Original Article

Design of Antisense Oligonucleotides against *twist1* Gene and Evaluation of Their Anti-Invasive Effects on Prostate Cancer Cell Lines

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Abstract

Introduction: Prostate cancer is one of the most common cancers and the second major cause of mortality in men. Different researches have shown that the overexpression of a gene called twist1 leads to initiation of metastasis process in this cancer. TWIST1 protein triggers this process through stimulating the transition pathway of cells from epithelial to mesenchymal tissue.

Materials and Methods: In this study, four oligonucleotides of antisense RNA have been designed for twist1 gene, and its anti-metastatic effect was examined in two cell lines PC3 and LNCaP. The antisense oligonucleotides (ASOs) were designed as single strands with a length of 20 nucleotides and chosen from ASOs suggested by Soligo program. The ASOs were synthesized in phosphorothioated form. MTT assay was used for evaluating the ASOs cytotoxicity effect on PC3 and LNCaP cell lines. The cell lines were transfected with 500 nmol of antisense oligonucleotides using cationic polymer turbofect and incubated for 48 hours, and then, their invasive ability were measured by CytoSelect™ Cell Invasion Assay Kit.

Results: The anti-invasive effect of ASOs in LNCaP and PC3 had a significant difference. This effect was more significant in LNCaP cell line as compared to PC3. The most anti- invasive effect was observed in LNCaP cell line (50%).

Conclusion: According to the results, Antisense oligonucleotides were effective in decreasing the invasion ability of two cell lines PC3 and LNCaP and therfore can be considered as a good candidate for preventing the prostate cancer metastasis.

Keywords: Prostate cancer, TWIST1, Antisense oligonucleotides, LNCaP, PC3

1. Introduction

Prostate cancer is the most common cancer and the fifth cause of cancer-induced mortality among men in developed countries [1, 2]. The main cause of mortality induced by prostate cancer is its metastasis to other tissues of the body. More than any other solid tumor, prostate cancer metastasizes to the bone tissue. Around 65-75% of men with advanced prostate cancer suffer from bone metastasis [3]. One of the genes that initiates metastasis is *twist1* gene, which codes a transcription factor with a basic helix-loop-

helix structure [4]. Normally, the maximum expression of this gene occurs in the placenta and during embryonic development, when the cells move and differentiate, whereby different tissues and organs are formed [5]. After the birth and in adulthood, this gene can act as an oncogene [6]. The overexpression of this gene in cancer cells such as prostate, breast, lungs, uterus, skin, stomach, melanoma, osteosarcoma, rhabdomyosarcomas, and other tumors confirms this issue [7-10]. Twist1 suppresses the expression of E-cadherin and increases Epithelial Mesenchymal Transition (EMT) which occurs in the primary stage of metastasis, and is a process whereby the epithelial cells lose their polarity and adhesion capability [11]. Unlike most solid tumors, prostate cancer has usually a poor response to chemotherapeutic agents. Thus, developing new drugs for the treatment of prostate cancer and preventing initiation of metastasis are inevitable [11]. When the overexpression of a special gene is known as a cause of disease progress, a single-strand nucleic acid (DNA, RNA, or its chemical analog) can be synthesized, forming an RNA/DNA double strand, causing diminished level of mRNA and targeting protein [7, 8]. One of the inhibitors for gene expression which has attracted researchers' attention is antisense oligonucleotides. An antisense oligonucleotide is a short single strand deoxyribonucleic acid with typical length of 20 nucleotides, with complementary sequence of target gene's mRNA [12, 13]. Antisense drugs become hybridized with a special mRNA and can prevent its translation to protein. Indeed, they can cause inhibition of transference of genetic information from DNA to the protein [14, 15]. The most important advantage of antisense drugs is that these molecules have the maximum effect on the target cell, while creating the minimum side effects compared to other drugs [16]. Numerous antisense drugs are in different stages of clinical experiments, and some of them have been approved by the Food and Drug Organization of the United States [17, 18]. Other advantages of RNA antisense drugs are their easy design and formulation approach in comparison to chemical drugs [13]. In this research, four antisense oligonucleotides was designed for the mRNA of *twist1* gene, and their cytotoxicity and inhibitory effect were evaluated on invasive ability of two cell lines of prostate cancer, PC3 and LNCaP.

2. Materials and Methods

2.1. Antisense oligonucleotides design

Since antisense nucleotide strands are rapidly attacked by all types of nucleases, the designed oligonucleotides were synthesized in phosphorothioate form. This is because a phosphorothioated ASO not only enhances nuclease resistance, but also is better absorbed by the surface proteins of cells [19]. To design the antisense oligonucleotides, the sequence of mRNA of twist1 gene with the identification number of NM-000474.4 was accessed through NCBI database with the address of https://www.ncbi.nlm.nih.gov. Then, it was entered in sfold Web server, which was created by Y Ding in 2004 for rational design of nucleic acids with the address of sfold.wadsworth.org, where the requested information was recorded in the soligo part. Different antisense oligonucleotides were proposed by the mentioned server. Four different oligonucleotides were chosen considering different factors such as ΔG of the hybridization reaction, GC content, and presence or absence of special motifs. Next, the similarity of oligonucleotide sequences with other genes of human genome was compared by online BLAST to ensure that they will only attach to their particular sequence in *twist1* gene. Eventually, the mentioned oligonucleotides, TW6, TW5, TW4 and TW3, were synthesized by SBS Genetech Co. Ltd in phosphorothioated form (Table 1).

Table 1. Antisense Oligonucleotide sequences, GC% and ΔG

Name	Sequence (5´-3´)	GC%	ΔG
TW3	CGTCCTGCATCAT**CTCT**CGA	55%	-9.8
TW4	TGG**CTCT**TCCTCGCTGTTGC	60%	-8.8
TW5	ACGTCCTGCATCAT**CTCT**CG	55%	-10.3
TW6	CTGG**CTCT**TCCTCGCTGTTG	60%	-9.1

2.2. Tumor cell lines, culture and transfection

The human prostate cancer cell lines PC3 (grade IV, adenocarcinoma and derived from metastatic bone) and LNCap (carcinoma and derived from metastatic left supraclavicular lymph node) were provided from Avicenna Research Institute (Fig.1). The tumor cell culture media were removed from the culture flasks. Then the cells were washed two times by adding of 2-3ml of phosphate‑buffered saline (PBS (1x); Inoclon). Subsequently, an equal volume of 0.25% Trypsin-EDTA (1-2ml) (Inoclon) was added and the cells were incubated at 37°C in humidified atmosphere containing 5% CO2 for approximately two minutes. The cells were observed under the microscope. When ≥ 90% of the cells were detached, 3-5ml of RPMI- 1640 (Bio-Idea) with 10% fetal bovine serum (FBS. Gibco) was added for inactivation of trypsin. Detached cells were transferred to a 15-mL tube and centrifuged at 1200rpm at 25°C for 5 minutes. The cell pellets were re-suspended in a complete culture medium (10% FBS and 1% penicillin/streptomycin) (Inoclon). The suspension was gently dispersed by pipetting and transferred into new flasks. Finally, tumor cells were incubated at 37°C in humidified atmosphere containing 5% CO2. There are currently several commercial transporter reagents for DNA and protein into cells, which their formulation is based on lipid, polymer, or peptide that facilitate their cellular uptake. Nucleic acids cannot enter into the cells without the aid of transfection reagents. The transporter reagent used in this study was TurboFect (Thermo Fisher Scientific) that is a cationic polymer, has high cellular absorption and enters the cell via micropinocytosis. Adding serum to the culture medium can reduce the absorption of TurboFect by up to 50% [20].

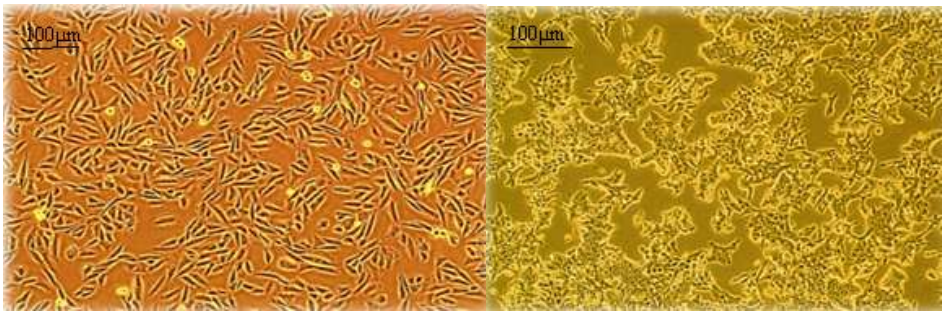


Figure1. The human prostate cancer cell lines PC3 (left) and LNCap (right)

2.3. MTT assay

Cell toxicity of ASOs in LNCaP and PC-3 cell lines was determined by using the colorimetric MTT cell viability assay. About 8000 cells were seeded in each well of 96- well plate and incubated overnight. The next day, the cells were starved for 2-3 hours and treated with different amounts of each ASOs (0, 62.5, 125, 250, 500 and 1000 nmol), TurboFect (0, 0.625, 1.25, 2.5, 5 and 10 µl were used respectively for antisense oligonucleotides concentrations mentioned above) and serum-free RPMI 1640. Before transfection, Turbofect and ASOs were incubated for 20 min at room temperature. Also according to TurbFect's toxic effect on cells, the cell lines were treated with same concentrations of TurboFect without ASOs.

Subsequently, the old media was replaced with new culture media with 10% FBS. After 48 hours incubation at 37°C in 5% CO_2 atmosphere, 200 µl of 0.5 mg/ml MTT (Merk) was added to each well and incubated at 37°C for 4 hours. Then, media was discarded and 100 µl of DMSO (Inoclon) added into wells carefully. The plate was covered with tinfoil and cells were agitated on orbital shaker for 1 hour. Finally, absorbance measured at 560 nm with ELISA Reader (BioTek).
Experiments were repeated 3 times.
Data was evaluated with Graph Pad Prism 6.

2.4. Cell Invasion Assay

Cell invasion assay was performed using the CytoSelect™ Cell Invasion Assay Kit, containing polycarbonate membrane inserts in a 24-well plate. Inserts had 8 µm pore size membranes. The invasion chamber plate was warmed up at 25°C for 10 minutes. By adding 300 µl of serum-free media to the inner compartment basement membrane layer of the cell culture, inserts were rehydrated and incubated at 25°C for 1 hour. Then, rehydration medium was removed from the inserts, carefully. A cell suspension containing 75×10^4 cells/ml was prepared in FBS free medium (300 µl of this suspension was added to the inside of the inserts and starved for 1 hour). Cells were treated with 500 nmol of each antisense oligonucleotide. Then, 500 µL of media containing 10% fetal bovine serum added to the lower well of the invasion plate. After 48 hours incubation at 37°C in 5% CO2 atmosphere, the media from the inside of the insert was discarded. Non- invasive cells were removed from the upper surface of the membrane by swab. Inserts were stained with 400 µL of cell stain solution and incubated for 10 minutes at 25°C. The stained inserts were washed with water, air dried and stained with adding 200 µL of extraction solution per wells, then incubated 10 minutes on a shaker. 100 µL of each sample was transferred to a 96-well plate and OD_{560} was measured with ELISA Reader (BioTek).
Experiments were repeated 3 times.
Data was evaluated with Graph Pad Prism 6.

3. Results

According to the present study, cytotoxicity of different concentrations of antisense oligonucleotides (0, 62.5, 125, 250, 500 and 1000 nmol), and carrier molecule, TurboFect, (0, 0.625, 1.25, 2.5, 5 and 10 µl respectively added for different concentrations of antisense oligonucleotides) was evaluated by MTT assay. As shown in Fig. 2, at concentration of 1000 nmol of antisense oligonucleotides (TW3, TW4, TW5, TW6) on PC3 cell line, 34%, 23%, 31% and 59% of the cells were dead respectively, 14% of which were due to the Turbofect effect. As observed in Fig. 3, at concentration of 1000 nmol of the same antisense oligonucleotides on LNCaP cell line, 28%, 30%, 37% and 33% of cells were dead, 11% of which were due to the Turbofect effect. Therefore, 500 nmol of the antisense oligonucleotides was select for invasive assay without interference of cytotoxic effect of them.

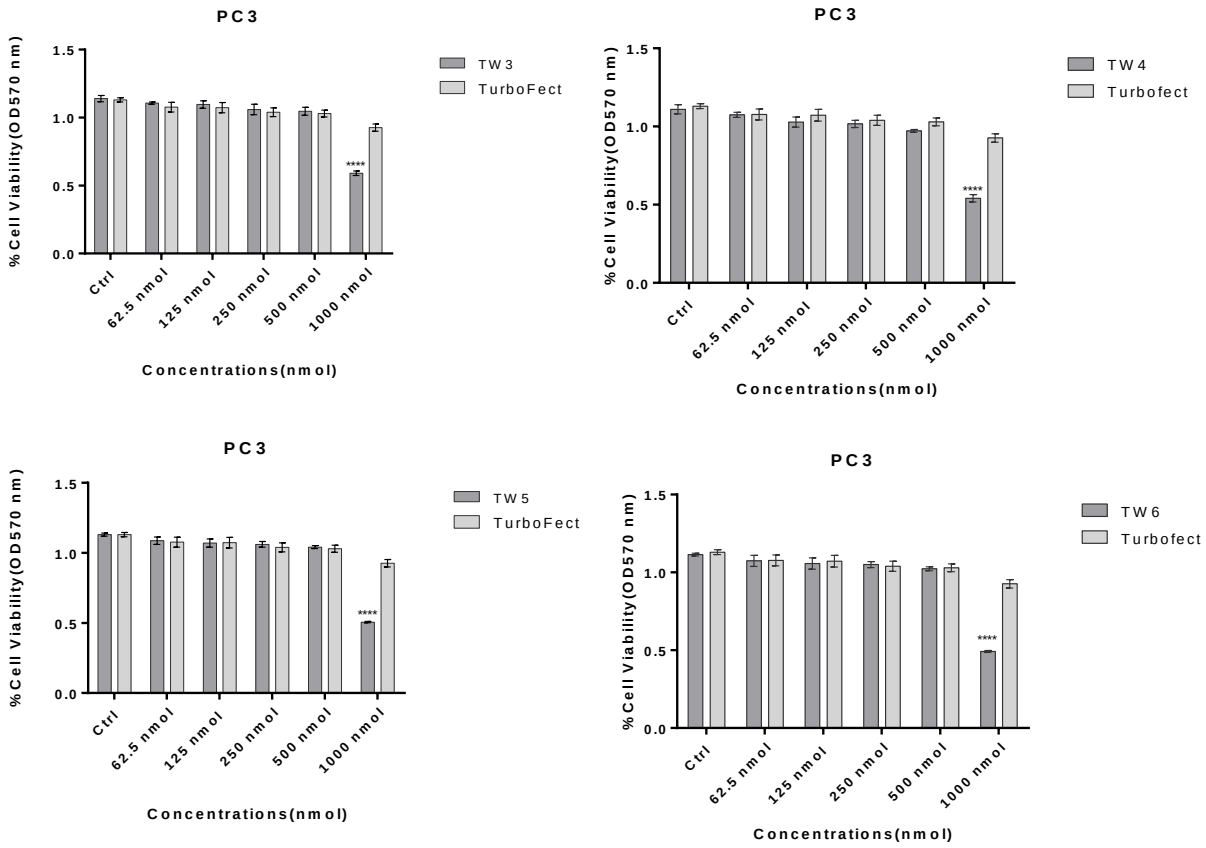
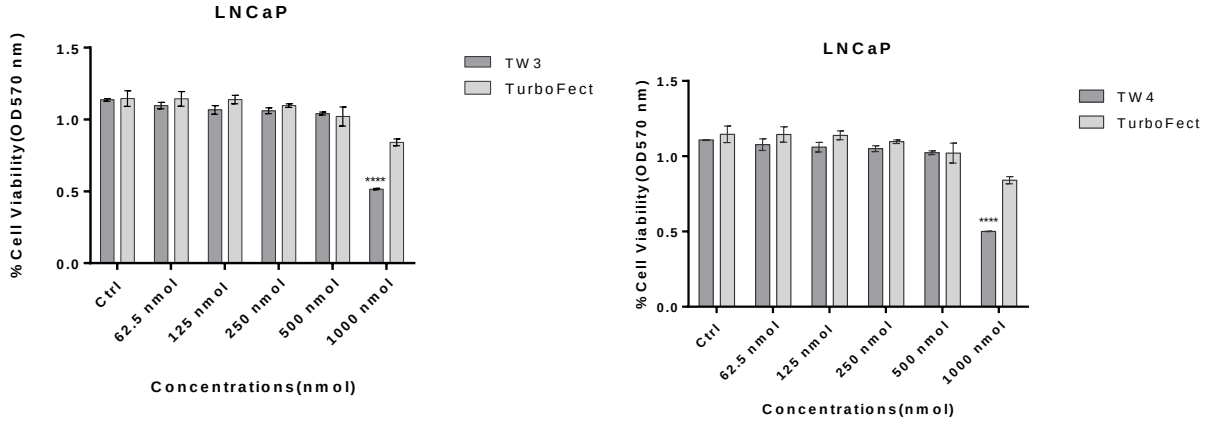


Figure 2. Determination of the toxicity of antisense oligonucleotides (TW3, TW4, TW5 and TW6) & TurboFect on PC3 cell line by MTT assay

PC3 cells (100 µl) were plated in a 96well plate and cultured with various concentrations of each antisense oligonucleotide (0, 62.5, 125, 250, 500 and 1000 nmol). For antisense oligonucleotide delivery into cells, TurboFect was used in 0, 0.625, 1.25, 2.5, 5 and 10 µl respectively. After 48 h, the cells were treated with MTT for 4 h at 37˚C, and absorbance was measured at 560 nm. Data are presented as mean ± SEM (n=3). (****P < 0.0001 different from the control).



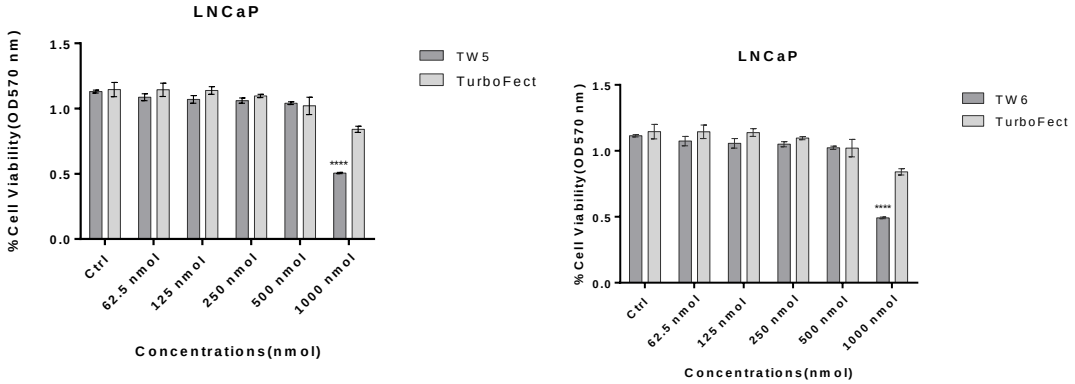


Figure 3. Estimation of antisense oligonucleotides (TW3, TW4, TW5, and TW6) & TurboFect toxicity on LNCaP cell line by MTT assay

LNCaP cells (100 µl) were plated in a 96well plate and cultured with various concentrations of antisense oligonucleotides (0, 62.5, 125, 250, 500 and 1000 nmol). For antisense oligonucleotide delivery into cells, TurboFect was used in 0, 0.625, 1.25, 2.5, 5 and 10 µl respectively. After 48 h, the cells were treated with MTT for 4 h at 37˚C, and absorbance was measured at 560 nm. Data are presented as mean ± SEM (n=3). (****P < 0.0001 different from the control).

Subsequently, the concentration of 500 nmol of antisense oligonucleotides was used to examine anti-invasive effect of them on PC3 and LNCaP cell lines using Cell Invasion Assay Kit. The invasive potential of PC3 and LNCap cell lines transfected with the 500 nm of antisense oligonucleotides (TW3, TW4, TW5, TW6 and TurboFect alone) was examined. As shown in Fig. 4, TurboFect, TW5, TW3, TW6 and TW4 reduced LNCaP cells invasion ability 8, 16, 58, 10 and 35% respectively in comparison to control cells. These results were 4, 15, 30, 28 and 27% on PC3 cells (data was shown in Fig. 5). The antisense oligonucleotide, TW3, had the main anti- invasive effect on LNCap cells. However this oligonucleotide showed similar effect with TW6 and TW4 on PC3 cells. Results are representative of three separate experiments.

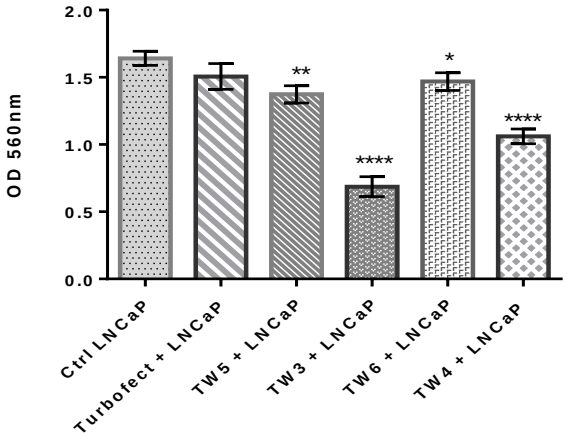


Figure4. Effects of Antisense oligonucleotides (TW3, TW4, TW5 and TW6) on LNCaP cell invasion.

LNCaP cells were seeded at 75×10^4 cells/well and allowed to invade toward 10% FBS for 48 hours in the presence and absence of antisense oligonucleotides. Invasive cells on the bottom of the invasion membrane were stained and quantified at OD 560 nm. Data are presented as mean ± SEM (n=3). (*P < 0.05, ***P < 0.001 and ****P < 0.0001 different from the control).

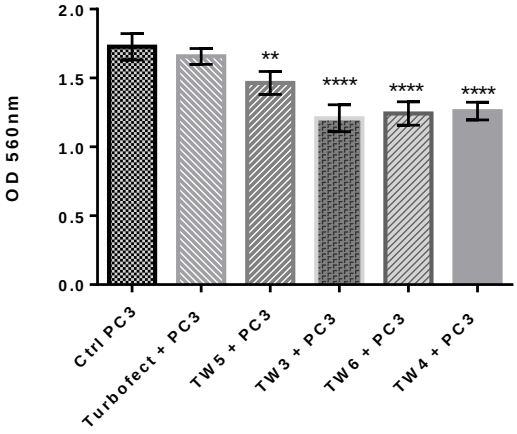


Figure 5. Effects of Antisense oligonucleotides (TW3, TW4, TW5 and TW6) on PC3 cell invasion.
PC3 cells were seeded at 75×10^4 cells/well and allowed to invade toward 10% FBS for 48 hours in the presence and absence of antisense oligonucleotides. Invasive cells on the bottom of the invasion membrane were stained and quantified at OD 560 nm. Data are presented as mean ± SEM (n=3). (**P<0.01 and ****P<0.0001 different from the control).

4. Discussion

Antisense technology is used for different therapeutic and non-therapeutic goals nowadays. There are several reports about different antisense molecules used against prostate cancer however many of them are about anti-apoptotic effect of antisense molecules and not anti-invasive effect of them. In 2001, different antisense oligonucleotide drugs have been used to control various cancers by Chi et al. For example, the combination of Genasense antisense oligonucleotide designed against bcl-2 mRNA and mitoxantrone was used for the treatment of hormone refractory prostate cancer in phase 1 clinical trial study. There was a synergistic effect between antisense oligonucleotides and mitoxantrone in those patients for increasing of chemo sensitivity of prostate cancer cells [21]. Another case was the antisense oligonucleotide therapy targeting *clusterin* gene which led to apoptosis in prostate cancer cells [22]. Moreover, there was a study about the role of STAT3 abnormal signaling in Hepatocellular carcinoma (HCC). The results showed inhibition of vascular endothelial growth factor expression by antisense oligonucleotide targeting STAT3 reduced cell proliferation and migratory potential of HCCs [23]. In 2002, Khatib and colleagues reported that down regulating the expression of hepatic endothelial E-Selectin gene by C-raf antisense oligonucleotide can reduce the colorectal carcinoma liver metastasis by 86% [24]. Another experiment was done by Margheri, representing the significant decrease in urokinase receptor signaling pathway in prostate cancer bone metastasis mouse model through using antisense oligonucleotides [25].

The results of the present study demonstrated that *twist1* antisense oligonucleotides enforce an anti-invasive effect on both early and advanced metastatic prostate cancer cells. Treatment of prostate cancer cells (LNCaP & PC3) with 500nmol of different antisense oligonucleotides leads to different inhibitory effects on their invasion ability. The maximum effect was related to TW3 antisense oligonucleotide with 50 and 26 % anti-invasive effect on LNCaP and PC3 cell lines, respectively. These results manifest that using the appropriate antisense drug for *twist1* gene can prevent metastatic process in cancerous cells effectively. Particularly, combination of two or more effective antisense oligonucleotides against genes have an important effect in metastasis of cancerous cells could be more efficient in

prevention of cancer progression into other tissues. In addition, improvement of antisense oligonucleotides stability could increase their anti-invasive activity on prostate cancer cell lines. These antisense oligonucleotides could be examined in other cancer cell lines as well.

5. Conclusion

According to the results obtained from this study, using *twist1* specific antisense oligonucleotides can be a proper way for decreasing metastasis rate of prostate cancer cells in primary and also advanced stages. Discovering the potential role of these types of drugs for the treatment of various cancers has made new hopes for preventing cancer cells metastasis in patients.

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Conflict of interest

The authors declare no conflict of interest.

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