Original Article

S100A4 siRNA Inhibits Human Pancreatic Cancer Cell Invasion *In Vitro*

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Abstract

Objective Pancreatic cancer is one of the most deadly cancers, which is characterized by its high metastatic potential. S100A4 is a major prometastatic protein involved in tumor invasion and metastasis which precise role in pancreatic cancer has not been fully investigated. We knocked down the S100A4 gene in the Bxpc-3 pancreatic cancer cell line via RNA interference to study the changes in cell behavior.

Methods Real-time polymerase chain reaction and western blotting were used to detect mRNA and protein expression levels of S100A4, matrix metalloproteinase (MMP)-2, E-cadherin and thrombospondin (TSP)-1. Transwell chambers were used to detect the migration and invasion abilities; a cell adhesion assay was used to detect adhesion ability; colony forming efficiency was used to detect cell proliferation; flow cytometry was used to detect apoptosis.

Results S100A4 mRNA expression was reduced to 17% after transfection with S100A4-siRNA, and protein expression had a similar trend. mRNA and protein expression of MMP-2 was reduced and that of E-cadherin and TSP-1 was elevated, indicating that S100A4 affects their expression. S100A4-silenced cells exhibited a marked decrease in migration and invasiveness and increased adhesion, whereas overall proliferation and apoptosis were not overtly altered.

Conclusion S100A4 and its downstream factors play important roles in pancreatic cancer invasion, and silencing A100A4 can significantly contain the invasiveness of pancreatic cancer.

Key words: Pancreatic cancer; S100A4; Matrix metalloproteinase-2; E-cadherin; Thrombospondin-1; RNA interference

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INTRODUCTION

Recent advances in cancer research have greatly improved the prognosis of many human cancers. Yet, pancreatic cancer remains one of the deadliest cancer types. The overall 5-year survival rate is disappointingly less than 5%. In the majority of pancreatic cancer patients, the tumors often have already metastasized at the time of diagnosis, making conventional treatment and management ineffective[1]. Therefore, overcoming the metastasis is the most urgent priority in pancreatic cancer research.

S100A4 (or mts1), a calcium-binding protein with two EF-hands, is known to be associated with the invasion and metastasis of many cancers. It is frequently overexpressed in metastatic tumors as well as normal cells with high mobility such as lymphocytes, macrophages and fibroblasts. The role of S100A4 in pathogenesis and metastasis have been extensively studied in various cancers including prostate cancer[2], breast cancer[3], and colon and other gastrointestinal cancers[4-6]. However, its role in pancreatic cancer is unclear.

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The goal of this study was to explore the feasibility of targeting metastasis-promoting factor S100A4 using siRNA knockdown in a pancreatic cancer cell line, to gain an insight into the possible containment of pancreatic cancer metastasis. We found that expression of S100A4 protein was effectively inhibited by specific siRNA in Bxpc-3 cells. More importantly, we found that S100A4 knockdown significantly slowed cell migration *in vitro*, suggesting that S100A4 may indeed constitute a candidate therapeutic target. We also observed that such functional alteration is possibly mediated through the modulation of expression of E-cadherin, matrix metalloproteinase (MMP)-2 and thrombospondin (TSP-1) genes.

MATERIALS AND METHODS

Cell Culture and Transfection

Human pancreatic cell line Bxpc-3 was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, and cultured at 37 °C in a humidified atmosphere with 10% CO_2. Exponentially growing cells were used in three experimental groups for mock transfection, transfection with nontargeting control siRNA, or with S100A4-specific siRNA. Transfection was carried out using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA according to manufacturer's instructions. Transfection efficiency was evaluated by measuring cotransfecting green fluorescent protein (GFP) expression through fluorescence-activated cell sorting (FACS).

siRNA Design

siRNA was designed and synthesized by Shanghai GenePharma Co. Ltd. Four different siRNAs for S100A4 silencing were initially designed for screening for the best silencing efficiency. The sequences of the most effective S100A4-siRNA used in this study and of the control siRNA were: S100A4-siRNA: sense strand: 5′-UGAGCAACUUGGAC AGCAATT-3′ (NM_002961; position 174-194); anti-sense strand: 5′-UUGCUGUCCAAGUUGCUCATC-3′. Negative control-si: sense strand: 5′-UUCUCCGA ACGUGUCACGUTT-3′; anti-sense strand: 5′-ACGUG ACACGUUCGGAGAATT-3′.

Western Blotting

Cells from different groups were washed with PBS and lysed. Protein concentration was estimated with Bradford Protein Assay kitBD, Franklin Lakes, USA. Cell lysates (50 μg) were electrophoresed on an 8% polyacrylamide gel (Bio-Rad) and transferred onto nitrocellulose membrane (Millipore, Billerica, MA, USA). After being blocked with Tris-buffered saline containing 5% skimmed milk and 0.1% Tween 20 for 2 h, membranes were incubated with the primary antibodies at 37 °C. After washing, the membranes were incubated with the secondary antibody at room temperature for 1 h. Protein bands were visualized with enhanced chemiluminescence reagent (ECL) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell Proliferation Assay

Cells from different groups were seeded in a 6-well plate at a density of 200 cells/well. Seven days later, colonies were fixed, stained and counted. Clonogenicity was calculated as number of clones/number of cells seeded × 100%.

Real-time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for Expression of S100A4, E-cadherin, MMP-2, and TSP-1

Total RNA was isolated from cells in different groups using an RNeasy RNA isolation kitPromega, Madison, USA, and cDNA synthesis was carried out according to the manufacturer's instructions. Real-time RT-PCR was carried out using a LightCyclerABI 7500, Carlsbad, CA. The results were calculated and presented as $\Delta\Delta$Ct.

Apoptosis Assay

Cells from different groups were stained with Annexin–fluorescein isothiocyanate and propidium iodide, followed by FACS.

Cell Adhesion Assay

Ninety-six-well plates were coated with fibronectin Sigma, Maryland, USA solution overnight at 37 °C. Next day, cells from different groups were seeded on fibronectin-coated plates at a density of 50 000 cells/well, followed by culture at 37 °C for 1 h. Fifty microliters of 5 mg/mL MTT solution was then added to each well, followed by an additional 4 h of culture. The supernatant was then removed, and 150 μL DMSO was added, followed by 5 min shaking before reading on a plate reader equipped with a spectrophotometer, at a wavelength of 570 nm.

Cell Migration and Invasiveness Assay

Transwell (8 μm pore size, Costar, Washington,

DC, USA) chambers were either preloaded with Matrigel (BD, Franklin Lakes, USABD) overnight for the cell invasiveness assay or without Matrigel for the cell migration assay. Overnight serum-free-DMEM-starved cells were added to the upper chambers with or without Matrigel, while NIH3T3 serum-free culture supernatant was added to the lower chambers. After 48 h culture, cell numbers in the lower chambers were counted.

Statistical Analysis

All experiments were conducted at least three times, and the values were expressed as mean±SD. Statistical significances were analyzed using SPSS 17.0 software, and $P<0.05$ was considered statistically significant.

RESULTS

Expression of S100A4 Gene in Bxpc-3 Cells

We aimed to establish whether we could contain the metastatic potential of pancreatic cancer through siRNA silencing of pro-metastasis factor S100A4. We investigated the expression of S100A4 gene in the Bxpc-3 pancreatic cancer cell line. Figure 1 shows that S100A4 gene was abundantly expressed at both the mRNA and protein levels. Figure 1A shows the determination of S100A4 at the RNA level

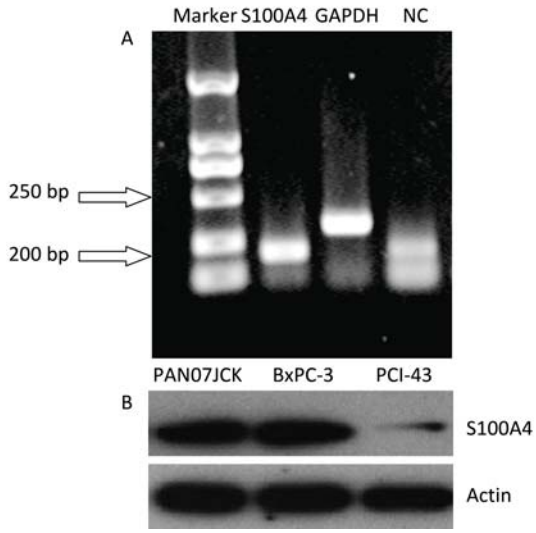

Figure 1. Expression of S100A4 in Bxpc-3 pancreatic cancer cell line. Figure 1A shows the high expression of S100A4 at the RNA level by RT-PCR. Figure 1B shows the protein level by western blotting.

in Bxpc-3 cells by RT-PCR. Agarose gel images show the resolved RT-PCR products. Figure 1B shows the determination of S100A4 at the protein level in Bxpc-3 cells by western blotting.

S100A4-siRNA Silences Expression of S100A4 Gene

We investigated whether S100A4-siRNA could silence abundantly expressed S100A4 in pancreatic cancer cells. We designed 4 sets of siRNA sequence and 1 set of non-targeting control siRNA sequencefor pilot screening. One siRNA that was complementary to the S100A4 gene sequence at position 174 showed the most effective S100A4 silencing (data not shown). This most potent siRNA was then used throughout this study. We transfected Bxpc-3 cells with S100A4-siRNA using the Lipofectamine 2000 method. FACS analysis showed that Bxpc-3 cells were transfected at >80% efficiency with siRNA deduced from the cotransfected GFP construct (Figure2A). Bxpc-3 cells expressed only about 17% S100A4 mRNA compared to the untransfected cells (Figure 2B). Figure 2B shows the evaluation of S100A4 knockdown at the RNA level by quantitative real-time RT-PCR. The values presented are $\Delta\Delta$Ct. More strikingly, the level of S100A4 protein became barely detectable by western blotting. Figure 2C shows the blotting images of S100A4 protein and the loading control β-Actin protein in Bxpc-3 cells. Our data suggest that silencing of S100A4 expression through siRNA knockdown in pancreatic cancer cells is indeed an effective approach to investigate the role of S100A4 in pancreatic cancer.

S100A4 May Alter Expression of MMP-2, E-cadherin, and TSP-1

We determined the expression of MMP-2, E-cadherin and TSP-1 at the RNA level using quantitative real-time RT-PCR. The values presented are $\Delta\Delta$Ct. The amount of MMP-2 transcript was markedly reduced upon S100A4 knockdown, while that of E-cadherin and TSP-1 mRNA was moderately and significantly increased, respectively (Figure 3A). To validate our observations at the protein level, we performed western blotting. Figure 3B shows the blotting images of S100A4 protein and the loading control β-Actin protein in Bxpc-3 cells. Expression of all three proteins was altered, suggesting that these genes were indeed regulated by expression of S100A4.

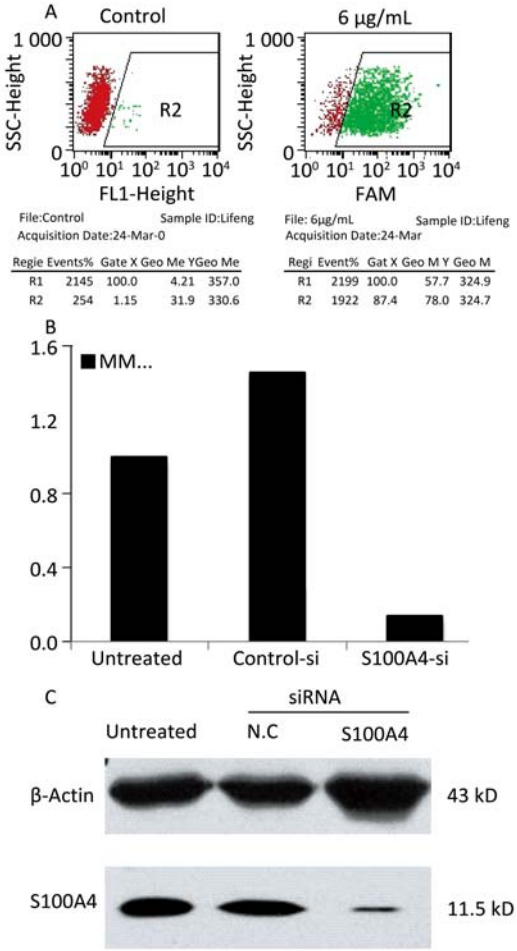

Figure 2. siRNA knockdown of S100A4 in Bxpc-3 pancreatic cancer cell line . Figure2A shows Bxpc-3 cells were transfected at >80% efficiency with siRNA deduced from the cotransfected GFP construct. Figure2B shows the evaluation of S100A4 knockdown at the RNA level by quantitative real-time RT-PCR. Figure2C shows the blotting images of S100A4 protein and the loading control β-Actin protein in Bxpc-3 cells

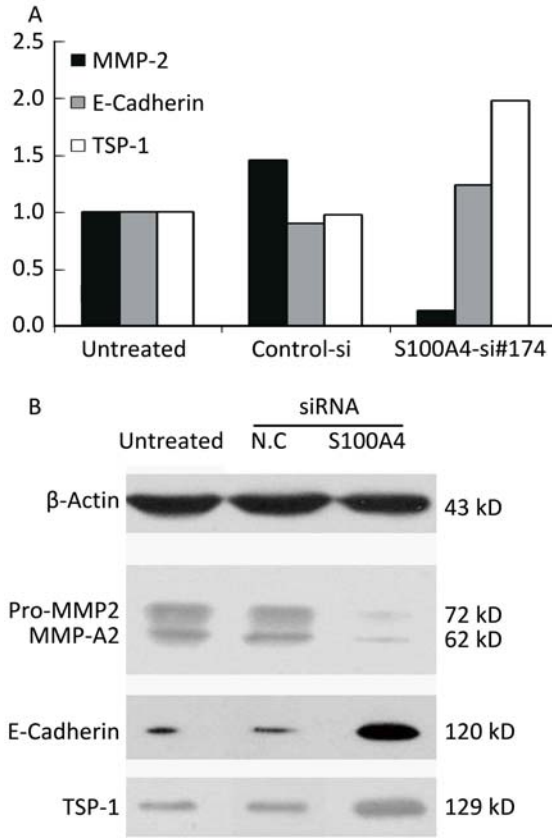

Figure 3. Expression of MMP-2, E-cadherin and TSP-1 in S100A4-silenced Bxpc-3 pancreatic cancer cell line. Figure 3A shows evaluation the expression of MMP-2, E-cadherin and TSP-1 in S100A4 silenced pancreatic cancer line Bxpc-3 cells at RNA level by quantitative realtime RT-PCR. Figure 3B shows evaluation the expression of MMP-2, E-cadherin and TSP-1 in S100A4 silenced pancreatic cancer line Bxpc-3 cells at protein level in Bxpc-3 cells by Western blotting.

Changes in Cell Function after Transfection

We determined the functional consequences of S100A4 silencing. We first investigated apoptosis. At 24 h and 48h and 72 h, all cells showed comparable apoptotic profiles (Figure 4A). We also investigated cell proliferation potential of Bxpc-3 cells before and after S100A4 knockdown, but no significant differences were observed (data not shown). Our data suggest that siRNA knockdown of S100A4 does not overtly change the cell proliferation and apoptosis properties of BxPc-3 cells.

We investigated cell migration after S100A4 silencing in Bxpc-3 cells by a cell migration assay in a Transwell system. Compared to nonspecifically targeted or untransfected cells, S100A4-silenced Bxpc-3 cells showed a marked decrease in cell migration (Figure 4B, P=0.000). We then aimed to establish if S100A4 silencing also led to reduced cell invasion, using a Matrigel-coupled Transwell culture system, and found that this was indeed the case (Figure 4C, P=0.003). Together, our data suggest that S100A4 plays an important role in cell invasion.

We investigated cell adhesion after S100A4 silencing of Bxpc-3 cells, using a cell adhesion assay in

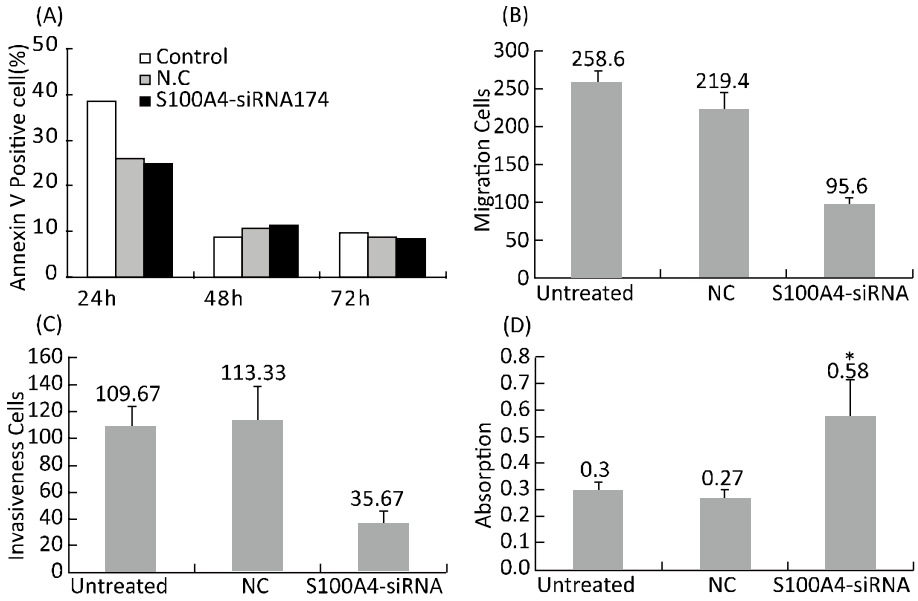

Figure 4. Functional study of S100A4-silenced Bxpc-3 pancreatic cancer cell line. Figure 4A shows evaluation of cell apoptosis upon S100A4 silencing in pancreatic cancer line Bxpc-3 cells by Annexin V/PI assay. Data represents the average of three independent experiments. Figure 4B shows evaluation of cell migration upon S100A4 silencing in pancreatic cancer line Bxpc-3 cells by cell migration assay in a transwell system. Data represents the average of three independent experiments. Figure 4C shows evaluation of cell invasiveness upon S100A4 silencing in pancreatic cancer line Bxpc-3 cells using matri-gel coupled transwell culturing system. Data represents the average of three independent experiments. Figure 4D shows evaluation of cell adhesion upon S100A4 silencing in pancreatic cancer line Bxpc-3 cells by cell adhesion assay in Fibronectin coated plates. Data represent the average of three independent experiments.

fibronectin-coated plates. Figure 4D shows that S100A4-silenced Bxpc-3 cells did have a significant increase in cell adhesion.

DISCUSSION

Pancreatic cancer is one of the most deadly types of cancer, as a result of its highly metastatic nature. The major challenge in combating pancreatic cancer is therefore to identify the molecules involved in metastasis for therapeutic intervention, as well as for diagnostic/prognostic biomarkers. Our present study met this challenge by demonstrating *ex vivo* that cell mobility and invasiveness are readily contained through the siRNA silencing of S100A4 protein. Although S100A4 silencing has been proven to be an effective means to reduce cancer metastasis in other cancer types, its expression and function in highly metastatic pancreatic cancer have not been explored, and the utility of silencing the protein has not been established. Therefore, we believe that our present study lays the foundation for containment of life-threatening pancreatic cancer.

We showed that S100A4 gene was abundantly expressed at both the mRNA and protein levels, which is consistent with the observation that S100A4 is overexpressed in laser-dissected pancreatic cancer tissues[7]. Our results raise the possibility that S100A4 gene could be used as a potential therapeutic target through RNA silencing.

Previously, it has been shown in other cell types that a consequence of downregulation of S100A4 is that expression of MMP-2, MMP-7, MMP-9, E-cadherin and TSP-1 may also be altered[8]. It is not known exactly how S100A4 protein modulates expression of MMP-2, E-cadherin and TSP-1 genes in pancreatic cells. Our results suggest that transcriptional regulation mechanisms are involved. S100A4 is a nonenzymatic protein; therefore, its functions in various cellular processes may largely rely upon its binding with other partner proteins. S100A4 is an intercellular as well as an extracellular protein. Intracellularly, it is localized in both the cytoplasm and nucleus. However, there is no evidence to date that S100A4 executes its function intracellularly. In contrast, it is also known that S100A4 functions through extracellular events

leading to the activation of signal molecules in cancer cells. This is largely based on the observation that S100A4 knockout mice show a great reduction in fostering tumor xenografts[8].

Taking our results together, our study shows that prometastatic protein S100A4 is readily silenced by siRNA in pancreatic cancer cells. Furthermore, S100A4 silencing greatly modifies cell mobility and invasiveness, which are important events in cancer behavior. Our present study provides an additional and complementary explanation that tumor-cell-derived S100A4 protein may play an indispensable role in pancreatic cancer invasion. Our data are consistent with previous studies of other cancer types showing that S100A4 regulates cancer invasion by modulation of MMPs through its extracellular activity[9], by activating downstream pathways that may be involved in the activation of extracellular signal-regulated kinase 1/2, heterotrimeric G proteins, phospholipase C, protein kinase C, nuclear factor-κB, and elevation of intracellular calcium[10-15]. A recent study has reported that tumor-cell-derived RANTES stimulates the externalization of S100A4 via microparticle shedding from the plasma membrane of tumor as well as stromal cells[16]. TSP-1 is an antiangiogenic factor, thus, our finding that its expression was negatively regulated by S100A4 protein suggests a possible role for S100A4 in modifying tumor angiogenesis. It has been recently shown that increased expression of S100A4, combined with decreased expression of E-cadherin, predicts a poor clinical outcome of pancreatic cancer, which further emphasizes the biological significance of our studies[17]. Although S100A4 silencing in pancreatic cancer cells results in suppression of the surface molecule E-cadherin, leading to improved cell mobility, E-cadherin-mediated cell adhesion alone may not be sufficient to make cancer more metastatic. Earlier studies have shown that S100A4-silenced cells also express reduced amount of MMP-2, an enzyme that can proteolytically degrade extracellular matrix components, which clears the way for cancer migration, validating earlier findings that S100A4 modulates expression of MMP-2 and the invasiveness of pancreatic cancer.

We believe that our study provides a framework for further exploration of other therapeutic agents such as antibodies specifically neutralizing extracellular S100A4 protein. Our data are compatible with the notion that S100A4 and its downstream factors play important roles in pancreatic cancer invasion, and silencing A100A4 could significantly contain the invasiveness of pancreatic cancer. Our study lays the foundation for the development of effective therapeutic agents and diagnostic biomarkers for this deadly cancer.

REFERENCES

1. Hidalgo M. Pancreatic cancer. N Engl J Med, 2010; 362, 1605-17.
2. Saleem M, Kweon MH, Johnson JJ, et al. S100A4 accelerates tumorigenesis and invasion of human prostate cancer through the transcriptional regulation of matrix metalloproteinase 9. Proc Natl Acad Sci U S A, 2006; 103, 14825-30.
3. de Silva Rudland S, Martin L, Roshanlall C, et al. Association of S100A4 and osteopontin with specific prognostic factors and survival of patients with minimally invasive breast cancer. Clin Cancer Res, 2006; 12, 1192-200.
4. Garrett SC, Varney KM, Weber DJ, et al. S100A4, a mediator of metastasis. J Biol Chem, 2006; 81, 677-80.
5. Heizmann CW. The multifunctional S100 protein family. Methods Mol Biol, 2002; 172, 69-80.
6. Taylor S, Herrington S, Prime W, et al. S100A4 (p9Ka) protein in colon carcinoma and liver metastases: association with carcinoma cells and T-lymphocytes. Br J Cancer, 2002; 86, 409-16.
7. Ikenaga N, Ohuchida K, Mizumoto K, et al. S100A4 mRNA is a diagnostic and prognostic marker in pancreatic carcinoma. J Gastrointest Surg, 2009; 13, 1852-58.
8. Boye K, Maelandsmo GM. S100A4 and metastasis: a small actor playing many roles. Am J Pathol, 2010; 176, 528-35.
9. Kikuchi N, Horiuchi A, Osada R, et al. Nuclear expression of S100A4 is associated with aggressive behavior of epithelial ovarian carcinoma: an important autocrine/paracrine factor in tumor progression. Cancer Sci, 2006; 97, 1061-9.
10. Hsieh HL, Schafer BW, WeigleB, et al. S100 protein translocation in response to extracellular S100 is mediated by receptor for advanced glycation endproducts in human endothelial cells. Biochem Biophys Res Commun, 2004; 316, 949-59.
11. Kiryushko D, Novitskaya V, Soroka V, et al. Molecular mechanisms of Ca^{2+} signaling in neurons induced by the S100A4 protein. Mol Cell Biol, 2006; 26, 3625-38.
12. Novitskaya V, Grigorian M, Kriajevska M, et al. Oligomeric forms of the metastasis-related Mts1 (S100A4) protein stimulate neuronal differentiation in cultures of rat hippocampal neurons. J Biol Chem, 2000; 275, 41278-86.
13. Pedersen KB, Andersen K, Fodstad O, et al. Sensitization of interferon-gamma induced apoptosis in human osteosarcoma cells by extracellular S100A4. BMC Cancer, 2004; 4, 52-9.
14. Schmidt-Hansen B, Ornas D, Grigorian M, et al. Extracellular S100A4(mts1) stimulates invasive growth of mouse endothelial cells and modulates MMP-13 matrix metalloproteinase activity. Oncogene, 2004; 23, 5487-95.
15. YammaniRR, Carlson CS, Bresnick AR, et al. Increase in production of matrix metalloproteinase 13 by human articular chondrocytes due to stimulation with S100A4: Role of the receptor for advanced glycation end products. Arthritis Rheum, 2006; 54, 2901-11.
16. Forst B, Hansen MT, Klingelhofer J, et al. Metastasis-inducing S100A4 and RANTES cooperate in promoting tumor progression in mice. PLoS One, 2010; 5, 10374-79.
17. Oida Y, Yamazaki H, Tobita K, et al. Increased S100A4 expression combined with decreased E-cadherin expression predicts a poor outcome of patients with pancreatic cancer. Oncol Rep, 2006; 16, 457-63.