay revealed that T-DVA inhibited the migration behavior of prostate cancer cells. Furthermore, the expression of matrix metalloproteinase (MMP)-9 and vascular endothelial growth factor (VEGF) was also decreased with T-DVA. On the contrary, T-DVA increased the tissue inhibition of metalloproteinase (TIMP)-1 and (TIMP)-2. Taken together, our findings indicate that the T-DVA possesses anti-migration activity on prostate cancer cells. This is the first study of DVA to report the anti-migration activity on prostate cancer.

Keywords: Deer velvet antler, prostate cancer, PSA, migration

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Introduction

Sika deer velvet antler (DVA) is one of the most popular traditional medicines in China and Korea. Velvet antler has been recorded in Chinese medical classics, such as ShenNong Ben Cao Jing 2000 years ago. It is believed to nourish the Yin, possess body strengthening, immunomodulatory and anti-aging effects, tonify the kidney, invigorate the spleen, strengthen bones and muscles, and promote blood flow (Wu *et al*., 2013). In Korea, it is generally referred to as "Nokyong" and is one of the most popular Korean traditional medicines. The benefits of DVA are supported by extensive in vivo and in vitro studies (Sunwoo *et al*., 1997). Numerous studies have also demonstrated that velvet antler possess anti-cancer, anti-inflammatory, anti-stress, and anti-aging (Takikawa *et al*., 1972; Wang *et al*., 1988; Zhang *et al*., 1992). Meanwhile, it is generally believed that the use of velvet antler should be avoided in men with hormone-sensitive diseases, such as prostate cancer. To the best of our knowledge, there is no scientific evidence of the bioactivity and safety of velvet antler for managing diseases in men.

Prostate cancer is one of the most common noncutaneous cancers in men and the second leading cause of cancer-related deaths in men (Ferlay *et al*., 2013). With its increased incidence in recent years, the mortality from prostate cancer has increased (Tormey, 2014). In addition, there has been a recent trend in Asia toward an increasing incidence of prostate cancer, with a more rapid increase than that reported from high-rich countries. The presence of prostate cancer is confirmed on the detection of PSA, and the basis of symptoms, physical examination result, or biopsy findings. PSA is one of the most important biomarkers currently used to check for signs of the pres-

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ence of prostate cancer. In addition, MMPs are a trigger mechanism in tumor migration, and contribute to tumor cells into the blood vessels and distant metastasis (McCawley and Matrisian, 2001; Morgan *et al*., 2010; Nishimura *et al*., 2008). Hence, MMPs considered potential markers of migration and metastasis of malignant tumor cells. Meanwhile, there are natural inhibitors of MMPs, a group of peptidases involved in degradation of the extracellular matrix, that is, TIMPs. They are the glycoproteins, and thought to be thought to be a metastasis suppressor. Moreover, VEGF is considered to be the main factor that promotes angiogenesis of prostate cancer, is also related to metastasis of prostate cancer (Cho *et al*., 2001).

Numerous studies have demonstrated that the progression of cancer can be retarded by using plants and the chemical substances (Mehta and Pezzuto, 2002). However, little is known about inhibition of prostate cancer cells by compound from animal sources such as velvet antler.

Therefore, in the present study, we investigated the effects of DVA on prostate cancer cells.

Materials and Methods

Materials

Seven sika DVAs were harvested and collected about 50 d after casting at the same farm (Fanrong farm, China). DVAs were lyophilized, divided into three sections; T-DVA, M-DVA, and B-DVA. 10 g of each section were put into 100 mL of 70% ethanol. They were subjected to extraction 70% ethanol solution for 2 h and repeated three times. Then DVA extracts were filtered (0.25 μm) and lyophilized (yield: top, 3.87%; middle, 3.61%; base, 2.66%) in a freeze dryer for 5 d. LNCaP, androgen-dependent human prostate cancer cells were obtained from Korean Cell Line Bank (Korea; KCLB numbers: 21740). DHT (dihydrotestosterone) was purchased from Sigma (USA). RPMI 1640 media for the cells and TRIzol reagent for RNA extraction were received from Invitrogen (USA). Antibodies for primary antibodies and the peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (USA).

DPPH radical scavenging activity

Antioxidant activity plays a crucial role in anti-cancer (Li *et al*., 2007; Valko *et al*., 2006; Waris and Ahsan, 2006). Therefore, to select one of segments from 3 segments of DVA, DPPH radical scavenging activity of 3 segments were measured according to a slightly modified method of Blois (1958). DPPH solution (1.5×10^{-4} M, 100 μL) was mixed with or without DVA (100 μL), after which the mixture was incubated at room temperature for 30 min. After standing for 30 min, absorbance was recorded at 540 nm by microplate reader and the percentage of scavenging activity was calculated using the following equation:

$$\text{Inhibition (\%)} = (A_{control} - A_{sample}) / A_{control} \times 100$$

Where $A_{control}$ is absorbance of reaction mixture without sample and A_{sample} is absorbance of reaction mixture with sample at 540 nm. In addition, the activity was also calculated was as mmol trolox equivalent at 1,000 μg/mL.

Cell culture

The human prostate cancer cell line, LNCaP cells were cultured in RPMI 1640 media, supplemented with 10% FBS, 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37°C. The cells were seeded at a density of 5×10^5 cells in a 6-well culture plate. After 24 h, the cells were treated with 125 to 1,000 μg/ mL of T-DVA extract in media for 24 h, and then harvested.

Gap closure migration assay

We performed the migration assay using the Radius^{TM} 24-well from Cell Biolabs, Inc (USA). For the analysis, slowly added 500 μL of Radius^{TM} gel pretreatment solution to each well by carefully pipetting down the wall of the well, and then covered the plate and incubated at room temperature for 20 min. Carefully aspirated the Radius^{TM} gel pretreatment solution from the wells, and added 500 μL of Radius^{TM} wash solution to each well. Harvested and resuspended cells in culture medium at 0.2×10^6 cell/mL. Carefully aspirated the Radius^{TM} wash solution from the wells, and added 500 μL of the cell suspension to each well by carefully pipetting down the wall of the well. Transfered the plate to a cell culture incubator for 24 h to allow firm attachment. After 24 h, aspirated the media from each well, and washed 3 times with 0.5 mL of fresh media. Prepared sufficient 1X Radius^{TM} gel removal solution for all wells by diluting the stock 1:100 in culture media. Aspirated the media from the wells and added 0.5 mL of 1X Radius^{TM} gel removal solution from each well and washed 3 times with 0.5 mL of fresh media. After the final washing was complete, added 1 mL of complete medium with T-DVA extract (125 to 1,000 μg/mL) to each well, and take a photo on 0, 8, 12, 24 h, respectively. To compare the differences in migratory gap, images were captured at the same size, and gap closure was determined

after the indicated times (0, 8, 12, and 24 h) using Cell Profiler™ software (Broad Institute, USA).

RNA isolation and mRNA expression analysis

For the RT-PCR, the total cellular RNA was isolated from cells using TRIzol according to the manufacturer's protocol. The first-strand complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase (Invitrogen, USA). PCR was previously described with exception of primer sets of following primers PSA (s 5'-AGC CCC AAG CTT ACC ACC-3'; as 5'-GCT GAC CTG AAA TAC CTG-3'), MMP-9 (s 5'-CGA CGT CTT CCA GTA CCG AG-3'; as 5'-GTT GGT CCC AGT GGG GAT TT-3'), TIMP-1 (s 5'-CAA GAT GAC CAA GAT GTA TAA AGG-3'; as 5'-AAC AGT GTA GGT CTT GGT GAA G-3'), TIMP-2 (s 5'-CAG CTT TGC TTT ATC CGG GC-3'; as 5'-ATG CTT AGC TGG CGT CAC AT-3'), and VEGF (s 5'-GGG GCA GAA TCA TCA CGA AG-3'; as 5´-TTT CTC CGC TCTGAG CAA GG-3'). GAPDH (s 5'-CCA TGG GGA AGG TGA AGG TC-3'; as 5'-AAA TGA GCC CCA GCC TTC TC-3') was used for internal control. The conditions for RT-PCR were similar ones that have been previously described.

Western blot analysis

Cell extracts were prepared by the detergent lysis procedure as described elsewhere (Kim *et al*., 2009b). Samples of protein (40 μg) were electrophoresed using Novex 4-12% Bis-Tris gel (Life Technolgies, USA), and then transferred to nitrocellulose membranes for 7 min in the iBlot dry blotting system (Life Technolgies, USA). Immunodetection was done using an enhanced chemiluminescence detection kit (Amersham Pharmacia, USA).

Statistical analysis

The experiments shown are, in fact, summaries of the data sourced from at least three experiments. All of data are presented using the mean±SE. Statistical analyses were performed using SAS statistical software (SAS Institute, USA). Treatment effects were analyzed using one-way analysis of variance, followed by Dunnett's multiple range tests. The results $p<0.05$ was used to indicate significance.

Results and Discussion

The selection of one from three sections; T-DVA, M-DVA and B-DVA

Generally, the DVA was divided into three segments, each of which is composed of different nutrients and biological compounds. Among the segments, T-DVA contained the highest amount of organic compounds. M-DVA contained equal amounts of both organic and inorganic compounds. B-DVA possessed the highest amount of inorganic compounds. However, which segments possess the highest inhibitory activity on prostate cancer cells is unclear.

Meanwhile, based on the report that many medical investigations and clinical observations showed that the disease-resistance ability was attributable to its antioxidant property (Dreher and Junod, 1996; Li *et al.,* 2007; Waris and Ahsan, 2006). Valk *et al.* (2006) also insisted that antioxidant property played an important role in preventing diseases such as cancer, and coronary heart disease. Hence, the DPPH radical scavenging activity assay was used to evaluate the antioxidant activities of different segments. As shown in Fig. 1, the antioxidant activity of the T-DVA was significantly higher than those of M-DVA and B-DVA, and the corresponding trolox equivalent (TE) values were 53.44, 40.33, and 34.37 μM at 1,000 μg/mL, respectively. In addition, the result was same tendency as Kim *et al.* (2009a), and we assume this is from the highest amount of organic compounds in T-DVA. Consequently, T-DVA was examined the cytotoxicity on normal liver cells, THLE-2. Finally, we found that T-DVA had no cytotoxic effect on THLE-2 (data not shown). Hence, T-DVA was selected for the anti-prostate cancer experiment. Therefore, T-DVA was selected for the further experiments.

The effect of T-DVA on the expression of PSA in human prostate cancer cells

The expression of PSA was investigated using RT-PCR

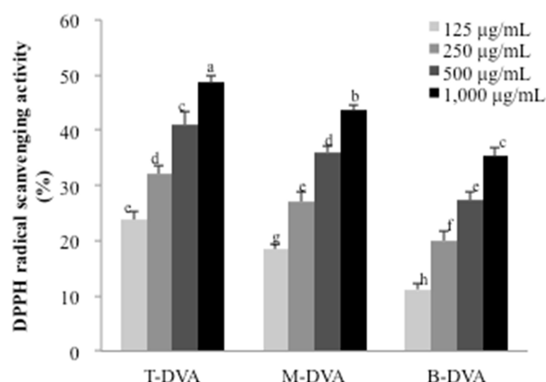

Fig. 1. The effect of DVA on DPPH radical scanvenging activity. [a-h]Values with different superscriot are significantly different of $p<0.05$ as analyzed by Dunnertt's multiple range tests.

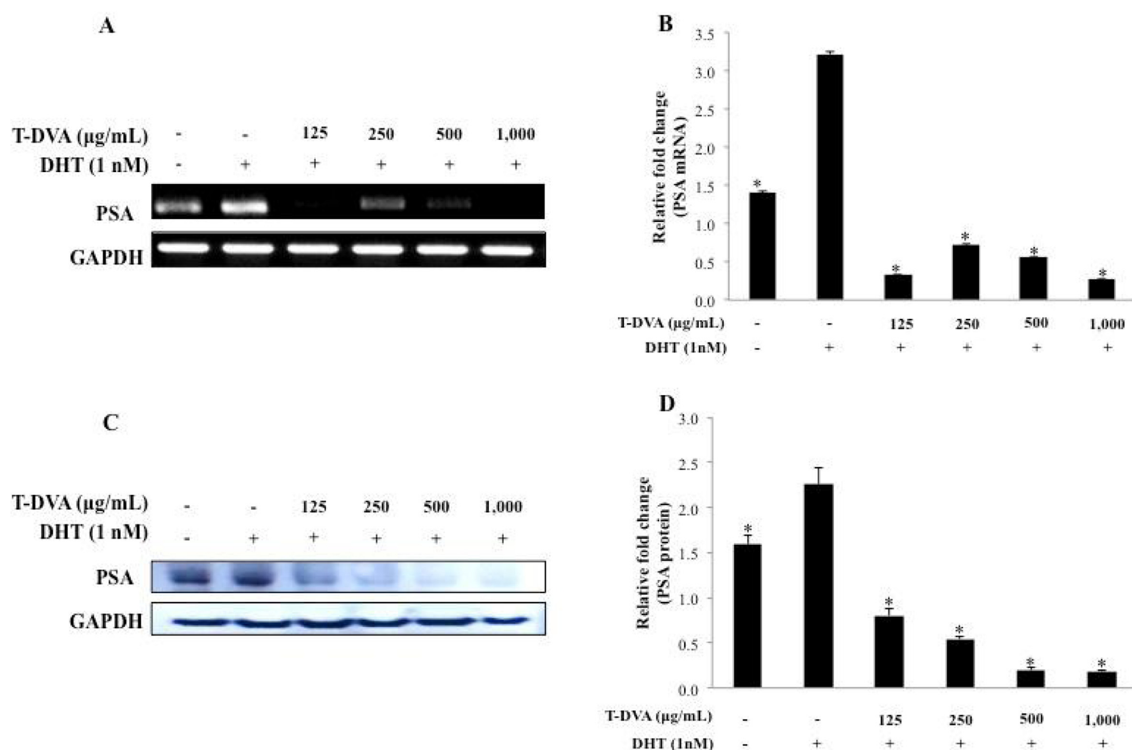

Fig. 2. The expression of PSA using RT-PCR and Western blot.mRNA (A, B) and protein (C, D) expression of PSA which were quantified by Image Guage (Fujifilm, Japan). Cells were treated with or without DHT and various concentrations of T-DVA for 24 h. The mRNA and protein levels from whole cell lysates were analyzed by RT-PCR or Western blot, respectively. GAPDH and β-actin was used as a loading control. The results were similar in three independent experiments. *Significant difference from the DHT alone group shown at $p<0.05$.

after treatment with or without dihydrotestosterone (DHT, 0.1 nM) and T-DVA (125 to 1,000 μg/mL) for 24h. DHT is a sex steroid and androgen hormone. DHT plays a role in the development and exacerbation of prostate cancer by enlarging the prostate gland. Therefore, prostate cancer growth is highly dependent on DHT (Freedland *et al.,* 2005). PSA is a member of the kallikrein-related peptidase family and is secreted by the epithelial cells of the prostate gland. It is present in small quantities in the serum of men with healthy prostates, but its level is often elevated in cases of prostate cancer or other prostate disorders (Catalona *et al.,* 1994). Hence, in current clinical practice, most oncologists monitor PSA levels more frequently (Burgio *et al.,* 2014). Accordingly, we estimated the expression of PSA. In Fig. 2(A) and 2(B), treatment with DHT significantly increased PSA mRNA expression. However, the T-DVA significantly decreased PSA mRNA expression levels compared to DHT-alone group (Fig. 2(A), 2(B)). In addition, the protein expression of PSA was also estimated. As shown in Fig. 2(C) and 2(D), treatment with the T-DVA significantly suppressed the protein expression of PSA against DHT-alone treatment.

The T-DVA inhibited the migratory behavior of human prostate cancer cells

To examine the effect of the T-DVA on cell migration, we performed migration space closure assay using the Radius^{TM} 24-well. To compare the differences in migratory behavior, the images were printed at the same size, and the gap closure was determined after the indicated time (8 and 24 h) compared to control. At 24 h, the gap closed approximately 32.5% (DHT) and 8.53% (T-DVA 1,000 mg/mL), respectively (Fig. 3). The result indicated that T-DVA possessed potent potential to suppress metastasis progress of prostate cancer cells.

Cell migration is an essential process in the development and maintenance of multicellular organisms (Ueno *et al.,* 2004). Tissue formation during embryonic development, wound healing and immune responses require an orchestrated movement of cells in particular directions to specific locations (Yun *et al.,* 2010). Errors during the process have serious consequences, including intellectual disability, vascular disease, tumor formation and metastasis (Guo *et al.,* 2013). An understanding of the mechanism by which cells migrate may lead to the development

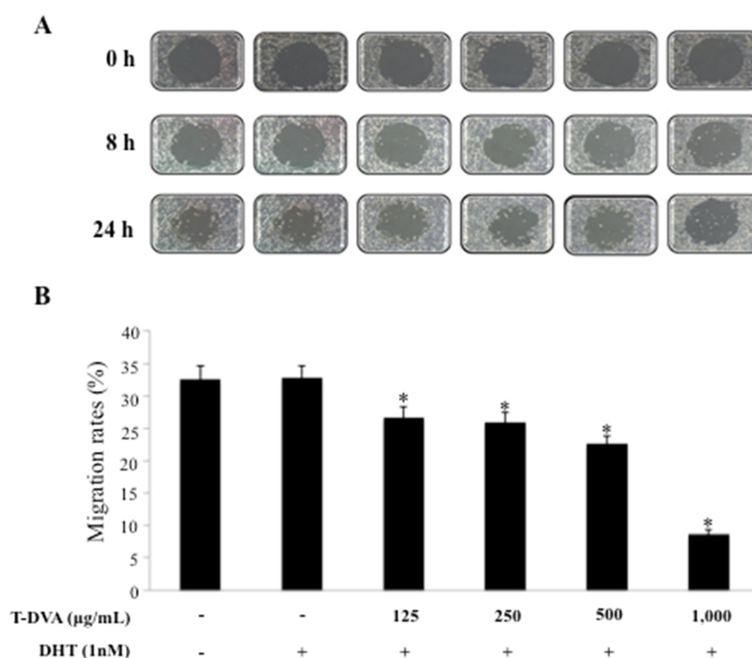

Fig. 3. Migration rates of T-DVA extract-treated LNCaP cells. A, Cell migration time course; B, images were captured at the same size, and gap closure was determined after the indicated times (0, 8, 12, and 24 h) using CellProfiler^{TM} software. Values not sharing a common letter are significantly different at $p<0.05$ by Dunnett's multiple range tests.

of novel therapeutic strategies for controlling, for example, invasive tumor cells.

The effect of T-DVA on the expression of migration-related genes in human prostate cancer cells

We also investigated the expression of migration-related genes. Initially, the mRNA expression levels of MMP-9, VEGF, TIMP-1, and TIMP-2 for 48h were estimated. As shown in (Fig. 4(A)-4(E)), treatment of LNCaP cells with the T-DVA significantly decreased the expression of MMP-9 and VEGF in the DHT-treated T-DVA group. On the contrary, the expression of TIMP-1 and TIMP-2 was increased in the DHT-treated T-DVA group. For further identification, the protein expression of MMP-9, VEGF, TIMP-1 and TIMP-2 was also investigated by western blot (Fig. 4(F)-4(K)). T-DVA-treated groups showed a dose-dependent decrease in MMP-9 and VEGF levels; however, the levels of TIMP-1, and TIMP-2 increased in the T-DVA-treated groups.

MMP-9 can be involved in the development of several human malignancies, as degradation of collagen IV in the basement membrane and extracellular matrix facilitates tumor progression, including migration, metastasis, growth and angiogenesis (Guo *et al.,* 2013). VEGF is a potent angiogenic factor and was first described as an essential growth factor for vascular endothelial cells (Itakura *et al.,* 2000). Not only is VEGF a major player in leukemias and lymphomas, it is also highly expressed in a variety of solid malignant tumors, and associated with progression of malignant diseases (Bellamy *et al.,* 2001). Overexpression of VEGF in tumors is associated with increased angiogenesis, proliferation and metastasis. Meanwhile, TIMP-1 and TIMP-2 are tissue inhibitor of metalloproteinases (Jinga *et al.,* 2006). They are critical to the maintenance of tissue homeostasis by suppressing the proliferation of quiescent tissues in response to angiogenic factors. They also inhibit protease activity in tissues undergoing remodeling of the extracellular matrix (Kamphorst *et al.,* 2015).

Conclusions

DVA has been one of the most important medicines in Asia for more than 2000 years. In the present study, anti-migration activities of DVA have been studied in prostate cancer cells. Firstly, the levels of the key marker of prostate cancer, PSA, were reduced. Furthermore, T-DVA inhibited the migration rate and expression of migration related-genes. It also increased the levels of TIMP-1 and TIMP-2. This is the first study to report the anti-migration activity of DVA on prostate cancer cells. However, further work needs to be done to further elucidate the mechanism underlying DVA's anti-prostate cancer activity.

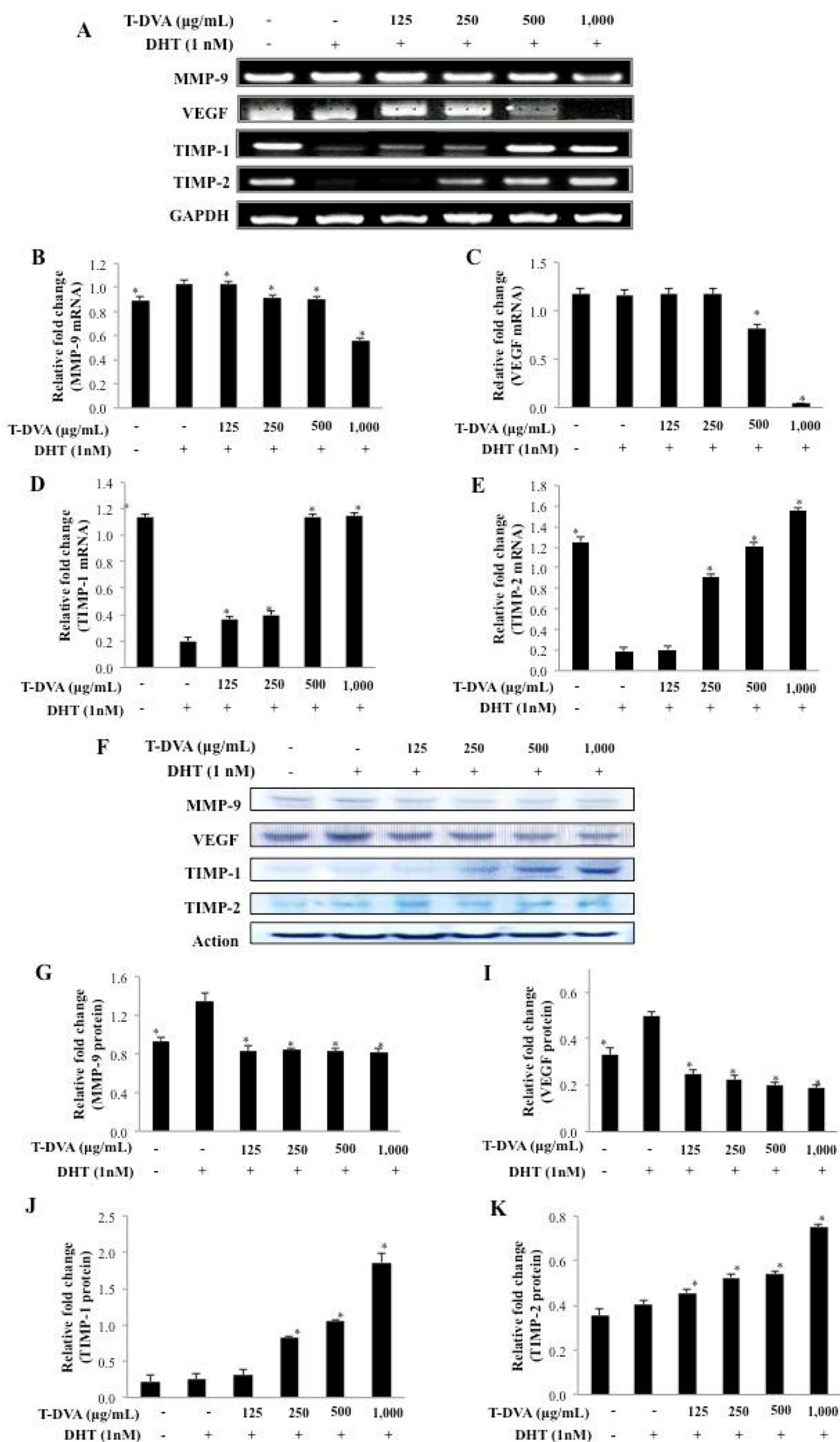

Fig. 4. The expression of migration-related genes using RT-PCR and Western blot.mRNA (A to E) and protein (F to K) expression of migration-related genes which were quantified by Image Guage (Fujifilm, Japan). Cells were treated with or without DHT and various concentrations of T-DVA for 24 h. The mRNA and protein levels from whole cell lysates were analyzed by RT-PCR or Western blot, respectively. GAPDH and β-actin was used as a loading control. The results were similar in three independent experiments. *Significant difference from the DHT alone group shown at p<0.05.

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