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Antimetastatic Activity of Tinospora Cordifolia Involves Inhibition of Cell Migration and Invasion Regulated By Twist and Snail Genes

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Abstract: *VEGF-mediated signalling facilitates survival and metastasis of tumour cells. The metastatic cascade follows tumour cell dissemination, passage through the blood and/or lymphatic system, and colonisation at a distant site. Increased cell motility of cancer cells at the leading tumour edge has been attributed to the epithelial–mesenchymal transition (EMT) which facilitates their release and invasiveness. Breast cancers which exhibit properties of EMT are highly aggressive and resistant to therapy. In the present study, two molecules from hexane and methanolfractions (T1 and T2)from the plant Tinospora cordifoliawere chosen to verify the anti-metastatic activity. Our data show that inMCF-7 cells,T1 treatment significantly suppressed the proliferation, migration and invasion of MCF-7cells when compared to that of T2. EMT-related genes, Twist and Snail, were downregulated by T1 with increased transcription of E-cadherin. Overall, our results demonstrate that T1 down-regulates Twist and Snail genes involvedin proliferation, migration and invasion.*

Keywords: *Epithelial–Mesenchymal Transition, Tinospora cordifolia, Metastasis, Vascular endothelial growth factor (VEGF).*

I. Introduction

Metastasis is the major cause of cancer associated death [1]. Metastasis occurs as a multistep process during which cancer cells detach from the primary tumor and intravasate circulation to disseminate and to invade surrounding tissues to form the secondary tumors [2,5]. Epithelialto mesenchymal transition (EMT) is defined as the ability of epithelial cells to convert from a polarized morphology to a loose mesenchymal phenotype [6]. Recent literature supports the idea that EMT is the key mechanism by which tumor cells gain invasive and metastatic ability, as EMT enables separation of individual cells from the primary tumor mass as well as promotes cellmigration [7,8]EMT is characterized by the down-regulation of epithelial markers and up-regulation of mesenchymal markers [9,11].The characterization of E-cadherin regulation duringEMT has provided important insights into the molecularmechanisms involved in the loss of cell-celladhesion and in the acquisition of migratory propertiesduring carcinoma progression [12].Many transcription factors that drive EMT progression do so through direct inhibition of E-cadherin transcription. Twist and Snail have all been shown to bind specific E-boxes on the E-cadherin promoter to silence its transcription and promote EMT [13, 15]. In cancer, loss E-cadherin is associated with increased migration and invasiveness [16]. Recent research suggests that VEGF also playsan important role in inducing EMT in human pancreatic cancer cells [17].VEGF is an essential factor in breast cancer progression that signals to cancer cells in a way that facilitates their survival [18, 19].Thus, blocking the VEGF induced EMT process is a promising therapeutic strategy for limiting the spread of cancer progression and metastasis.

Tinospora cordifolia [(Willd.) Miers ex Hook. F. & Thoms] is commonly known, as "Amrita" or "Guduchi" is an important drug of Indian Systems of Medicine (ISM) and used as indigenous cure in traditional system of medicine for treatment of various kinds of diseases [20]. T. cordifolia is widely used in veterinary folk medicine and Ayurvedic systems of medicine for its general tonic, antiperiodic, antispasmodic, anti-inflammatory, antiarthritic, antiallergic and antidiabetic properties [21, 24]. Previously we have reported that crude extract or hexane fraction of T. cordifolia has the proapoptotic potentials [25]. The aim of this study was to investigate the potential of T. cordifolia to inhibit VEGF-triggered EMT in breast cancer cells. We suggest that T. cordifolia may inhibit VEGF-induced EMT and this effect is accompanied by the inhibition of the Snail and Twist signaling pathways.

II. Materials And Methods

2.1 Cell culture

MCF-7 cell line was obtained from National Centre for Cell Science (NCCS); Pune, INDIA. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA). Culture medium was supplemented with 10% Fetal Bovine Serum (FBS) and 100units/ml Streptomycin and Penicillin from GIBCO

laboratories, Grand Island, NY, USA.). The cells were cultured in 25 cm^3 tissue culture flask (NUNC, USA). Incubation was carried out in a humidified atmosphere of 5% CO_2 at 37°C. After reaching confluency, the cells were passaged and used for the experiments. [^{3}H] thymidine was from the Baba Atomic Research Centre, Mumbai, India. Mammalian transfection kit, Luciferase reporter assay kit and β-galactosidase assay kits were from Promega, USA. All other reagents were of the highest analytical grade.

2.2 Plant Material: The mature stems of T. cordifolia were collected from University of Mysore campus, Mysore, India. Fresh stems were dried in shade. The voucher specimen was deposited in the herbarium collections maintained in the Department of Botany, University of Mysore, Mysore.

Preparation of hexane fraction: The hexane fraction from T. cordifolia crude extract was essentially prepared as described by Thippeswamy et al. [25]. In brief, polarity-based fractionation and activity guided purification were adopted in order to identify the fraction containing anti angiogenic activity. Shade dried stems (1 kg) were powdered and extracted with different solvents on basis of polarity, using soxhlet apparatus. Solvents such as hexane, benzene, chloroform, ethyl acetate and methanol were used for extraction. In order to identify the fraction containing anti-angiogenic activity, the solvents were evaporated using a rotary evaporator. The hexane fraction (T1)andmethanol fraction (T2), which showed anti-angiogenic activity in vitro assays, wereeluted and both the fractions werechosen to verify their anti-metastatic potential.

2.3 Cell Proliferation Assay

In vitro cell proliferation assay was carried out using [^{3}H] thymidine incorporation into DNAin rapidly multiplying cells as described earlier[26]. The MCF-7 cells (50,000cells/well) were seeded to 12-well plates in their defined media and grown in 5% CO_2 at 37°C for 48h. On the third day, [^{3}H] thymidine (1μCi /ml medium) was added and the effect of with or without VEGF (10ng) or T1 or T2(60μg/ml) was tested on proliferation of cells. After 48 h, the cells were trypsinised and washed with phosphate-buffered saline (PBS) and high molecular weight DNA was precipitated using ice-cold trichloroacetic acid (10 %). Scintillation fluid (5 ml) was added to all the samples and radioactivity was measured in a liquid scintillation counter (Perkin Elmer Tri-Carb 2900 TR model).

2.4 Transient transfection

MCF-7 cells were seeded in six well plates and cultured to 60-70% confluency. On the subsequent day, cells were transfected with pGL3 plasmid containing human gene construct of E-cad (-624bp) or Twist (-824bp) or Snail (-900bp)with a luciferase reporter gene constructusing calcium phosphate transfection kit according to manufacturers instruction (Promega, USA). The transfected cells were used for the respective assays.

2.5 Cell Migration and Invasion Assay

In order to verify if T1 inhibits VEGF induced migration and invasion of MCF-7 cells, transwell migration assay was performed as described earlier [27]. Briefly, Twist and snail transfected MCF-7 (2x 10^4 cells per well) cells were treated with mitomycin C (10 ng/ml) for 2 h and seeded onto the top chamber of transwell which was precoated with 0.1% of gelatin for 30 min at 37°C. The bottom chamber of transwell was filled with basal media and was treated with growth stimulators with or without VEGF (10ng/ml) or T1 or T2(60μg/ml) followed by overnight incubation at 37°C, 5% CO_2. The non migrated cells were swabbed using cotton bud and fixed with 4% ice cold paraformaldehyde for 30 min. Then the cells that had migrated into membrane were stained using haematoxylin and counted manually. The cells were photographed under an Olympus inverted microscope (CKX40; Olympus, New York, USA) connected with a digital camera at 40X magnification.

2.6 Cell Migration Assay (Scratch Assay)

MCF-7 Cells (1×10^5) were transfected with Twist or Snail plasmids as mentioned above and cultured to a confluent monolayer in DMEM media. The assay was performed as described earlier [28] the wells were washed gently with PBS and cells were serum starved overnight and treated with mitomycin-C (10ng/ml) for 2h. The monolayer was wounded by vertically scratching the surface with a sterile 10 to 200-μl sterile micro-pipette tip in presence of PBS. The wells were washed again gently and the detached cells were aspirated along with PBS. Fresh basal media was added followed by treatment with or without VEGF (10ng/ml) or T1 or T2 (60μg/ml) and incubated at 37°C and 5% CO_2. The initial wound healing and the movements of cells in the scratched area were photographically monitored at 0 and 24h after the treatment under an Olympus inverted microscope (CKX40; Olympus, New York, USA) connected to a digital camera at 40X magnification.

2.7 VEGF promoter and luciferase reporter gene analysis

To determine the effect of T1and T2 on expression of E-cadherin, Twist and Snail genes, a transient transfection assay was performed. In brief, MCF-7 (2×10^5 cells per well) were seeded in six-well plates and cultured to 60-70% confluency. On the subsequent day, cells were transfected (calcium phosphate transfection kit, Promega, USA) with 2 µg of E-cad, Twistor Snail, promoter–luciferase reporter constructs and 2 µg of the β-galactosidase expression vector RSV-β-gal as an internal control. The transfected cells were incubated either with or without VEGF (10ng) or T1 or T2(60µg/ml). Cells were washed once with PBS and were serum starved for 24h. Cells were washed once again with PBS and lysed using reporter lysis buffer. Luciferase (Luc) activity of the cell extract was determined using the luciferase assay system. β-galactosidase (β-Gal) activity was determined by measuring hydrolysis of O-nitrophenyl β-D-galactopyranoside using 50 µL of cell extract at 37°C for 2 h. Absorbance was measured at A_{405}. Luciferase activity was determined using 50 µL of cell extract. The reaction was initiated by injection of 100 µL of luciferase assay substrate. Relative Luc activity (defined as E-cad, Twistor Snail, reporter activity was calculated as RLU (relative light units per 50 µL cell extract)/β-Gal activity (A_{405} per 50 µL cell extract per 2 h).

III. Statistical Analysis

Unless stated otherwise, all experiments were performed in triplicates. Wherever appropriate, the data were expressed as the means were compared using one-way analysis of variance. Statistical significance of differences between control, T1, VEGF and VEGF plus T1 treated cells was determined by Duncan's multiple range test (DMRT). For all tests, $P< 0.05$ was considered statistically significant. All of the analyses were performed using the SPSS for Windows, version 13.0 (SPSS Inc.).

IV. Results

Effect of T1 and T2 on VEGF Induced Proliferation of MCF-7 Cells in vitro

The proliferation potential of MCF-7 cells in response to VEGF was assessed using the [^{3}H] thymidine incorporation assay. As is shown in Figure (1), the basal level of proliferation of MCF-7 cells was increased by 44% by VEGF (10ng). Addition of T1 (60µg/ml) alone orT1 with VEGF reduced proliferation of MCF-7 cells by 41% and 78%respectively, when compared to T2, which reduced VEGF induced proliferation only by 22% and 17%. These results suggest that T1 is more potent anti-proliferative compound when compared to the effect of T2 on VEGF induced proliferation of MCF-7 cells.

T1 inhibits VEGF induced cell migration in MCF-7 cells over expressing Twist and Snail genes

Expression of Twist and Snail is required formigration, invasion and metastasisof tumor cells. The data shown in the Figure (2A, B) indicates that there is migration of MCF-7 cells with concominent wound closure at 24 h in presence of VEGF. In contrast, addition of T1 (60µg/ml) inhibited VEGF-induced cell migration.However, T2 showed less inhibitory effect on migration as compared to T1. Quantitative analysis of wound healing assay Figure (2C, D) shows that MCF-7 cells overexpressing Twist and Snail proteins induce migration of more number of cells in presence of VEGF as compared to that of control.

VEGF induced invasion of cells overexpressing Twist and Snail genes is inhibited by T1

Given that induction of Twist and Snail genes is required for activating the EMT program, we next verified whether Twist and Snail play an essential role inVEGF mediated tumor cell invasion and metastasis. The result shown in Figure 3 indicated that higher invasion of cells is in VEGF treated cells. T1 was able to significantly inhibitVEGF induced cell migration and invasion in MCF-7 cells with forced expression of Twist and Snail genes as compared to that of T2 (60µg/ml)Figure (3).These results show that T1 inhibits cellmotility and is anti-invasive in breast cancercells.

T1 treatment increases E-cadherin and inhibits Twist and Snail gene expression in MCF-7 cells

The data on transient transfection and gene promoter luciferase reporter analysis revealed that, luciferase activity in MCF-7 cells with E-cadherin promoter reporter construct was significantly increased upon treatment with T1 (60µg/ml) in comparison with controlat 24 hour time points, as is shown inFigure (4A). These resultsindicated that T1 administration increased transcriptionof E-cadherin in MCF-7 cells. In contrast, Twist and Snail promoter activity was substantially decreased on treatment with T1 (60µg/ml). However, VEGF induced expression of both Twist and Snail genes that were inhibited by T1 Figure (4B, C). Whereas, T2 had less effect on the either E-cadherin gene expression orTwist or Snail genes inhibition. These findings indicated that Twist and Snail transcriptionfactors are important mediators of signaling in response to E-cadherin loss and operates upstream of certain cellular functions that contribute to metastatic competence.

V. Discussion

The role of EMT in cancer cell invasion and metastasis is strongly supported by several cellular models [29,30]. VEGF has recently been shown to influence metastatic potential on cancer cells through its ability to induce cell migration and invasion. Twist and Snail transcription factors which are known to repress E-cadherin expression are sufficient in various cellular contexts to induce EMT. Twist had previously been shown to be essential for the metastasis of mouse mammary tumor cells [31]. Also, over expression of Snail in pancreatic cancer cells lead to lymph node invasion and distant metastasis [32]. Recent studies have shown that, Caveolin-1 downregulate Snailexpression and sensitize pancreatic cancer cells tochemotherapy[33].Previous studies have shown that herbal extract of T. cordifoliahas inhibitoryeffect on proliferation, VEGF and other cytokine productionand induction of apoptosis in different cell lines [34-36]. In addition, we have shown that T. cordifolia showed significant antiproliferative activity and induction of apoptosis in EAT cells [25] and also T. cordifolia inhibits MTA-1 or VEGF induced expression of VEGF.In this study, we observed that anti-metastatic activities of hexane fraction (T1)was more pronounced when compared with methanol fraction (T2) from T. cordifolia as determined by the proliferation, migration, invasion and promoter reporter analysis assays. Taken together, these results suggest that the ability of T1 to inhibit tumor cell proliferation, migration and invasion is associated with the EMT process, possibly by inhibiting the activation of VEGF signaling and regulating the expression of EMT markers such as E-cadherin, Twist and Snail genes.

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Legends

Figure 1: Effect of T1 on proliferation of MCF-7 cell in vitro. Cells were treated with and without VEGF (10ng) or T1 or T2 (60µM) in the presence of ^{3}[H] thymidine (1µCi/ml). After 48h of incubation, the incorporated ^{3}[H] thymidine into the cells was quantified by scintillation counting. All data are presented as the mean from three different experiments with triplicates and mean of± S.E.M.

Figure(2.a): T1 inhibited VEGF induced snail transfected cell migration in MCF-7 cells. Snail transfected cells treated with or without VEGF (10ng), T1 or T2 (60µM).**(C, D)**. Quantification of the cells involved in wound closure. The cells that moved in the wounded area was counted and expressed as movement of control.

Figure (2.b): T1 inhibited VEGF induced Twist transfected cell migration in MCF-7 cells. Twist transfected cells treated with or without VEGF (10ng), T1 or T2 (60µM).**(C, D)**. Quantification of the cells involved in wound closure. The cells that moved in the wounded area was counted and expressed as movement of control

Figure (3A)T1 inhibited VEGF induced Snail transfected cell migration and invasion in MCF-7 cells. Snail transfected cells treated with or without VEGF (10ng), T1 or T2 (60µM). **(C, D)**Quantification of the cells migration and invasion was counted and expressed as movement of control.

Figure (3B): T1 inhibited VEGF induced TWIST transfected cell migration and invasion in MCF-7 cells. TWIST transfected cells treated with or without VEGF (10ng), T1 or T2 (60µM). **(C, D)**Quantification of the cells migration and invasion was counted and expressed as movement of control.

Figure (4.a): T1 upregulates E-cadherin promoter luciferase reporter activity. The luciferase activity was measured in cells alone or with or without VEGF (10ng) or T1 or T2for 24h. The experiments were repeated thrice. The data was presented in means of ±SEM.P<0.05 vs. control. Results revealed that T1 and T2 enhance VEGF induced E-cadherin promoter luciferase reporter activity.

Figure (4.b): T1 down regulates snail promoter luciferase reporter activity. The luciferase activity was measured in cells alone or with or without VEGF or T1 or T2 for 24h. The experiments were repeated thrice. The data was presented in means of ±SEM.P<0.05 vs. control. Results revealed that T1 significantly inhibits VEGF induced snail promoter luciferase reporter activity.

Figure (4.c): T1 down regulates Twist promoter luciferase reporter activity. The luciferase activity was measured in cells alone or with or without VEGF or T1 or T2 for 24h. The experiments were repeated thrice. The data was presented in means of ±SEM.P<0.05 vs. control. Results revealed that T1 inhibits VEGF induced Twist promoter luciferase reporter activity.

Fig. 1

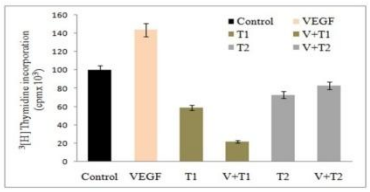

Fig.2

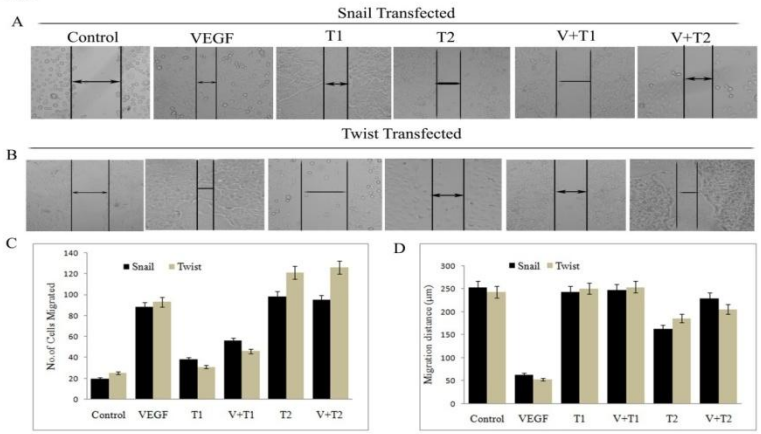

Fig 3

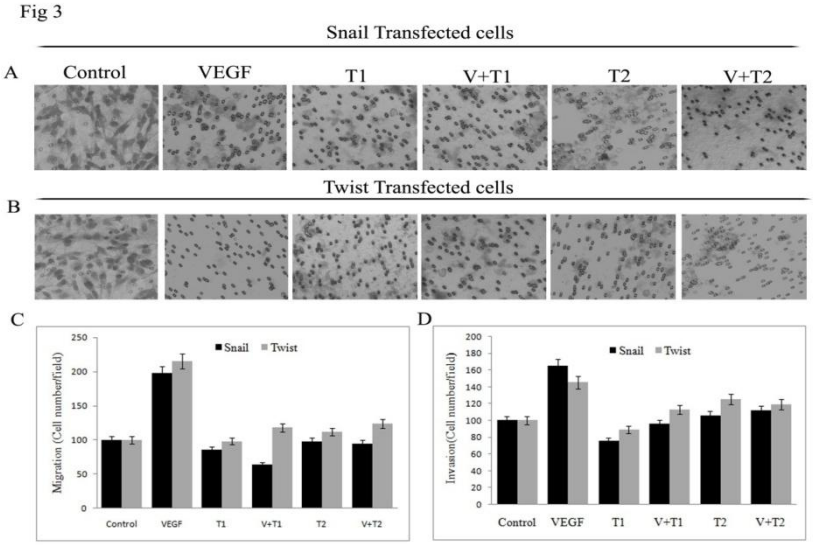

Fig 4