ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
April 2024; 23(2):220-230.
DOI: 10.18502/ijaai.v23i2.15327

The Evaluation of the N-cadherin Promoter's ability to Block EMT by Specific Expression of Diphtheria Toxin in EMT-induced A549 Cell Lines

Zohreh Mehmandoostli[1], Mahmood Dehghani Ashkezari[1], Seyed Morteza Seifati[1], Vida Sadeghi[2,3], Reza Falak[4,5], and Gholam Ali Kardar[2,3]

[1] *Department of Biology, Medical Biotechnology Research Center, Ashkezar Branch, Islamic Azad University, Ashkezar, Yazd, Iran*
[2] *Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran*
[3] *Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran*
[4] *Immunology Research Center, Iran University of Medical Sciences, Tehran, Iran*
[5] *Department of Immunology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran*

Received: 14 May 2023; Received in revised form: 18 December 2023; Accepted: 2 January 2024

ABSTRACT

During epithelial to mesenchymal transition, the ability of cancer cells to transform and metastasize is primarily determined by N-cadherin-mediated migration and invasion. This study aimed to evaluate whether the N-cadherin promoter can induce diphtheria toxin expression as a suicide gene in epithelial to mesenchymal transition (EMT)-induced cancer cells and whether this can be used as potential gene therapy.

To investigate the expression of diphtheria toxin under the N-cadherin promoter, the promoter was synthesized, and was cloned upstream of diphtheria toxin in a pGL3-Basic vector. The A-549 cells was transfected by electroporation. After induction of EMT by TGF-β and hypoxia treatment, the relative expression of diphtheria toxin, mesenchymal genes such as N-cadherin and Vimentin, and epithelial genes such as E-cadherin and β-catenin were measured by real-time PCR. MTT assay was also performed to measure cytotoxicity. Finally, cell motility was assessed by the Scratch test.

After induction of EMT in transfected cells, the expression of mesenchymal markers such as Vimentin and N-cadherin significantly decreased, and the expression of β-catenin increased. In addition, the MTT assay showed promising toxicity results after induction of EMT with TGF-β in transfected cells, but toxicity was less effective in hypoxia. The scratch test results also showed that cell movement was successfully prevented in EMT-transfected cells and thus confirmed EMT occlusion.

Our findings indicate that by using structures containing diphtheria toxin downstream of a specific EMT promoter such as the N-cadherin promoter, the introduced toxin can kill specifically and block EMT in cancer cells.

Keywords: Cancer; Diphtheria toxin; Epithelial-mesenchymal transition; N-cadherin; Promoter

Corresponding Author: Gholam Ali Kardar, PhD; Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran.
Tel: (+98 912) 0199 469, Fax: (+98 21) 642 8995, E-mail: gakardar@tums.ac.ir

INTRODUCTION

Although more than a century of research has been dedicated to identifying and treating cancer, it remains the leading cause of death worldwide.[1,2] In response to external stimuli, primary tumor cells undergo phenotypic changes during tumor development. The epithelial to mesenchymal transition (EMT) is one of the most important changes.[3,4] This phenomenon increases cancer cells' aggressiveness and migratory capability, which is required for cancer metastasizing and spreading to other tissues.[5] Cancerous tumor metastasis also depends on reversible transmission from mesenchymal to epithelial (MET), which is also required for reciprocating modes of transmission.[6,7] According to recent research, a small number of cancer cells may cause EMT-induced signals such as TGFB due to impaired cell adhesion[8] mediated by E-cadherin,[9] which eventually leads to invasion and metastasis,[10] although this has been disputed.[11,12]

The EMT occurs when epithelial genes like E-cadherin are downregulated, and mesenchymal genes like N-cadherin and Vimentin are upregulated.[8] Oxygen deprivation and hypoxia, as well as other signaling pathways, can cause EMT. Deficiency of oxygen due to the increase of cytoplasmic HIF-1α results in the induction of EMT-related genes such as TGF-β, TWIST, and lysyl oxidase (LOX).[13] The heterodimer of HIF-1α/β can be attached directly to the hypoxia-responsive element (HRE) specified on several genes like the TWIST promoter.[14-16] HIF-1α also increases the expression of HDAC3, which is involved in the formation of histone methyltransferase complexes such as Vimentin and N-cadherin, which are markers of mesenchymal cells.[17] The EMT is also induced by the release of Transforming growth factor- β (TGF- β), which promotes the return of cells to a mesenchymal state.[18] The TGF-β belongs to the TGF superfamily, composed of TGF-βs and other related proteins such as bone morphogenetic proteins (BMPs). Inhibition of tumor growth occurs whenever TGF- β signaling blocks the induced cell cycle and increases apoptosis, while prometastatic production and EMT suppress tumor cell proliferation.[19,20]

A delivery structure that expresses a toxic protein in cancer cells must encode a level of regulation to limit its expression in normal cells. It is common for bacterial toxins to be delivered as recombinant proteins attached to a targeting moiety.[21] The cytotoxic nature of toxins, including adenine dinucleotide phosphate (ADP) ribosylation, makes them excellent treatments for cancer.[22] ADP-ribosyltransferase, a component of Pseudomonas exotoxin (PE), has entered clinical trials for anticancer drugs.[23] Various antibodies can be conjugated with various toxins or fragments, making them a viable option for developing cancer drugs. Diphtheria toxin (DT-A) is another source of ribosyltransferase, which is used to inactivate cancer cell factors in cancer treatment.[24,25] On the other hand, immunoconjugate diphtheria toxin and its fusion with therapeutic agents have increased its ability to penetrate and target cancer cells.[26]

Since EMT is associated with a poor prognosis,[27] it is essential to know and understand its underlying genes in vitro and in vivo. Also, no effective treatment has been developed in clinical conditions.[28] Therefore, to identify the mechanisms involved and provide an appropriate therapeutic approach, the different modes of EMT and the expression conditions of the genes involved should be examined.[8] A bacterial toxin is used to treat cancer based on the creation of a protein toxin attaching to the cancer cell and identifying specific antigens on its surface that eventually cause the cancer cell's death.[29] In this regard, a study showed that immunotoxin (VGRNb-DT) obtained by chemical conjugating diphtheria toxin with the vascular endothelial growth factor receptor 2 (VEGFR-2) inhibited cell growth in prostate cancer.[30] The H19 protein is found in various tumor cells, including liver cells, bladder cells, and colon cancer cells.[21] In addition, DT-A expression and its effect on luciferase activity were investigated in human liver cancer cells, mouse bladder cancer cells, and T24P bladder cancer cells.[31] In recent years, diphtheria toxin has become increasingly popular as an immunotoxin. It was also studied whether PDTA-PBH19 immunotoxin expression affected the 814 bp promoter flanking the 5'-region of the H19 gene in bladder, choriocarcinoma, colorectal, and ovarian cancer cells.[32] Furthermore, immunotoxins PRAD51-DTA and PRAD51C-DTA have been studied on the promoters of Rad51, Rad51C, and XRCC2 regulatory elements in cervical and breast cancer.[33-35] In some studies were investigated diphtheria Surp1430-DTA immunotoxin in ovarian, gastric, non-small, small-cell lung, and breast cancers.[36]

Despite the effectiveness of this approach, disadvantages such as possible drug leakage into the bloodstream, Capillary leakage syndrome in blood neoplasms, or thrombocytopenia [37], The development of immunotoxins necessitates the development of safer and more efficient methods.[38,39] A study was conducted

using diphtheria immunotoxin as a nanoparticle structure to inhibit CXCR4+ lymphocyte proliferation, showing that immunotoxin-induced cell death has cytotoxic effects.[29] However, more studies will be needed to establish more effective and safe therapy because of the complexity of the mechanisms in this field and the diversity of genes involved in signaling the EMT process in cancer.

Using a specific expression of diphtheria toxin in EMT-induced A549 cell lines, this study evaluated the capability of N-cadherin promoters to block EMT.

MATERIALS AND METHODS

pGL-DTA Construction

Generay Biotech (China) provided the pGH vector containing DT-A and a pGL3-Baic vector containing the N-cadherin promoter. As part of the preparation of the PGL-DTA vector, the pGH vector containing the inserted DT-A was digested twice with NcoI and xbaI enzymes (Jena Bioscience, France), and then the DT-A was purified from an agarose gel (GENETBIO, Korea). In addition, xbaI was used to linearize pGL3-Baic vectors containing the N-cadherin promoter before partial digestion with NcoI. DT-A was successfully replaced with luciferase fragments cleaved from the transporter. The pGL-DTA vector was modified to include the DT-A fragment downstream of the N-cadherin promoter, using a T4 ligase enzyme (Sigma, Germany) to complete the construct.

Cell Line

In this study, A549 cell line, as a common EMT cell model,[40] was purchased from the National Cell Bank of the Pasteur Institute (Tehran, Iran) and cultured in a high glucose DMEM medium. We supplemented the medium with 10% fetal bovine serum (FBS) (Gibco, USA), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Gibco, USA). The cell culture was kept in an atmosphere of 37°C humidified with 5% CO2 and cells were passaged every 3-4 days. Cells were divided into two groups for the investigation.

The first study

1) Control (not treated and not transfected),
2) TGF-β1+ (treatment with 5ng/ml TGF-β1),
3) Transfected (transfected with pGL3 -DTA), and
4) Transfected + TGF-β1 (transfected with pGL-DTA and treated with 5 ng/ml TGF-β1).

The second study

1) Hypoxia (induced by hypoxia conditions), and
2) Transfected +hypoxia (transfected with pGL-DTA and cells cultured in hypoxia conditions).

Electroporation and Determination of Transfection Efficiency

The electroporation mix was prepared by dissolving the 2×10^5 cells in 100 uL of PBS buffer with 2 ug of plasmid (pGL-3Basic vector-NCAD-DT Toxin). Then, cells were transferred to a sterile 0.2 cm cuvette (Bio-Rad, USA) and electroporated using the Genepulser Xcell Bio-Rad electroporation system's default program (10 msec, 150 V, square wave pulse). The transfected cells were gently resuspended in 1 ml of prewarmed high-glucose DMEM medium supplemented with 20% FBS, then plated in 24- and 96-well plates for RT-PCR and MTT, respectively, at 37°C and 5% CO2. An electroporation system (Bio-Rad) was used to transfect the pIRES2-EGFP plasmid into A549 cell lines (2×10^5 cells) of the same length as the PGL-DTA vector. Afterward, the cells were seeded into 24-well culture plates. Fluorescence microscopy was performed 48 hours after transfection of the cells. By counting the number of GFP-positive cells determined the percentage of transfected cell populations.

EMT Induction: Treatment with TGF-β1 or Hypoxia Condition

Hypoxia was accomplished by replacing the medium in the first study the day after transfection with DMEM containing 5% FBS and 5 ng/mL recombinant human TGF-β1 (Calbiochem, Billerica, MA, USA) and incubating the cells at 37°C with 5% CO2 for a further 48 hours. In the second study, under standard hypoxia conditions (<1% O2, 5% CO2, 94% N2), which was prepared with a hypoxia chamber (STEMCELL Technologies, USA), the medium of cells was replaced by a DMEM medium supplemented with 5% FBS.

Proliferation Assay

For assessment of cell proliferation following transfection and EMT induction was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (Sigma, USA). First, plasmids were electroporated into cells as described previously. Following seeding, cells adhered for 24 hours in a growth medium supplemented with 10% FBS in two sets of 96-well plates. In order to induce EMT by TGF-β1

treatment, a fresh medium containing 5% FBS and 5 ng/ml TGF-β1 medium was added to the cells, and cells were incubated at 37°C for an additional 48 h. Under hypoxic conditions (<1% O2, 5% CO2, 94% N2), the cells were incubated for 48 h at 37 °C with a fresh medium containing 5% FBS. After transfection and EMT induction, MTT assays were conducted using 20 uL of MTT stock reagent (5 mg/mL) per well. After incubation for 4 hours at dark, the medium was removed, and 100 µL DMSO was added to each well. An absorbance measurement at 570 nm and reference at 630 nm was performed after 15 minutes of incubation by using a microplate reader.

Real-time PCR

Total RNAs of the A549 cell line were isolated using a RiboExTM kit (Geneall, Portugal), and the total RNAs were separated by chloroform phase separation and precipitated with isopropanol. Also, the quality and quantity of isolated RNAs were evaluated using a nanodrop spectrophotometer, with absorption at the optimal density of 260 and 280 (OD). RNA samples (1 µg) were amplified with SuperScript™ double-stranded cDNA synthesis kit for cDNA synthesis using an oligo-dT primer (InvitrogenTM, USA). Then the amount of cDNAs was measured using the Real-time PCR method with primer pair for GAPDH and studied genes using SYBR Green PCR Master Mix (Takara, USA). Real-time PCR was performed using a Rotor-Gene Q kit (USA). Finally, the $2^{-\Delta\Delta CT}$ method was used to quantify the relative expression of genes. Amplification of the GAPDH gene was used as an internal control for real-time PCR, and fold changes (FC) in gene expression were also normalized with the GAPDH gene. The sequence of primers used for Real-time PCR is shown in Supplementary Table 1.

Scratch Test

To simulate cell motility, scratch or direct manipulation of cells was implemented as a complementary assay to monitor the effect of TGF-β1 and hypoxia on the migration of the cells. 24 h after the transfection, seeding the cells, and before replacing the medium, two crossed gaps were created by scratching the monolayer of the cells with a p100 pipette tip. After 48h, the cell migration was observed under the light microscope, and photos have been provided for qualitative evaluation of cell migration.

Statistical Analysis

Three separate experiments were conducted in triplicate (n=9), and the results were expressed as mean±standard deviation. The results means were compared using the Mann-Whitney U test. The analysis was performed using SPSS (Statistical Package for Social Sciences, Inc., Chicago, IL) version 20, and the display was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical significance was defined as a $p<0.05$.

RESULTS

Diphtheria Toxin Expression under N-cadherin Promoter

As shown in Figure 1, Diphtheria toxin subunit A, 585 bp, cloned downstream of human N-cadherin promoter, which was about 2900 bp.

Morphological Changes after EMT Induction

After cell treatment with TGF-β1, or exposing cells to hypoxia for 48 h, significant morphological changes in A549 cells were observed (Figure 1), demonstrating a fibroblast-like morphology (Figure 2B and C) compared to the basic epithelial form of A549 cells (Figure 2A).

Transfection Efficiency

Figure 3 shows the expression of GFP in transfected cells as an indicator of transfection efficiency through electroporation. The approximate efficiency of transfection was low and estimated to be about 20%.

pGL-DTA Affects the Relative Expressions of Epithelial and Mesenchymal Markers to Block EMT

Figure 3 shows the effect of TGF-β1 and hypoxia as independent factors inducing EMT on epithelial and mesenchymal markers after 48 hours. As expected, after EMT induction, the mesenchymal markers expression, N-cadherin and vimentin, were increased and epithelial markers like β-catenin and E-cadherin decreased. Thus, the pGL-DTA construct was successfully expressed, and EMT induction was confirmed. In transfected groups, the expression of DT-A was significantly increased and consequently, the expression of mesenchymal markers, decreased and expression of epithelial markers increased in comparison with the EMT group. However, despite our expectation, E-cadherin did not in line with others and not increase (data not shown). Figure 3).

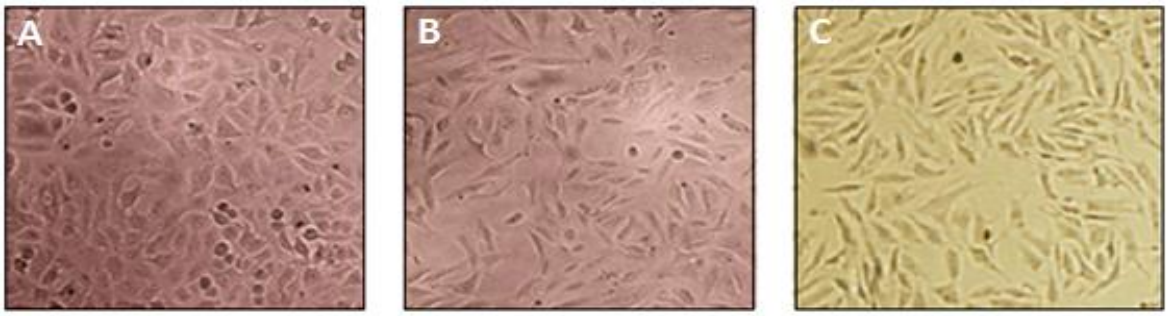

Figure 1. Morphologic changes of A549 cells induced by TGF-β1 or exposure to hypoxia for 48 h. A: Untreated A549 cells, B: A549 cells treated with TGF-β1 for 48 h (5 ng/mL), C: A549 cells exposed to hypoxia (<1% O2, 5% CO2, 94% N2) for 48 h. (Images at 20× magnification).

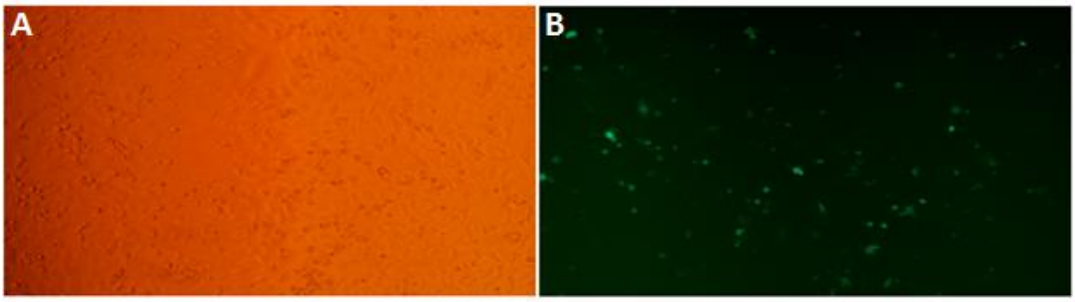

Figure 2. Schematic view of EGFP-transfected A549 cells by inverted fluorescence microscopy after 48 h. A: Transfected cells light field B: Transfected cells fluorescent field (Images at 10× magnification).

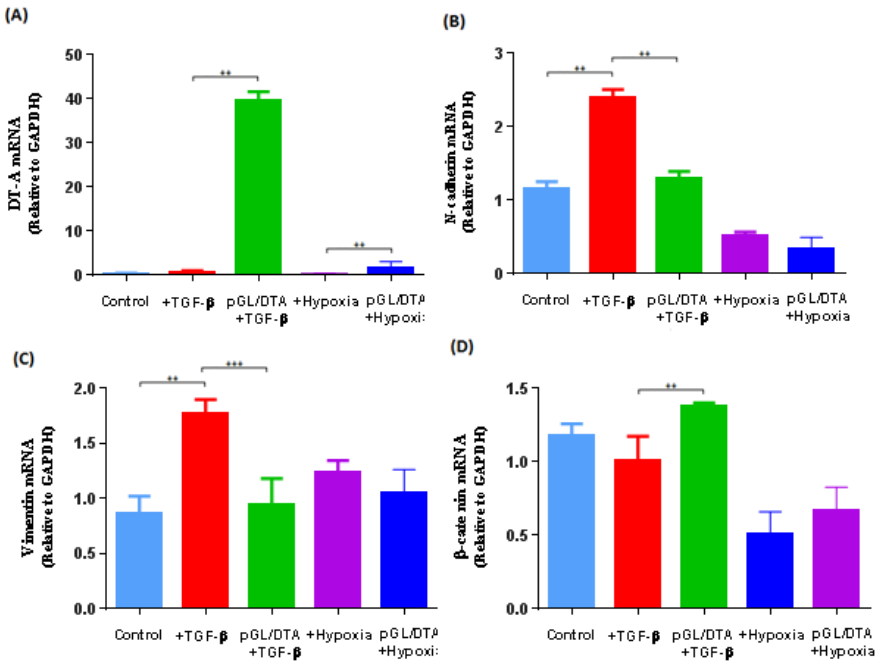

Figure 3. Real-time PCR analysis for relative expressions of (A) DT-A, (B) N-cadherin, (C) Vimentin, and (D) β-catenin. Results are presented as the mean ±standard error of the mean from triplicate experiments. (p<0.01, *** p<0.001). As you mentioned the data were shown based on 20% transfection**

Moreover, the decrease in N-cadherin expression indicates that the pGL-DTA structure effectively blocks EMT. This result was repeated in vimentin (Figure 4C). Transfected cells also produced more N-cadherin and Vimentin mRNA when hypoxia was applied, confirming the effectiveness of the pGL-DTA construct in inhibiting EMT (Figure 3B and 3C).

For epithelial markers, the results showed that the expression of E-cadherin in transfected cells decreased after treatment with TGF-β1. Also, the expression of β-catenin decreased after treatment with TGF-β1, which confirms EMT. However, β-catenin expression was increased in transfected cells, suggesting that pGL-DTA inhibited EMT (Figure 4). Also, β-catenin mRNA expression was increased in transfected cells exposed to hypoxia compared to non-transfected cells (Figure 3D).

pGL-DTA Hinders the Migration of Cells Induced by TGF-β1 and Hypoxia

After treatment cells with TGF-β1 for 48 hours, the migration of cells (Figure 4c) was significantly higher compared to the control group (Figure 4a). Despite high cell migration in the TGF-β1-treated group (Figure 4b), the movement of transfected cells was suppressed entirely (Figure 4d). Similar results were obtained in cell groups under hypoxia (Figure 4a). Transfected cells showed significantly less migration under hypoxic conditions than non-transfected cells (Figure 4f). These data support the ability of TGF-β1 and hypoxia to induce EMT separately and that the presented construct can block EMT by inhibiting cell migration.

Cytotoxicity Assessment of pGL-DTA on EMT-Induced Cells

Figure 5 shows the results of the MTT test that A549 cells treated with TGF-β1 did not show growth suppression compared to the control group. On the other hand, the proliferation of cells transfected with pGL-DTA followed by treatment with 5 ng/mL TGF-β1 was significantly lower than cells treated with TGF-β1 ($p<0.01$), indicating toxicity in the present structure. Also, based on the data obtained from hypoxia group cells, the effect of pGL-DTA cytotoxicity on transfected cells under hypoxia conditions was significantly higher than non-transfected cells ($p>0.05$).

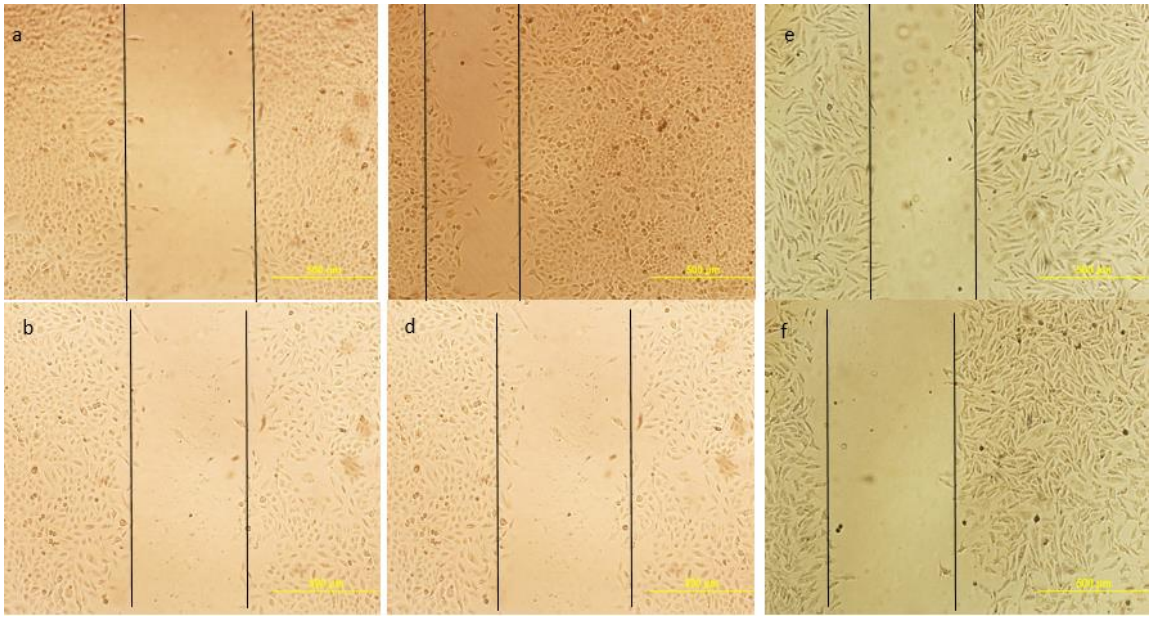

Figure 4. Microscopic view of cell migration in A549 cell lines. (a) Control A549 cells (b) Transfected A549 cells (c) A549 cells treated with TGF-β1 for 48 h (d) Transfected A549 cells treated with TGF-β1 for 48 h. (e) A549 cells under hypoxia condition for 48 h (f) Transfected A549 cells under hypoxia condition for 48h.

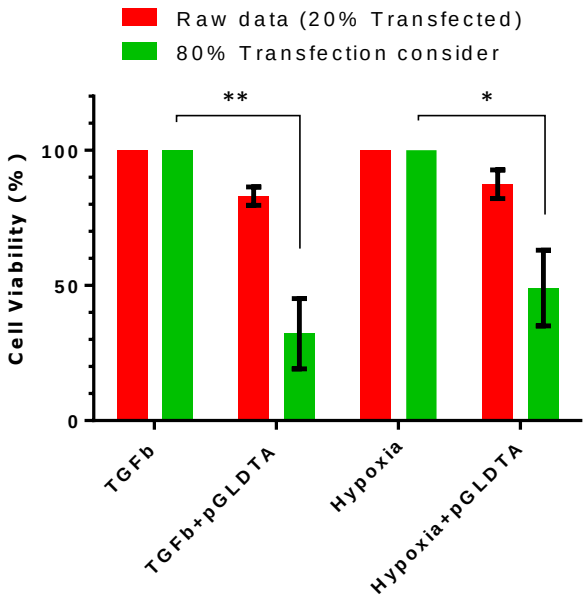

Figure 5. Cytotoxic effect of pGL-DTA on EMT-induced A549 cells after 48 h. Data represent the mean (± SD) of percent viability from three independent experiments performed in triplicate. Error bars indicate ±SD. (*p< 0.05, **p< 0.01). As was explained in the result, the transfection efficiently was only 20% and consequently the toxin expression was shown no significant difference. However, with considering transfection in 80% of cells, the result will be significant.

DISCUSSION

Despite immense advances in treatment methods, cancer-related deaths remain significant worldwide. Understanding the molecular pathways of cancer and developing effective treatment methods will lead to a reduction in the death rate.[41] Suicide gene therapy strategies using bacterial toxins, including diphtheria toxin, have provided good therapeutic potential for cancer treatment.[21] In this regard, a DNA plasmid containing the H19 promoter regulatory sequences was shown to induce toxicity in bladder cancer cells by expressing diphtheria toxin intracellularly.[42] Moreover, other models based on plasmids capable of directing the expression of the diphtheria toxin chain have previously been developed for cancer treatment in liver[43] and ovarian cells.[44] Two studies have shown that diphtheria toxin induces cell death in cancer tumors when alpha-fetoprotein promoters and prostate-specific antigens are used in some liver and prostate cancer cells.[45,46]

Epithelial mesenchymal transition, which is primarily involved in the differentiation of normal cells during early development, is required for metastasis.[47] It can reprogram malignant tumors into mesenchymal-like cells in most cancers.[48] In this process, epithelial cells migrate and spread to the adjacent tissues to create metastasis. Therefore, understanding the molecular pathways of EMT is essential for achieving cancer treatment goals.[49]

Extracellular factors such as hypoxia and TGF-β can activate the EMT process. However, it has been reported that TGF-β can induce pro- and anti-apoptotic effects on epithelial cells.[50] Human cancer cells also become significantly invasive under hypoxic conditions, which stimulates the EMT process independently.[51] An important feature of EMT is the decreased expression of E-cadherin and increased expression of N-cadherin during cancer development.[52]

Studies on the N-cadherin promoter are limited compared to E-cadherin. A potential E-box sequence in the first intron of the N-cadherin gene at +2,627 to +2,632 bp has been found as the specific binding site for Twist1. Mutagenesis in the E-box sequence eliminated Twist1's ability to induce N-cadherin promoter activity. Twist1 siRNAs confirmed its potential role in regulating N-cadherin promoter activity, resulting in a 50% decrease in N-cadherin mRNA expression. The role of Twist1 in regulating EMT is suspected to be related to hypoxia.[53]

This study focused on using a specific gene promoter, N-cadherin, to activate diphtheria toxin in cancer cells undergoing EMT. The goal was to induce cell death and inhibit EMT. The study found that all EMT inducers were successful in activating the process.[48] However, there was an unexpected response to E-cadherin expression.

This study suggests that inducing apoptosis in cancer cells and inhibiting EMT can be achieved by targeting the N-cadherin promoter with diphtheria toxin. The vector containing the N-cadherin promoter competes with transcription factors involved in EMT, attracting and engaging endogenous N-cadherin and Vimentin, which are mesenchymal markers. However, not all transcription factors involved in EMT may be inhibited, and unknown factors may influence E-cadherin expression, which was not as desired. The scratch test results indicate that EMT inhibition depends on the expression of N-cadherin and Vimentin, while E-cadherin may not be involved. Further research is needed to fully understand the complex interactions between the promoter, transcription factors, and mesenchymal markers involved in EMT. A study found that inhibiting EMT did not result in the expected increase in E-cadherin expression. The study blocked certain sequences on the E-cadherin promoter to prevent its expression from being reduced during EMT. However, this change did not lead to the desired expression pattern of E-cadherin. In fact, the expression of E-cadherin unexpectedly decreased, while other markers associated with EMT also showed decreased expression.[54]

In addition, cells that were transfected with pGL-DTA and treated with TGF-1 showed a lower rate of cell growth compared to cells treated with TGF-1 alone. This confirms that the current construct is toxic. There was also a significant difference between transfected and non-transfected cells in terms of cytotoxicity caused by pGL-DTA under hypoxia conditions (p>0.05). This finding is consistent with previous studies and suggests that oxygen deficiency in cancer cells, along with reactive oxygen species (ROS), leads to the selection of tumor cells with aggressive characteristics and high growth ability.[55]

In line with previous studies, the results obtained from the study conducted by Sadeghi et al. demonstrated that exposure to 150 mM and 200 mM CoCl2 did not have a significant impact on the viability of HEK293T cells after 24 and 48 hours.[56] This finding aligns with the notion that hypoxia can stabilize hypoxia-inducible factor (HIF), which in turn affects various cellular processes such as anaerobic metabolism, angiogenesis, invasion, and survival.[57] Additionally, the study conducted by Gupta et al. revealed that cancer cell line survival increased under hypoxic conditions after 24 and 48 hours. These findings further support the role of hypoxia in influencing cellular responses and highlight the potential implications for cancer cell survival and adaptation.[58]

In a similar study, the impact of the designed structure on cell viability did not demonstrate a significant effect on A549 cells, despite the inhibition of EMT induced by TGF-β1.[59] However, previous research has indicated that TGF-β1 is the most potent inducer of EMT.[60] Our findings support this, as we observed that TGF-β1 can induce EMT in the cell line by transforming polygonal cells into spindle-shaped cells, which aligns with previous findings.[61] Additionally, our study revealed a decrease in E-cadherin expression and an increase in N-cadherin, β-catenin, and Vimentin expression, further supporting the findings of the aforementioned study.[61]

The engineered pGL-DTA construct could block EMT by downregulating N-cadherin and Vimentin and upregulating β-catenin. Furthermore, results from cell migration indicate that N-cadherin has a regulatory role in preventing tumor cell migration and metastasis. Also, the cytotoxic effect of diphtheria toxin demonstrated that using specific event promoter, like an important molecule in EMT, can be a potential cancer treatment. Overall, pGL-DTA showed an inhibitory effect on EMT and concomitant killing of EMT-induced cells. It is necessary to conduct further research like animal model to gain a deeper understanding of this strategy effectiveness.

STATEMENT OF ETHICS

This study is by the Tehran University of Medical Sciences with the ethical code of IR.TUMS.VCR.REC. 1398.047.

FUNDING

This research was financially supported by a grant from Tehran University of Medical Sciences, Tehran, Iran [Grant No. 95-02-87-32022].

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

The Authors a special thanks to colleagues of Immunology Asthma & Allergy Research institute who helped us in this study.

REFERENCES

1. Monsuez J-J, Charniot J-C, Vignat N, Artigou J-Y. Cardiac side-effects of cancer chemotherapy. Int J Cardiol. 2010;144(1):3-15.
2. Davis AM, Scott TA, Morris KV. Harnessing Rift Valley fever virus NSs gene for cancer gene therapy Cancer Gene Ther. 2022:1-10.
3. Hay ED. An overview of epithelio-mesenchymal transformation. Cells Tissues Organs. 1995;154(1):8-20.
4. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. Nat Rev Mol Cell Biol. 2014;15(3):178-96.
5. Thiery JP. Epithelial–mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002;2(6):442-54.
6. Tsai JH, Donaher JL, Murphy DA, Chau S, Yang J. Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. Cancer cell. 2012;22(6):725-36.
7. Ocaña OH, Córcoles R, Fabra Á, Moreno-Bueno G, Acloque H, Vega S, et al. Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prrx1. Cancer cell. 2012;22(6):709-24.
8. Gupta S, Shetty DC, Gulati N, Juneja S, Jain A. Potentiated action on the progression of OSMF by hypoxia mediated signaling pathway by the epithelial mesenchymal transition and angiogenic apparatus. J Cancer Res Ther. 2023.
9. Mendik P, Kerestély M, Kamp S, Deritei D, Kunšič N, Vassy Z, et al. Translocating proteins compartment-specifically alter the fate of epithelial-mesenchymal transition in a compartmentalized Boolean network model. NPJ Syst Biol Appl. 2022;9(8):1-19.
10. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119(6):1420-8.
11. Chui MH. Insights into cancer metastasis from a clinicopathologic perspective: Epithelial-Mesenchymal Transition is not a necessary step. Int J Cancer. 2013;132(7):1487-95.
12. Bill R, Christofori G. The relevance of EMT in breast cancer metastasis: Correlation or causality? FEBS letters. 2015;589(14):1577-87.
13. Lu X, Kang Y. Hypoxia and Hypoxia-Inducible Factors: Master Regulators of MetastasisHypoxia and HIFs in Cancer Metastasis. Clini Cancer Res. 2010;16(24):5928-35.
14. Yang M-H, Wu M-Z, Chiou S-H, Chen P-M, Chang S-Y, Liu C-J, et al. Direct regulation of TWIST by HIF-1α promotes metastasis. Nat Cell Biol. 2008;10:295.
15. Ryan HE, Lo J, Johnson RS. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J. 1998;17(11):3005-15.
16. Chen ZF, Behringer RR. twist is required in head mesenchyme for cranial neural tube morphogenesis. Genes Dev. 1995;9(6):686-99.
17. Wu MZ, Tsai YP, Yang MH, Huang CH, Chang SY, Chang CC, et al. Interplay between HDAC3 and WDR5 is essential for hypoxia-induced epithelial-mesenchymal transition. Mol Cell. 2011;43(5):811-22.
18. Kitami K, Yoshihara M, Tamauchi S, Sugiyama M, Koya Y, Yamakita Y, et al. Peritoneal restoration by repurposing vitamin D inhibits ovarian cancer dissemination via blockade of the TGF-β1/thrombospondin-1 axis. Matrix Biol. 2022;109:70-90.
19. Wan CC, Nisar MF, Wu H. Pharmacological Activities of Natural Products through the TGF-β Signalling Pathway. Evid Based Complement Alternat Med. 2022; 2022: 9823258.
20. Seoane J, Gomis RR. TGF-β family signaling in tumor suppression and cancer progression. Cold Spring Harb Perspect Biol. 2017;9(12):a022277.
21. Shafiee F, Aucoin MG, Jahanian-Najafabadi A. Targeted diphtheria toxin-based therapy: a review article. Front Microbiol. 2019;10:2340.
22. Krueger KM, Barbieri JT. The family of bacterial ADP-ribosylating exotoxins. Clin Microbiol Rev. 1995;8(1):34-47.
23. Wolf P, Elsässer-Beile U. Pseudomonas exotoxin A: from virulence factor to anti-cancer agent. Int J Med Microbiol. 2009;299(3):161-76.
24. Mateyak MK, Kinzy TG. ADP-ribosylation of translation elongation factor 2 by diphtheria toxin in yeast inhibits translation and cell separation. J Biol Chemistry. 2013;288(34):24647-55.
25. Gofrit ON, Benjamin S, Halachmi S, Leibovitch I, Dotan Z ,Lamm DL, et al. DNA based therapy with diphtheria toxin-A BC-819: a phase 2b marker lesion trial in patients with intermediate risk nonmuscle invasive bladder cancer. J Urol. 2014;191(6):1697-702.

26. Asrorov AM, Muhitdinov B, Tu B, Mirzaakhmedov S, Wang H, Huang Y. Advances on Delivery of Cytotoxic Enzymes as Anticancer Agents. Molecules. 2022;27(12):3836.
27. Markiewicz A, Topa J, Nagel A, Skokowski J, Seroczynska B, Stokowy T, et al. Spectrum of epithelial-mesenchymal transition phenotypes in circulating tumour cells from early breast cancer patients. Cancers. 2019;11(1):59.
28. Brown MS, Muller KE, Pattabiraman DR. Quantifying the epithelial-to-mesenchymal transition (EMT) from bench to bedside. Cancers. 2022;14(5):1138.
29. Falgàs A ,Garcia-León A, Núñez Y, Serna N, Sánchez-Garcia L, Unzueta U, et al. A diphtheria toxin-based nanoparticle achieves specific cytotoxic effect on CXCR4+ lymphoma cells without toxicity in immunocompromised and immunocompetent mice. Biomed Pharmacother. 2022;150:112940.
30. Shajari S, Farajollahi MM, Behdani M, Tarighi P. Production and Conjugation of Truncated Recombinant Diphtheria Toxin to VEGFR-2 Specific Nanobody and Evaluation of its Cytotoxic Effect on PC-3 Cell Line. Mol Biotechnol. 2022:1-9.
31. Ohana P, Bibi O, Matouk I, Levy C, Birman T, Ariel I, et al. Use of H19 regulatory sequences for targeted gene therapy in cancer. Int J Cancer. 2002;98(5):645-50.
32. Lavie O, Edelman D, Levy T, Fishman A, Hubert A, Segev Y, et al. A phase 1/2a, dose-escalation, safety, pharmacokinetic, and preliminary efficacy study of intraperitoneal administration of BC-819 (H19-DTA) in subjects with recurrent ovarian/peritoneal cancer. Arch Gynecol Obstet. 2017;29.751-61:(3)5
33. Hine CM, Seluanov A, Gorbunova V. Rad51 promoter-targeted gene therapy is effective for in vivo visualization and treatment of cancer. Mol Ther. 2012;20(2):347-55.
34. Chen C, Yue D, Lei L, Wang H, Lu J, Zhou Y, et al. Promoter-operating targeted expression of gene therapy in cancer: current stage and prospect. Mol Ther Nucleic Acids. 2018;11:508-14.
35. Cao Y, Xu Y, Zhang L, Li Z, Jiang Y, Tian X, et al. Utilization of Rad51C promoter for transcriptional targeting of cancer cells. Oncotarget. 2014;5(7):1805.
36. Lin B, Gao A, Zhang R, Ma H, Shen H, Hu Q, et al. Use of a novel integrase-deficient lentivirus for targeted anti-cancer therapy with survivin promoter-driven diphtheria toxin A. Medicine. 2015;94(31).
37. Alkharabsheh O, Frankel AE. Clinical activity and tolerability of SL-401 (Tagraxofusp): recombinant diphtheria toxin and interleukin-3 in hematologic malignancies. Biomedicines. 2019;7(1):6.
38. Aruna G. Immunotoxins: a review of their use in cancer treatment . J Stem Cells Regen Med. 2006;1(1):31.
39. Donaghy H, editor Effects of antibody, drug and linker on the preclinical and clinical toxicities of antibody-drug conjugates. MAbs. 2016;8(4):659-71.
40. Gholami MD, Falak R, Heidari S, Khoshmirsafa M, Kazemi MH, Zarnani A-H, et al. A truncated snail1 transcription factor alters the expression of essential EMT markers and suppresses tumor cell migration in a human lung cancer cell line. Recent Pat Anticancer Drug Discov. 2019;14(2):158-69.
41. Steeg PS, Theodorescu D. Metastasis: a therapeutic target for cancer. Nat Clin Practice Oncol. 2008;5(4):206-19.
42. Sidi AA, Ohana P, Benjamin S, Shalev M, Ransom JH, Lamm D, et al. Phase I/II marker lesion study of intravesical BC-819 DNA plasmid in H19 over expressing superficial bladder cancer refractory to bacillus Calmette-Guerin. J Urol. 2008;180(6):2379-83.
43. Zhang D-M, Lin Z-Y, Yang Z-H, Wang Y-Y, Wan D, Zhong J-L, et al. lncRNA H19 promotes tongue squamous cell carcinoma progression through β-catenin/GSK3β/EMT signaling via association with EZH2. Am J Translat Res. 2017;9(7):3474.
44. Mizrahi A, Czerniak A, Ohana P, Amiur S, Gallula J, Matouk I, et al. Treatment of ovarian cancer ascites by intra-peritoneal injection of diphtheria toxin A chain-H19 vector: a case report. Journal of Med Case Rep. 2010;4(1):1-5.
45. Watson-Hurst K, Becker D. The role of N-Cadherin, MCAM, and β3 integrin in melanoma progression, proliferation, migration and invasion. Cancer Biol Ther. 2006;5(10):1375-82.
46. Fu J, Qin L, He T, Qin J, Hong J, Wong J, et al. The TWIST/Mi2/NuRD protein complex and its essential role in cancer metastasis. Cell Res. 2011;21(2):275-89.
47. Kim Y-N, Koo KH, Sung JY, Yun U-J, Kim H. Anoikis Resistance: An Essential Prerequisite for Tumor Metastasis. Int J Cell Biol. 2012;2012:306879.
48. Matouk IJ, Raveh E, Abu-lail R, Mezan S, Gilon M, Gershtain E, et al. Oncofetal H19 RNA promotes tumor metastasis. Biochim Biophys Acta. 2014;1843(7):1414-26.
49. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene. 2010;29(34):4741-51.
50. Caja L, Bertran E, Campbell J, Fausto N, Fabregat I. The transforming growth factor-beta (TGF-β) mediates

acquisition of a mesenchymal stem cell-like phenotype in human liver cells. J Cell Physiol. 2011;226(5):1214-23.
51. Yang M-H, Wu M-Z, Chiou S-H, Chen P-M, Chang S-Y, Liu C-J, et al. Direct regulation of TWIST by HIF-1α promotes metastasis. Nat Cell Biolo. 2008;10(3):295-305.
52. Thiery JP, Boyer B. The junction between cytokines and cell adhesion. Curr Opin Cell Biol. 1992;4(5):782-92.
53. Alexander NR, Tran NL, Rekapally H, Summers CE, Glackin C, Heimark RL. N-cadherin gene expression in prostate carcinoma is modulated by integrin-dependent nuclear translocation of Twist1. Cancer Res. 2006;66(7):3365-9.
54. Gholami MD, Falak R, Heidari S, Khoshmirsafa M, Kazemi MH, Zarnani A-H, et al. A truncated snail1 transcription factor alters the expression of essential EMT markers and suppresses tumor cell migration in a human lung cancer cell line. Recent Pat Anticancer Drug Discov. 2019;14(2):158-69
55. Kurnit KC, Draisey A, Kazen RC, Chung C, Phan LH, Harvey JB, et al. Loss of CD73 shifts transforming growth factor-β1 (TGF-β1) from tumor suppressor to promoter in endometrial cancer. Cancer Lett. 2021;505:75-86.
56. Sadeghi F, Kardar GA, Bolouri MR, Nasri F, Sadri M, Falak R. Overexpression of bHLH domain of HIF-1 failed to inhibit the HIF-1 transcriptional activity in hypoxia. Biol Res. 2020;53(1):1-11.
57. Sullivan LB, Chandel NS. Mitochondrial reactive oxygen species and cancer . Cancer Meta. 2014;2(1):1-12.
58. Gupta R, Chetty C, Bhoopathi P, Lakka S, Mohanam S, Rao JS, et al. Downregulation of uPA/uPAR inhibits intermittent hypoxia-induced epithelial-mesenchymal transition (EMT) in DAOY and D283 medulloblastoma cells. Int J Oncol. 2011;38(3):733-44.
59. Ko H, So Y, Jeon H, Jeong M-H, Choi H-K, Ryu S-H, et al. TGF-β1-induced epithelial–mesenchymal transition and acetylation of Smad2 and Smad3 are negatively regulated by EGCG in human A549 lung cancer cells. Cancer lett. 2013;335(1):205-13.
60. Sheppard D. Transforming growth factor β: a central modulator of pulmonary and airway inflammation and fibrosis. Proc Am Thorac Soc. 2006;3(5):413-7.
61. Li C, Song L, Zhang Z, Bai X-X, Cui M-F, Ma L-J. MicroRNA-21 promotes TGF-β1-induced epithelial-mesenchymal transition in gastric cancer through up-regulating PTEN expression. Oncotarget. 2016;7(41):66989.