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shRNA-mediated downregulation of α-N-Acetylgalactosaminidase inhibits migration and invasion of cancer cell lines

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ABSTRACT

Objective(s): Extracellular matrix (ECM) is composed of many kinds of glycoproteins containing glycosaminoglycans (GAGs) moiety. The research was conducted based on the N-Acetylgalactosamine (GalNAc) degradation of ECM components by α-N-acetylgalactosaminidase (Nagalase) which facilitates migration and invasion of cancer cells. This study aims to investigate the effects of Naga-shRNA downregulation on migration and invasion of cancer cell lines.

Materials and Methods: In this study, MCF-7 cell line (human mammary carcinoma cell line) and A2780 (human ovarian carcinoma cell line) were used. The level of normalized Naga expression and Nagalase protein were evaluated by quantitative polymerase chain reaction and enzyme-linked immunosorbent assay/western blotting, respectively. Migration and invasion were determined using transwell assays, and statistical analysis was carried out by ANOVA test.

Results: Response to transduction by shRNA compared to the control group, migrative and invasive properties of the transfected cells were significantly inhibited.

Conclusion: These results indicate that Nagalase may have an important role in migration and invasion of cancer cells and can be considered as a candidate for further studies.

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Introduction

Cancer has been known as the cause of more than 8 million deaths annually around the world (1). Various treatment strategies have been used so far, however, none of them is a certain therapy for cancer treatment. Therefore, cancer diagnosis in early stages or in the pre-metastatic stage can greatly affect the treatment process (2, 3). In this regard, several tumor markers have been proposed, and their efficiency has been evaluated. α-N-AcetylGalactosaminidase (Nagalase) serum level elevation has been observed in cancer patients in many studies particularly in primary and metastatic stages (4-6). Although much evidence confirm elevated levels of Nagalase in serum of breast, colon, and prostate cancers, other studies endorse this subject in gastrointestinal system cancer, genital system, neural system, respiratory system and various leukemias (7-9). Nagalase (E.C. 3.2.1.49) cleaves α-N-Acetylgalactosaminyl moieties from Thr/Ser in glycoprotein O-linkage. This enzyme was detected not only in humans, but in all eukaryotes and prokaryotes even in viruses. Nagalase deficiency causes a rare hereditary lysosomal storage disease named Schindler/Kanzaki, a heterogeneous genetic disorder which is known more as a metabolic disease (10). Nagalase deficiency has a wide spectrum of clinical symptoms. neuroaxonal dystrophy in an early stage is called Schindler disease and angiokeratoma corporis diffusum in late stage is called Kanzaki disease (11, 12).

Although researchers are not sure about the role of various factors in cancer metastasis, it can be assumed that altered glycosylations play an important role in the occurrence of metastasis cascade (13). There is some evidence that suggests Nagalase acts as an extracellular matrix-degrading enzyme (14, 15). It seems degradation of glycoproteins/ proteoglycans containing GalNAc side chains have an important role in this process, because of its considerable amount in extracellular matrix (ECM). ECM composition and the structure of ECM components in cancerous tissues and the correlation of ECM with macrophage activity and chemotaxis to target tissue are not understood completely. Evidence also shows changes in the GalNAc components of the extracellular matrix

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and extracellular membrane attachment component such as integrins in cancer tissues or cells (16, 17). These findings confirm that the presence of Nagalase in many pathogens such as bacteria, parasites and viruses may be effective in their spreading in the body (15, 18, 19). One of the most important role of Nagalase has been investigated in macrophage suppression (20). Macrophages play an indispensable role in recovery process of most diseases. Several mechanisms are involved in the activation of macrophages which are not exactly identified. Vitamin D binding protein=DBP (and known as group specific component=Gc) is derived by the activation of β-galactosidase and sialidase membrane-bound enzymes which activate B cell and T cell lymphocytes, respectively, is called DBP-macrophage activating factor (DBP-MAF) or Gc-MAF (21). Gc-MAF is administered as a therapeutic agent in cancer immunotherapy and many evidence support its high efficacy in cancer patients or cell lines (6, 22, 23). Deglycosilation of DBP by Nagalase secreted by cancer cells inhibits the production of MAF (8, 20). In cell lines, the same results were seen after addition of Gc-MAF (24-26). Altogether, there are several evidence indicating that serum level of Nagalase have a significant relationship with tumor burden (regardless of its type), in that parallel to tumor size shrinkage, the level of Nagalase in serum decreases. (27). That is why some investigators proposed Nagalase as a diagnostic and prognostic marker of cancer (5, 6, 27). Based on the evidence mentioned above, in this study the role of Nagalase in migration and invasion of MCF-7 (breast cancer cell line) and A2780 (ovarian cancer cell line) was investigated.

Materials and Methods

Cell culture

Human breast cancer cell line (MCF7) and human ovarian cancer cell line (A2780) were obtained from BuAli Research Center (Mashhad University of Medical Sciences, Mashhad, Iran). All cells were cultured in RPMI1640 medium (Caisson, USA) with 10 % (v/v) fetal bovine serum-FBS (Gibco, USA), streptomycin (100 μg/ml) and penicillin (100 U/ml) (CMG, Iran). The cells were incubated at 37 °C in a humidified atmosphere and 5% CO_2. The cells were sub-cultured when reached the confluency of 80-90% using 0.25% Trypsin-EDTA (Gibco, USA).

Vector construction and transfection

All cells were transfected with Naga shRNA plasmid (h) including 3 target-specific lentiviral vector plasmids each encoding 19-25 nt (plus hairpin) shRNAs (sc-75860-SH, SantaCruz Biotechnology) to knock-down Naga gene expression. In order to neutralize the external interfering factors the control shRNA plasmid-A, encoding a scrambled shRNA sequence that will not lead to the specific degradation of any known cellular mRNA (sc-108060, SantaCruz Biotechnology), and copGFP control plasmid which contains the full-length copGFP gene-natural green monomeric GFP-like protein from copepod (*Pontellina* sp.) with optimized human codons for high level expression of the fluore-scent protein from the CMV promoter in mammalian cells, (sc-108083, SantaCruz Biotechnology) were used. The sequences in the shRNA expression cassettes were verified to correspond to the target gene with 100% identity. The sequences of shRNAs are listed in Table 1. Cell viability was assessed by trypan blue staining before transfection. Transfection was performed according to the manufacturer's instructions. After transfection, cells stably expressing shRNAs were isolated using puromycin selection.

Briefly, cells were seeded in a 24-well plate at a density of 1×10^5 cells/well in 500 μl antibiotic-free normal growth medium supplemented with 10% FBS 24 hr before the transfection. For each well, 2.5 μl (250 ng) of the shRNA were mixed with 22.5 μl transfection medium (sc-108062, SantaCruz Biotechnology) and incubated at room temperature for 45 min with 1.5 μl transfection reagent (sc-108061, SantaCruz Biotechnology) and 23.5 μl transfection medium. After washing the cells with Briefly, cells were seeded in a 24-well plate at a density of 1×10^5 cells/well in 500 μl antibiotic-free normal growth medium supplemented with 10% FBS 24 hr before the transfection. For each well, 2.5 μl (250 ng) of the shRNA were mixed with 22.5 μl transfection medium (sc-108062, SantaCruz Biotechnology) and incubated at room temperature for 45 min with 1.5 μl transfection reagent (sc-108061, SantaCruz Biotechnology) and 23.5 μl transfection medium.

After washing the cells with transfection medium, 50 μl of transfection mixtures were added to each well with 200 μl transfection medium. 6-8 hr after transfection,

Table 1. Three different shRNA plasmids of Naga shRNA plasmid, corresponding small interfering RNA (siRNA) sequences

Hairpin sequence (5′ → 3′)	siRNA sequences (5′ → 3′)
GATCCCGAGATGAAACCAACTTCATTCAAGAGATGAAGTTGGTTT	Sense: CGAGAUGAAACCAACUUCAtt
CATCTCGTTTTT	Antisense: UGAAGUUGGUUUCAUCUCGtt
GATCCCCTTGCTGTTGACTCTGAATTCAAGAGATTCAGAGTCAAC	Sense: CCUUGCUGUUGACUCUGAAtt
AGCAAGGTTTTT	Antisense: UUCAGAGUCAACAGCAAGGtt
GATCCGGACATTTATTCCCTTCTATTCAAGAGATAGAAGGGAATA	Sense: GGACAUUUAUUCCCUUCUAtt
AATGTCCTTTTT	Antisense: UAGAAGGGAAUAAAUGUCCtt

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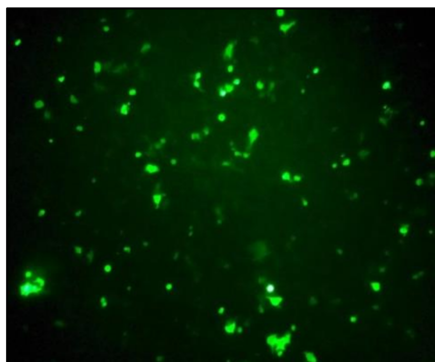
the medium was replaced with fresh RPMI medium containing 20% FBS. After 18-24 hr medium was changed using RPMI containing 10% FBS and 3 µg/ml puromycin (sc-108071, SantaCruz Biotechnology) for antibiotic selection. The cells were incubated at 37 °C with 5% CO_2. Transfection efficiency was assessed by the expression of green fluorescent protein (GFP) under a florescence microscope (Figure 1A). After 24 hr, live transfected cells were used for RNA or protein isolation as well as migration/invasion assay. To perform further examinations (qPCR, ELISA, Western blot, migration and invasion) each cell line was transfected in 12 wells. To obtain statistically reliable results, experiments were performed in triplicate. Control wells transfected by scrambled plasmid were used during all steps mentioned above, and their culture medium was exchanged along with tests.

Puromycin screening

To eliminate the un-transfected cells within the cell population, the transfected cells were treated with different doses of puromycin. The minimum lethal dose of puromycin to mortify un-treated cells in 24 hr was determined after microscopic screening. The results indicate that un-transfected cells will die at 3 µg/ml puromycin concentration. Therefore, after plasmid transfection and selection by puromycin treatment, (un-transfected cells) wells were washed gently to eliminate dead cells, and adherent living cells were used for further studies (Figure 1B).

(A)



(B)

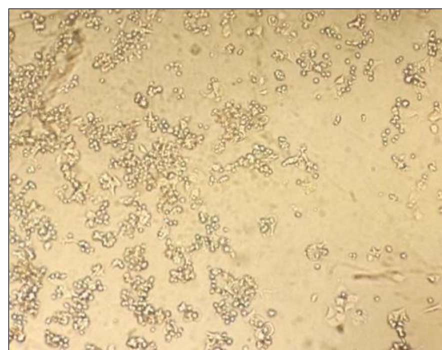


Figure 1. (A) The MCF7 cells in the Naga-shRNA group demonstrate green fluorescence under the exciting wavelength. The transfection efficiency was ≥50% for the other two transfection groups. (B) Cell death mediated by 3 µg/ml puromycin concentration (400X)

Quantitative RT-PCR analysis (RT-qPCR)

RNA was extracted by Total RNA extraction Kit-A101231 (ParsTous, Iran) according to the manufacturer's protocol. Then, RNA concentration was measured (NanoDrop One/One^c, Thermo Fisher Scientific Inc., US) and cDNA was synthesized by PrimeScript™ 1st strand cDNA Synthesis Kit-6110B (Takara Bio Inc., Japan) according to the manufacturer's instructions. The primers designed for Naga expression analysis were as follows: forward 5′-ACGCTTCCGCTGCAACATTAACTG-3′ and reverse 5′-CCACCGATCCAGCAGTCATCAATG-3′. Naga gene expression was normalized to β-Actin as internal control. The primer sequences for β-Actin were as follows: F5′-TCATGAAGTGTGACGTGGACATC-3′ and reverse 5′-CAGGAGGAGCAATGATCTTGATCT-3′ (Macrogen Inc., Korea) (28). The primer was synthesized based on sequences mentioned above and quantitative RT-PCR analysis was performed using RealQ Plus 2X MasterMix Green-without Rox™ (A323402, Amplicon, Denmark) in a Rotor-Gene Q 96-RT-PCR instrument (QiaGen Inc., Germany). Briefly, first-strand cDNA was reverse transcribed from 1 µg total RNA using MMLV reverse transcriptase (Takara, Japan). For each qPCR reaction, a master mix was prepared containing SyberGreen MasterMix, forward and reverse primers (5 pµ), and 10-50 ng template cDNA. The PCR cycling conditions were 5 min at 95 °C followed by 35 cycles of 95 °C for 10 sec, 64 °C for 10 sec, and 72 °C for 15 sec. Melt curve analysis confirmed specific amplification. The PCR products were kept at 4 °C. All RT-qPCR reactions were performed in triplicate. To evaluate the impact of transfection, the relative expression of Naga mRNA was calculated using ΔΔCT the method of comparative threshold cycle (Cp) with the following formula: $(2^{-\Delta\Delta Cp=2-\Delta Cp\ (target\ gene)-\Delta Cp\ (bActin)})$

Enzyme linked immunosorbent assay (ELISA)

In order to ensure the effectiveness of transfection, cell lysate was prepared by sonication (Hielscher-Ultrasound Technology, Germany), 10 cycles (5 sec on/5 sec off) by 70% amplitude on ice. Lysate was centrifuged in 10.000 g at 4 °C, and the supernatant was separated. α-N-acetylgalactosaminidase commercial ELISA kit were used (Cloud-Clone Corporation, China), and the assay was performed according to the manufacturer's instructions (detection range; 0.3-20 ng/ml).

Western blotting (WB)

Total protein was extracted as described above and protein concentrations were determined. Cell lysates (20–30 mg) were separated in a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes. The membranes were blocked in 5% skimmed milk at 37°C for 1 hr. The membranes were incubated with mouse anti-human Naga (dilution 1:200; 47kD; sc-393485, SantaCruz Biotechnology,

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US) and β-actin as internal control (dilution 1:1000; ~43 kD; sc-47778, SantaCruz Biotechnology, US) antibodies at 4˚C overnight. The membranes were then washed and incubated with the goat anti-mouse IgG antibody conjugated with horseradish peroxidase (dilution 1:1000, sc-2005, SantaCruz Biotechnology, US) as secondary antibody with shaking for 2 hr at room temperature. The intensity of protein bands was detected using chemiluminescence-substrate detection kit (B111420, ParsTous, Iran) according to the manufacturer's instructions. Results were scanned and analyzed using GelQuant.NET software provided by 'biochemlabsolutions.com'.

Transwell migration assay

Cell migration analyses were performed in 24-well 8 µm pore size polycarbonate transwell plates (SPL, Korea) according to the manufacturer's instructions. After 48 hr post-transfection, puromycin-selected cells in each group were cultured using low serum medium (5% FBS) and washed gently to remove non transfected cells (not for un-treated group). The bottom chamber was filled with culture medium containing 20% FBS. Cells (transfected with Naga-plasmid, scramble plasmid and untreated control) were trypsinized and suspended at a density of 5×10^4 cells/ml in serum-free medium, and 100 µl of cell suspension was plated in the upper chamber. The cells were wiped out gently from the upper surface of the chamber using a cotton swab after 24 hr incubation with 5% CO_2 at 37 °C. The cells that migrated to the lower surface of the membrane were washed with PBS, fixed in formaldehyde 3.7% for 2 min and methanol for 15 min. The cells were then stained with 0.1 % Giemsa for 10 min, and rinsed with distilled water several times. The cells were counted on the lower surface of the filters in 5 random microscopic fields using an FSX100 light microscope (Olympus, Japan) at 400X magnification.

For invasion assay, the transwell 24-well insert plate was precoated with 100 µl of matrigel ECM-gel (Sigma, US) diluted 1:4 with serum-free RPMI medium at 37 ºC overnight. 5×10^4 cells of three groups were seeded onto the insert as described for migration assay and then incubated for 24 hr in 37ºC. The cells that invaded the lower surface of the membrane were stained and counted as described above. Cell viability assay was used (trypan blue vital stain) before use in migration and invasion assay.

Statistical analysis

Independent experiments were performed in triplicate on each cell line in independent cultures. All data were analyzed using ANOVA test by statistical software GraphPad Prism-6 and expressed as mean ± standard error of the mean (SEM). The significance level was set at P-value<0.05.

Results

Naga-shRNA decreases the expression of nagalase

In the present study, a copGFP vector was used to silence Naga gene with shRNA. After transfection, based on ratio of fluorescence-positive cells, transfection efficiency was estimated to be more than 50% (Figure 1A). RT-qPCR was performed to evaluate the changes in mRNA expression. Based on melting curve analysis, all tests showed a single peak of the desired amplicons, and negative control group showed no peak, which confirms the results. RT-qPCR analysis revealed significant decrease of Naga-mRNA expression in Naga-shRNA groups (NG) compared to the negative control group (NC) and Scramble-shRNA group (SC) in both cell lines (all tests were performed in triplicates) (P<0.05). Despite this difference between the cell lines, MCF-7 cells transduced with Naga-shRNA had the lowest Naga-mRNA expression compared to the other two control groups (NC & SC) (P-value<0.05). While no significant difference of Naga-mRNA expression was observed between negative control group (NC) and scramble-shRNA group (SC), a little difference was detected in both cell lines (Figure 2).

Analysis of nagalase protein expression

Based on ELISA assay, in both cell lines nagalase expression in Naga-shRNA group, compared to negative control group (NC) and Scramble-shRNA group (SC), was significantly decreased (P<0.05), but no significant difference was observed between Negative control group (NC) and Scramble-shRNA group (SC)(P>0.05).

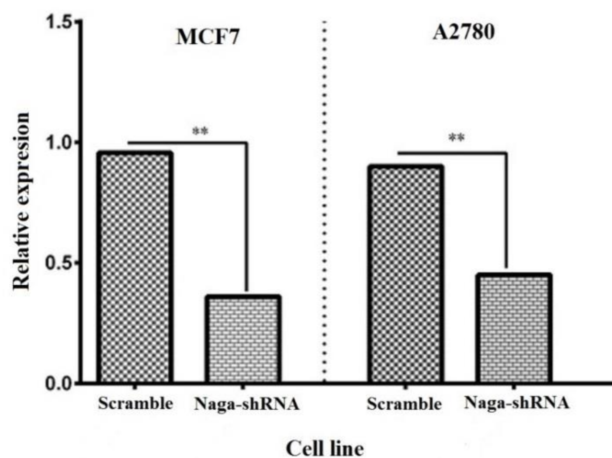

Figure 2. The relative Naga mRNA expression in cell lines following transfection with shRNA plasmid. The expression of mRNA was measured with reverse transcription quantitative polymerase chain reaction (RT-qPCR). Naga mRNA expression was significantly suppressed by shRNA vector compared to scramble-plasmid controls. Naga group (cells transfected with Naga-plasmid); Scramble group (cells transfected with scramble plasmid). Data are shown as mean±SEM of triplicate experiments in compare to control group (un-treated cells) and β-actin was included as an internal control. ** P-value <0.01

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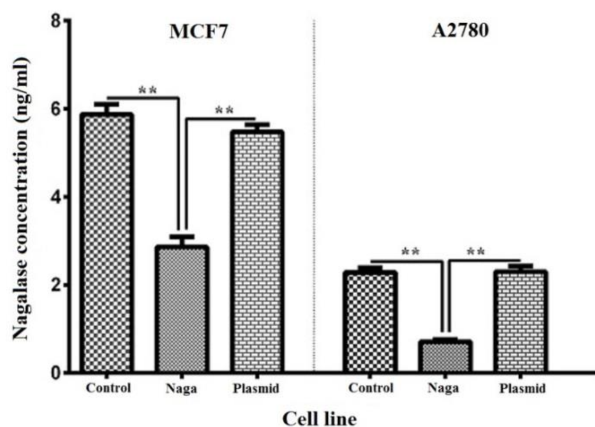

Figure 3. The nagalase protein expression in cell lines following transfection with shRNA plasmid was examined by enzyme linked immunosorbent assay. Nagalase protein expression was significantly decreased by shRNA plasmid compared to controls. Control group (un-transfected cells); Naga group (cells transfected with Naga-plasmid); plasmid group (cells transfected with scramble pasmid). Data are shown as mean ± SEM of triplicate experiments. ** *P*<0.01

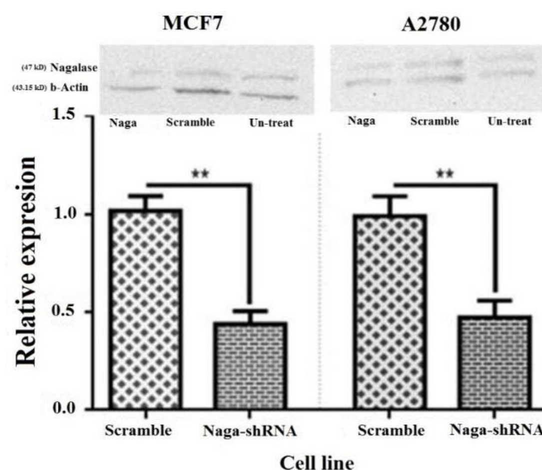

Figure 4. The relative nagalase protein expression assay by Western blot. Nagalase protein expression was significantly decreased by shRNA plasmid compared to controls. The average signal intensity was normalized by internal control. Naga group (cells transfected with Naga-plasmid); scramble group (cells transfected with scramble plasmid). Data are shown as mean ± SEM of triplicate experiments in compare to control group (un-treated cells) and β-actin was included as an internal control. ** *P*<0.01

The results demonstrated the expression levels of Nagalase protein in the MCF7 and A2780 cells were significantly decreased, 72 hr following transfection, which confirms the downregulation of the gene (Figure 3). Also nagalase and β-Actin (as internal control) expression was detected in these cells by Western blotting. As shown in Figure 4, Western blot analysis also showed a significant decrease in nagalase expression in both transfected cell lines compared to negative control group (NC) and scramble-shRNA group (SC).

Effect of naga silencing on cell migration and invasion

In our study, transwell assay and Matrigel transwell assay were used to evaluate migration and metastatic potential of cancer cells, respectively. Results showed lower migration and invasion capacities after Naga-shRNA treatment compared to both control groups (un-treated group and scramble shRNA group) in both cell lines, and these differences were significant in both MCF7 and A2780 (*P*-value<0.05). Regarding the number of migrated/metastasized cancer cells, no significant difference was observed between scramble-shRNA group and un-treated group in both cell lines (*P*-value>0.05) (Figure 5).

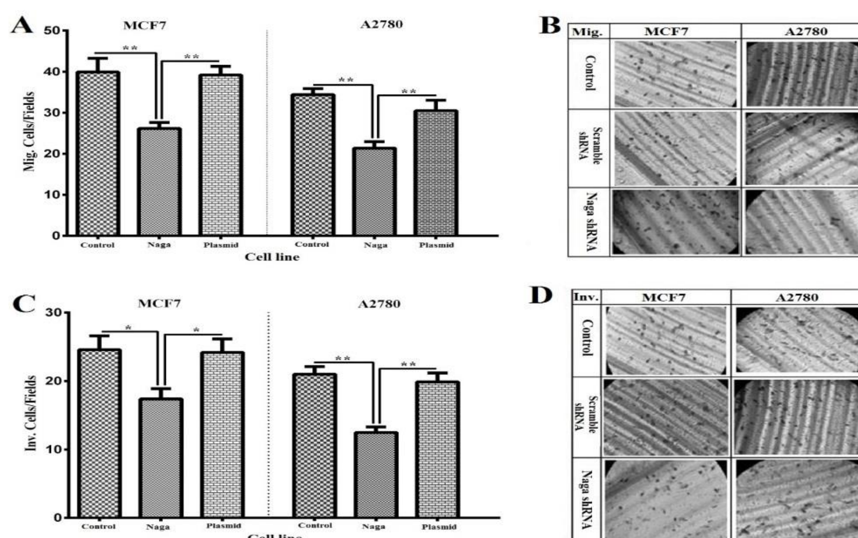

Figure 5. Cell migration and invasion evaluation by transwell assay. The comparison of the average amount of migrated/invaded cells of the groups. The cells penetrating the filter membrane were stained with geimsa and distinguished under a light microscope 400X. (A, B) migration and (C, D) invasion ability was significantly decreased by shRNA vector compared to controls in MCF7 & A2780. Control group (untransfected cells); naga group (cells transfected with Naga-Plasmid); plasmid group (cells transfected with scramble plasmid). Data are shown as mean ± SEM of triplicate experiments. * *P*-value <0.05, ** *P*-value <0.01

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Discussion

Extracellular matrix (ECM) remodeling by cancer cells, promote tumor growth or invasion (29). The ECM mostly contains glycoprotein/proteoglycan structures. glycosaminoglycan's (GAGs) are carbohydrate components (as N-acetylgalactosamines) of ECM binding to protein components and form glycoproteins of ECM (30). Cell surface proteoglycans and pericellular proteoglycans like extracellular proteoglycan that contain GalNAc (as GAG's) may be targets of nagalase (31). Many kinds of ligands on the surface of cells and ECM receptors that play important role in metastasis, migration, angiogenesis, proliferation and cell signaling are composed of GAGs (32). Also, several evidences have shown that stiffness of ECM encourages cell migration, named durotaxis (33). Due to the function of nagalase in glycoprotein destruction, it seems nagalase can affect durotaxis by ECM-degradation. All issues mentioned above, presents the role of nagalase in digestion of GalNAc's upon glycoproteins (34, 35). As proved, GalNAc's were digested by ECM-degrading enzyme, it is possible to be done by the nagalase enzyme (36).

In addition, macrophage–ECM interactions in the tumor microenvironment have the most important role in macrophage polarization and function (37). Also, researchers have proved the role of nagalase in macrophage activating factor (MAF) deactivation through deglycosilation (20, 38). Increased serum levels of nagalase in patients with different types of cancer; particularly metastatic process has been proven in many studies (4, 5, 8). Furthermore, glycosylation modification of antibodies is important for their stability and efficiency as cell surface receptors (39-42). Several studies have mentioned glycosylation modification may change antibody functions as can affect other glycoprotein (43-45). Therefore, it can be concluded that any change mediated by nagalase in addition to affecting the performance of ligands on cell surface or ECM, can affect antibodies.

Our results indicate that inhibition of nagalase using shRNA downregulation can be effective in suppression of migration and invasion in cancer cells. There are very limited studies that have evaluated the role of nagalase in ECM degradation/remodeling. However, they have not addresses enzymes/isoenzymes with similar function. Increased level of N-acetylgalactosaminyl transferase was reported in the tissue of breast cancer patients as well as breast cancer cell lines (46, 47). Similarly, Taniuchi and colleague confirmed these findings and stated the polypeptide N-acetylgalactosaminyl-transferases-T3 suppression induces apoptosis of PDAC as pancreatic cancer cell line (48). The role of β-N-acetylglucosaminidase (β-NAG) as a hexosaminidase was proven in breast cancer cell lines (MDA-MB435 and MCF7) invasion by Ramessur *et al* (49). Also, in a study conducted by Li *et al.* downregulation of N-acetylglucosaminyl transferase-V significantly suppressed tumor progression in MA782 cells as mouse mammary adenocarcinoma (50). In addition, multiple ECM degrading/modifying enzymes have been identified so far, and their similar effects in ECM degradation have been proven. Some of these enzymes are proteases such as MMPs (51), cathepsin-L (52), heparanase (53) and others are hexosaminidase in which the Nagalase is classified into them (54) .

Conclusion

Our findings showed that Naga downregulation by shRNA can be effective in migration and invasion potential of breast (MCF7) and ovarian (A2780) cancer cell lines. Although there is conflicting evidence in this context based on the results of the present study, due to the limitation on financial resources, it is not possible to examine other aspects of the nagalase function and will be investigated in future studies. Nevertheless, nagalase is suggested as a potential target for more investigation regarding occurrence and expansion of cancer.

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Conflict of interest

The authors declare that no conflict of interest exists.

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