

S100A4 and S100A6 Proteins Expression Promote Migration of Bladder Cancer Cells in Zebrafish

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Abstract

Background: Bladder cancer (BC) is one of the major causes of cancer-related mortality in the world. S100 family of small Ca-binding proteins has been implicated in the progression of different cancer types, including BC. **Objective:** The goal of this study is to develop a more rigorous understanding of the role of S100 proteins in BC progression and also to what extent S100s have the ability to increase the metastatic potential for BC. **Methods:** A selected panel of bladder cell lines (J82, T24, HT1376, and RT112) was transplanted into zebrafish embryos to investigate their migration behavior and metastatic potential. Characterizing the expression pattern of S100 proteins including (S100A4 and S100A6) in different BC cells was performed using quantitative polymerase chain reaction and Western blot. Assess the effect of S100 proteins on bladder cell migration *in vivo* was carried out using a zebrafish xenotransplant model. S100 proteins expression was modulated by small-interfering RNA approach. **Results:** High expression of mRNA and proteins levels of S100A4 and A6 were detected in T24 and J82 cell lines, which displayed the highest migration rate in zebrafish embryos. In addition, our data showed that the average migration rate of T24 cells transfected against S100A4, S100A6, and S100A4 and A6 were 17.3%, 10%, and 17.3%, respectively, which was lower than from siControl 41.9%. Likewise, the same effect of protein silencing on cell migration was observed in J82 cell lines. S100A4, S100A6, and S100A4 and A6 knockdown reduced the migration rate of J82 cells to 14.6%, 17%, and 14% compared to the control 37.8%. Furthermore, overexpressed S100A4 expression in RT112 cells significantly increased in migration ability by 29%, compared to control cells 7.3%. **Conclusion:** S100A4 and S100A6 play a role in BC cells invasion and migration. Silencing of these proteins affected dissemination of BC cells in zebrafish embryos highlighting their role in tumor progression.

Keywords: Bladder cancer, gene expression, quantitative reverse transcription-polymerase chain reaction, S100 proteins, zebrafish

INTRODUCTION

Bladder cancer (BC) is the fourth most common cancer among men and the ninth overall in the world. Males are more likely to develop BC than females. When diagnosed, 90% of BCs are urothelial carcinomas, whereas the remaining 10% are mostly squamous cell carcinoma and adenocarcinomas. Many risk factors contribute to the malignant transformation and the progression of BC, including smoking, heavy alcohol consumption, and occupational exposure to polycyclic aromatic hydrocarbons or aromatic amines.^[1]

Data on BC metastatic pattern are limited. Timely detection of metastasis is important for appropriate treatment. Lymph nodes, bones, lungs, liver, and peritoneum are the most common sites of metastasis from BC.^[2]

S100 proteins, a family of calcium-binding proteins, are multifunctional signaling proteins that are involved in numerous intra- and extracellular functions, such as protein phosphorylation, enzyme activation, interaction with cytoskeletal components, and calcium homeostasis.^[3] Deregulated expression of several members of the S100 protein family, including S100A2,^[4] S100A4,^[5] S100A6,^[6] S100A7,^[7] S100A11,^[8] S100A14,^[9] and S100P,^[10] has been

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reported in association with the progression of various human cancer.

In addition, S100 proteins are implicated in the control of many steps of tumor metastasis and some of them have been detected as metastasis markers such as S100A4. For examples, overexpression of S100A4 in two gastric cell lines AGS and SCM-1, significantly increased the invasive activity for these cells. While silencing S100A4 expression in MKN-45 and TMK-1 cells, which displayed high levels of endogenous S100A4, resulted in decrease the migration rate of cancer cells.^[11] Defects in the expression of S100A6 led to an increase or decrease in the migration capacity of osteosarcoma cells.^[6,12]

The intrinsic invasive abilities of cancer cells are important in the process of metastatic dissemination. While studies on the metastatic potential of human tumor cells using mice model are still in use, but, recently, transparent zebrafish embryos have become increasingly popular as a model system in cancer research.

Although there is considerable evidence that S100 proteins may play important roles in various types of cancer, there has been no analysis to date concerning the expression of the S100 proteins in BC. This study was aimed to develop a more rigorous understanding of the role of S100 proteins in BC progression and also to what extent S100s have the ability to increase the metastatic potential for BC.

MATERIALS AND METHODS

Tissue culture

BC cell lines including T24, J82, HT1376, and RT112 were obtained from the American Tissue Culture Collection and cultured in the 5% CO₂ and 37°C incubator in Roswell Park Memorial Institute medium supplemental with 10% fetal bovine serum and 1% penicillin and streptomycin.

Cells treatment and small interfering RNA transfection

To activate the expression of S100A4 protein depending on interleukin-11 (IL-11)/STAT3 manner,^[13] cells were seeded in 60 mm dishes in complete media. After 24 h, the media was replaced with serum-free media and cells left for further 24 h. The dishes then were incubated an additional 48 h with 200 ng/ml of IL-11 (PeproTech, UK) before harvesting. Small interfering RNA (siRNA) targeting the S100A4 and S100A6 and scramble siRNA sequence (siControl), were purchased from Dharmacon, UK. For gene silencing, cells were subjected to transfection with either si-target genes or siControl, while for S100A4 overexpression, cells were transfected with either control vector or pSV2neo-S100A4 plasmid using Ingenio® Electroporation solution (Bio Ingenio, UK) following the manufacturer's instructions. Briefly, 2×10^6 cells were transfected with 2 µg siRNAs or plasmid suspended with the transfection reagent. The final suspension was transferred into 4 mm cuvette and electroporated using Gene PulserX cell electroporator which was set at 250V and 250 µF.

Quantitative reverse transcription-polymerase chain reaction analysis

Total RNA from BC cells was isolated by Trizol (Invitrogen, USA) and purified using the RNeasy Mini Kit and RNase-free DNase Set (QIAGEN, USA) according to the manufacturer's protocols. The first-strand cDNA synthesis, primed with random primers, was performed using the protocol provided by the manufacturer ReverAid H minus First strand c DNA synthesis kit (Thermo Scientific, UK). Quantitative polymerase chain reaction (PCR) was performed using primers for S100A4 (Forward primer: CTAAAGGAGCTGCTGACCCG, reverse primer: TGTCCCTGTTGCTGTCCAAG) and S100A6 (Forward primer: GAAGGAGCTCACCATTGGCT, reverse primer: CACCTCTGGTCCTTGTTC). Real-time PCR reactions were performed in triplicate in a 20 µl reaction volume containing 1 SYBR green mix (Applied Biosystems, USA), 600 nmol/l primers, and 1 ng cDNA. Reactions were performed on an ABI7900HT Sequence Detection System device (PE Applied Biosystems) using the standard program (10 min at 95°C followed by 40 cycles of 15 s at 95°C, and 60 s at 55°C). All PCR reactions were performed in triplicate, positive and negative controls were included in each run. For each sample, the cycle threshold (CT) value for the gene of interest was determined, normalized to the geometric mean value of the housekeeping gene (Glyceraldehyde-3-phosphate dehydrogenase) (Forward primer: GTCAAGGCTGAGAACGGGAA, reverse primer: TCGCCCCACTTGATTTTGA). Conventional CT method was adopted to analyze the data.

Western blotting

Cells were washed with phosphate-buffered saline (PBS) and harvested with Loading Buffer lysis 1× (2.5 ml Tris HCl 1M pH 6.8, 5 ml 20% sodium dodecyl sulfate (SDS), 5 ml 100% Glycerol topped up with 100 ml distilled water), and proteins concentrations measured using a Pierce BSA Protein Assay Kit (Thermo Scientific, UK) according to the manufacturer's protocol. Lysates were analyzed by SDS-polyacrylamide gel electrophoresis (SDS; gel concentration 15%) and blotted onto nitrocellulose membranes. After blocking with 5% bovine serum albumin in a TBS buffer (1.8 ml Tween 20, 36 ml Tris HCl 1M pH 8 and 49 ml NaCl 5M, topped up with 1 L distilled; pH 8.0) with 0.1% Tween-20, the membrane was probed with the primary antibody. Then, the membrane was incubated with secondary antibody, followed by visualization using an ECL detection system (Thermo Scientific, UK). Protein loading was assessed by reprobing for tubulin.

Zebrafish embryo invasion assay

Zebrafish, handled in compliance with the Animals (Scientific Procedures) Act 1986, were kept in 28.5°C aquaria with 10 h dark, 14 h light cycles. Fertilized embryos were stored at 28.5°C in egg water ("Instant Ocean" Sea Salts 60 µg/ml distilled H₂O) containing methylene blue, to prevent fungal infection, until 48 h post fertilization (hpf).

To enable visualization, cells were stained for 1 h in RPMI containing CM-Dil (2 µg/ml final concentration),

washed twice with PBS, and detached with 1× trypsin/ethylenediaminetetraacetic acid. Cells were centrifuged and resuspended in 100 µl of fresh RPMI.

Dechorionated zebrafish embryos were anesthetized in 0.02% tricaine and immobilized for injection by placing in 1% low melting point agarose (in tricaine). Borosilicate glass capillary needles (1.0 mm OD, 0.78 mm ID) (Harvard apparatus) were pulled using a micropipette puller (Sutter Instrument, Novato, USA) and loaded with 10 µl of cell suspension. They were attached to a Picospritzer III injection apparatus (Intracel) set at a pressure of 500–1000 hPa and time of 0.3–0.8 s. Embryos were manually injected in the perivitelline cavity with ~100–150 cells in ~5 nl. After 1 h incubation, they were screened for successful injection using a Nikon total internal reflection fluorescence microscope, gently cut free from the agarose, and placed in egg water at 33°C.

Statistical analysis

All experiments were carried out in triplicate and repeated independently at least three times.

t-test for comparison of two groups or ANOVA for comparison of more than three groups was used for statistical analysis. All data and figures were analyzed and generated using the GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). *P* < 0.05 was considered to be statistically significant.

RESULTS

Migratory behavior of bladder cell lines in zebrafish embryos

Zebrafish have emerged as a useful tool to study tumor biology *in vivo* since, due to their visual clarity, they can be exploited by the use of fluorescent dyes to label cells and visualize invasion and metastasis. In these experiments, a selected panel of bladder cell lines was transplanted into zebrafish embryos to

investigate their migration behavior and metastatic potential. To this end, the cells were fluorescently labeled with fluorescence dye (Dilc12) and injected into the perivitelline space of 48 hpf zebrafish embryos. The process of invasion and migration of cancer cells in the living animal body were visualized 48 h postinjection (hpi) using fluorescence microscopy. The results showed that the average migration rates of T24 and J82 cells were 41.9% and 38%, respectively [Table 1 and Figure 1a], where these cells disseminated extensively throughout the body of the fish [Figure 1b] compared to other cell lines including HT 1376 and RT 112 which showed less invasion potential; 7% and 6.6%, respectively [Table 1 and Figure 1a].

Expression of S100A4 and S100A6 in selected bladder cancer cell lines

To analyze the role of S100A4 and S100A6 proteins in BC, we first examined the mRNA and protein expression levels of S100A4 and S100A6 in human BC cell lines using quantitative reverse transcription-PCR and Western blot. T24 and J82 cells expressed both transcripts at high levels compared to HT1376 and RT112 cell lines [Figure 2a]. Western blot analysis also

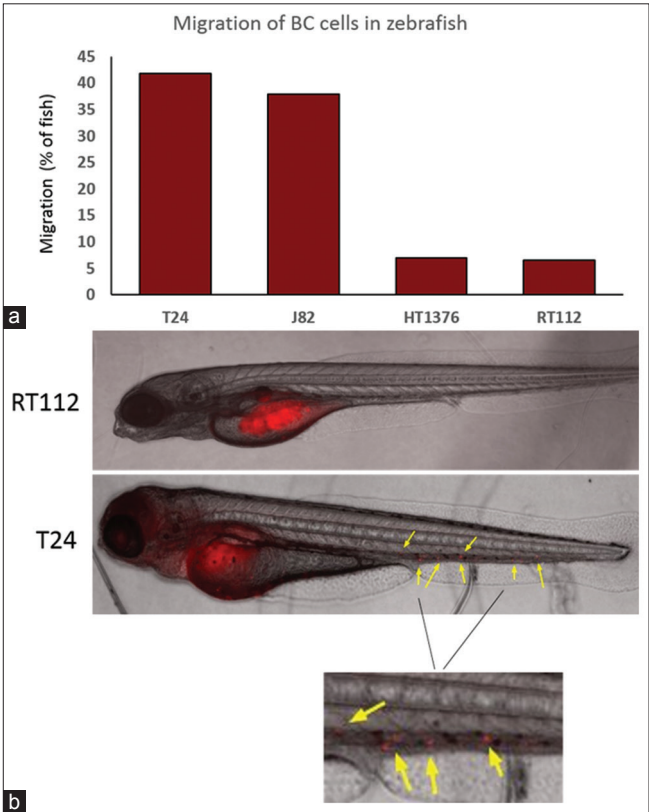


Figure 1: Migratory behavior of bladder cancer cell lines in zebrafish embryo. (a) Migration of bladder cancer cell lines in zebrafish. Cell migration was quantified in three independent experiments (b) Merged images of zebrafish embryos injected with T24 and RT112 cells 48 h postinjection. Cells were fluorescently labeled and injected into perivitelline space 48 h post fertilization zebrafish embryo. Fluorescence images were taken with 4× objectives. T24 cells were extensively disseminated through the fish body in comparison to RT112 cells. (Arrows indicate disseminated tumor cells)

Table 1: Migration of bladder cancer cells in zebrafish embryos		
Type of cells injected	Migration (%)	Average migration (%)
T24	40.4	41.9
	40	
	45.3	
J82	33.3	38
	44.4	
	36.3	
	36.3	
HT1376	11.1	7
	0	
	10	
RT112	10	6.6
	10	
	0	

Migration ability of the BC cancer cells was calculated as a percentage of the positive embryos with detected disseminated cells to the total number of embryos used in the experiment. Average of dissemination was calculated from three independent experiments. BC: Bladder cancer

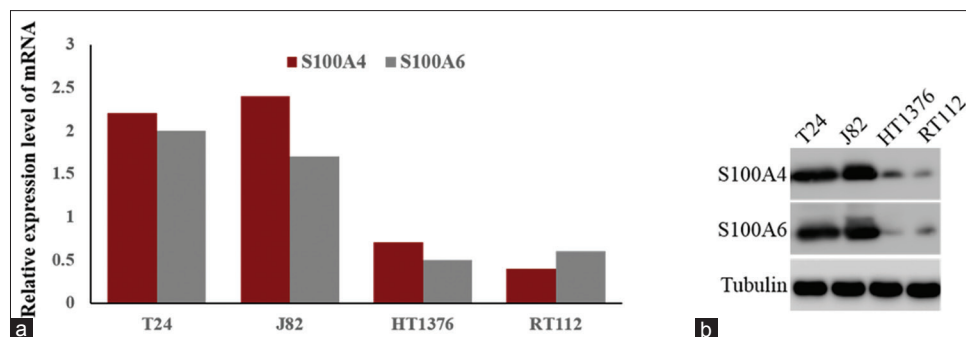


Figure 2: Expression of S100A4 and A6 in bladder cancer cells. (a) Quantitative polymerase chain reaction analysis of the expression of the selected S100A4 and A6 genes. The relative mRNA expression level was quantified using the ΔCT method, and mRNA level was normalized to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. (b) Western blot analysis was carried out to further verify the expression of S100 proteins. The lysates were collected from cells and resolved on polyacrylamide gel. Membranes were stained with antibodies to S100A4 and S100A6 as well as tubulin as loading control

demonstrated strong expression of S100A4 and A6 in both J82 and T24 cells. However, HT1376 and RT112 cells expressed low levels of both proteins [Figure 2b].

Silencing of S100A4 and S100A6 reduces Bladder cells (BC) cell migration in zebrafish

The proposed function of S100A4 and S100A6 has focused on their role in the regulation of cell migration, with their downregulation suppressing the ability of cell migration, while their upregulation promotes it. It can be shown from [Table 1 and Figure 1a] that J82 and T24 cell lines displayed the highest migration rate among selected bladder cell lines and that S100A4 and S100A6 were highly expressed in both these cell lines [Figure 2a and b]. It is possible, therefore, that a high ability of cells to migrate could be attributed to overexpression of these proteins. To determine the functional role of S100A4 and S100A6 in promoting cancer cell migration, expression of S100A4 and S100A6, either alone or in combination, was downregulated in T24 and J82 cells using specific siRNA. The transfected cells were fluorescently labeled and injected into zebrafish embryos. Fluorescence microscopy was performed 48 hpi and showed that the average migration rate of T24 cells transfected against S100A4, S100A6, and S100A4 and A6 were 17.3%, 10%, and 17.3%, respectively, which was lower than from siControl (39.7%) [Table 2]. Compared to siControl cells, the silencing in T24 cells of S100A4, S100A6, and both S100A4 and A6 together resulted in a significant decrease in cell migration ($P = 0.002$, $P = 0.001$, and $P = 0.002$, respectively) [Figure 3a].

Likewise, the same effect of protein silencing on cell migration was observed in J82 cell lines. S100A4, S100A6, and S100A4 and A6 knockdown reduced the migration rate of J82 cells to 14.6%, 17%, and 14% compared to the control 37.8% [Table 2]. There were the following significant differences between the control groups compared with the others: (siControl vs. siS100A4 [$P = 0.01$]), (siControl vs. siS100A6 [$P = 0.02$]), and (siControl vs. siS100A4+siS100A6 [$P = 0.01$]) [Figure 3a]. Cells in which S100A4 and S100A6 had undergone knockdown remained within the injection site and did not disseminate, whereas control cells disseminated into other parts

Table 2: Migration of T24 and J82 cells transfected with siControl, siS100A4, siS100A6 or combination of siS100A4&A6

Type of cells injected	Migration %	Average migration %
siControl		
T24	40 43 36.3	39.7
J82	44 36.3 33.3	37.8
siS100A4		
T24	14 13 25	17.3
J82	11 11 22	14.6
siS100A6		
T24	0 22 18	10
J82	20 20 11	17
siS100A4&A6		
T24	14 18 20	17.3
J82	0 20 22	14

Cell migration in zebrafish embryos was quantified in 3 independent experiments

of the body [Figure 3d]. The efficiency of transfection at both mRNA and protein level was then evaluated by quantitative real-time PCR and Western blot analysis. The mRNA and protein expression levels were significantly decreased in cells transfected with siS100A4, siS100A6, both individually and in combination, compared to control cells [Figure 3b and c].

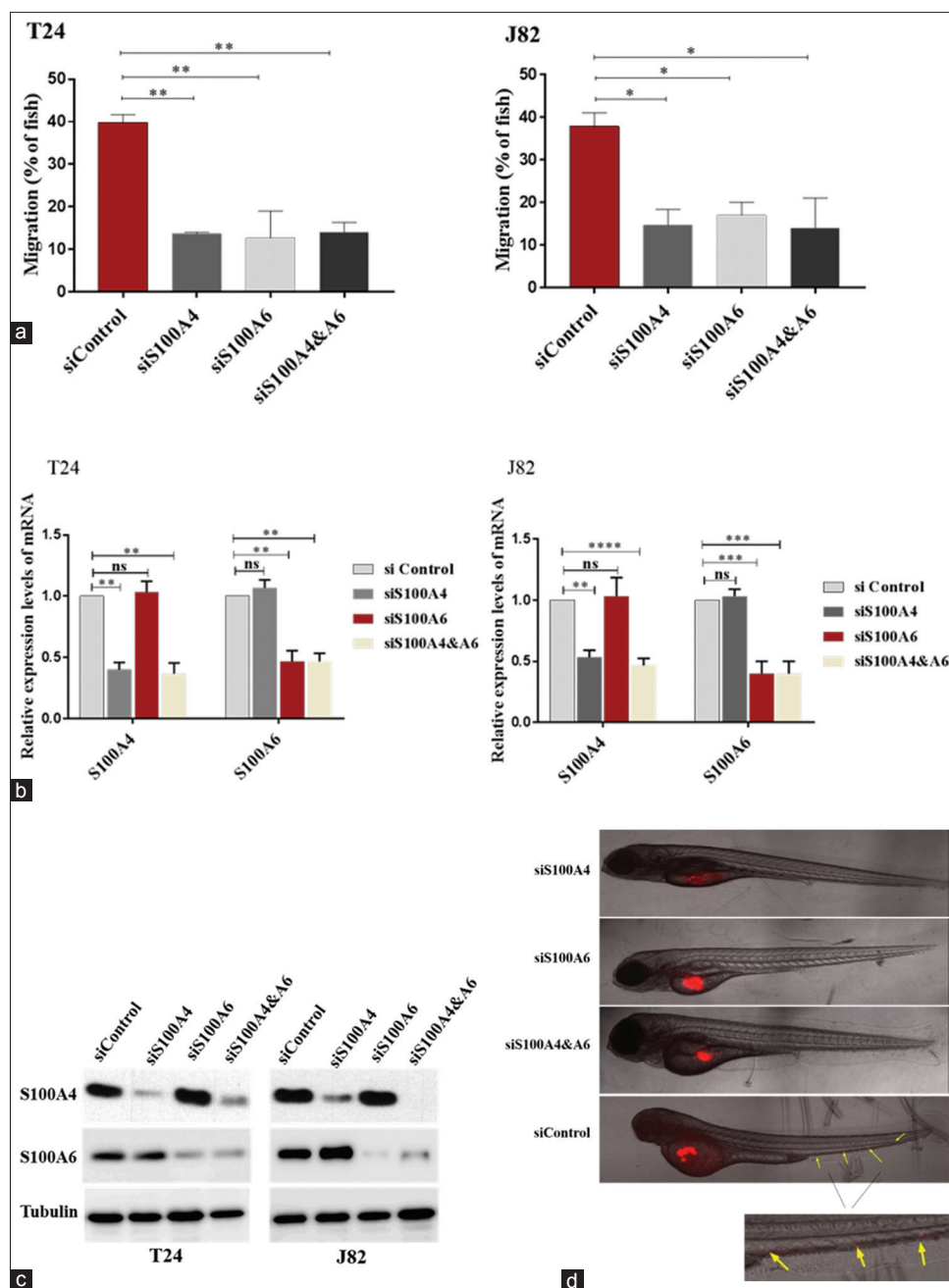


Figure 3: Silencing of S100A4 and S100A6 reduces bladder cancer cells migration in zebrafish. (a) Statistical analysis of knockdown of S100A4 or S100A6 on the migration of T24 and J82 cells in zebrafish. Bar charts with standard errors of the mean represent the average migration of control cells or cells with reduced expression of S100 proteins, * $P = 0.01$, ** $P = 0.002$. The results of 3 independent experiments are shown. (b) Depletion of S100A4 and S100A6 by small interfering RNA at mRNA level were confirmed by quantitative polymerase chain reaction. The relative mRNA level was evaluated by the $2^{-\Delta\Delta CT}$ method, and mRNA level was normalized to housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase. Bar charts with standard errors of the mean represent delta cycle threshold value, ** $P = 0.001$, *** $P = 0.0001$, **** $P < 0.0001$. The results from three representative experiments are shown. (c) Reduced expression of S100A4 and A6 was confirmed by Western blot. (d) Merged images of zebrafish embryos 48 h postinjection. T24 cells were transfected with small interfering RNAs targeting S100A4, S100A6, S100A4 and A6 and siControl and injected into zebrafish. Fluorescence images were taken 48 h postinjection with $\times 4$ objective. Arrows indicate migrated tumor cells

Taken together, these findings suggest that the silencing of S100A4 and A6 affected the migration ability of PC cells in zebrafish embryos, thus highlighting their role in tumor progression.

Effect of S100A4 Overexpression on RT112 Cells

To further analyze the role of S100A4 in BC, RT112 cells were treated with IL-11 (To activate expression of S100A4) or a control DMSO. The overexpression of S100A4 was

confirmed by performing Western blot analysis 48 h after treatment. We detected a significant increase in the level of S100A4 protein in S100A4-overexpressing cells, compared to control cells [Figure 4a].

Next, we explored the effect of S100A4 expression on cell invasion *in vivo*. To this end, the cells transfected cells were fluorescently labeled and injected into zebrafish as described previously. Zebrafish microinjection assay revealed a significant increase in migration ability of S100A4-overexpressing cells by 29%, compared to control cells 7.3% ($P=0.01$) [Table 3 and Figure 4b]. These data further support our hypothesis that S100A4 confers the invasive characteristics to cells during human BC development.

DISCUSSION

BC is one of the major causes of cancer-related mortality in the world and is characterized by its aggressive behavior.^[14] Better understanding of the molecular mechanisms responsible for the enhanced BC aggressiveness is required.

To this end, several studies so far have linked many S100 protein members with BC progression.^[15,16] Overexpression of S100A4 and S100A6 has been reported to be significantly associated with development of a metastatic phenotype in

many cancer types including breast cancer,^[17] hepatocellular carcinoma^[18,19] colorectal cancer,^[20] gastric cancer,^[21] and multiple myeloma.^[22] To date, in our knowledge, there are no studies that have investigated the association between S100A4 and S100A6 expression and invasive behavior of BC, indicating that more research is needed to highlight the role of these proteins in BC.

Injection of cancer cells into zebrafish embryos has provided new mechanistic insights into the processes by which cancer cells migrate. There is no conclusive evidence on the use of zebrafish as an animal model to study *in vivo* dissemination of BC cells. For this reason, establishing a zebrafish cancer model to study the effect of S100 proteins in promoting BC cells migration was one of the priorities of this study.

We observed that the migration rate of different BC cell lines varied in zebrafish significantly. Importantly, we found a statistically significant correlation between S100A4 and A6 expression and migratory potential of BC cells. Indeed, we demonstrated that T24 and J82 displayed both high-level migration rates in zebrafish and a high level of S100A4 and A6 expression, while HT1376 and RT112 cell lines showed low migration potential and very low level of S100 proteins expression. On the other hand, knockdown of S100A4 and A6 expression in T24 and J82 cells by siRNA revealed significantly decreased cell migration compared to control cells. In addition, overexpressed S100A4 expression in nonmotile RT112 cells using S100A4-overexpression plasmid resulted in increased migration ability of cells *in vivo*. This result is in accord with the other studies indicating that knockdown or induce expression of S100A4 or S100A6 led to diminish or promoter of breast cancer, renal carcinoma cells,^[23] gastric cancer,^[11] osteosarcoma cells,^[6,12] and pancreatic cancer.^[24]

Notwithstanding the fact that S100A4 and S100A6 play a critical role in the enhancement of cell invasion and migration of different human cancers, the precise mechanism of their function has remained elusive. One mechanism by which it is suggested S100A4 and S100A6 exert their oncogenic effect is based on their ability to interact with the cytoskeleton-related proteins, leading to the promotion of cancer cell migration^[25,26] Furthermore, binding of S100A4 or S100A6 to their specific receptor, RAGE promotes cancer cell migration via activation signaling pathways such as mitogen-activated protein kinase/extracellular signal-regulated kinase.^[27,28] In addition, it was found that the metastatic function of S100A4 correlates to its ability to induce expression of matrix metalloproteinases, which degrade the extracellular matrix, thereby enhancing cancer cells' migration.^[29]

From the research that has been undertaken, it is possible to conclude that S100A4 and S100A6 play a vital role in cancer cell migration in zebrafish. In addition, we could conclude that zebrafish xenografts are a useful tool for *in vivo* studies of BC cells migration.

Table 3: Migration of RT112 cells treated with interleukin-11 or DMSO (control)

Type of cells injected	Migration (%)	Average migration (%)
DMSO (control)	11	7.3
	11	
	0	
IL-11	30	29
	27.2	
	30	

Cell migration in zebrafish embryos was quantified in three independent experiments. DMSO: Dimethyl sulfoxide, IL: Interleukin

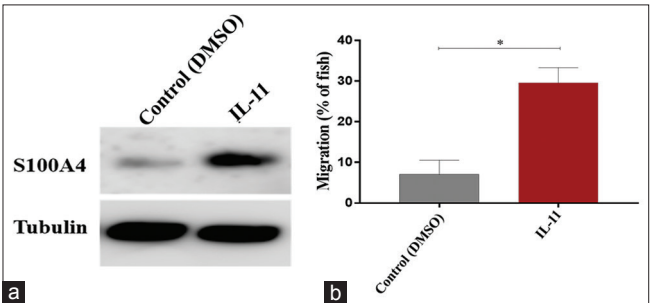


Figure 4: S100A4 overexpression promotes bladder cancer cells migration in zebrafish. (a) RT112 cell lysates were collected 48 h after treatment and loaded into acrylamide gel. The transferred membranes were stained with S100A4 antibody to confirm level of proteins expression. (b) Statistical analysis of the effect of overexpressed S100A4 on the migration of RT112 cells in zebrafish. Bar charts with standard errors of the mean represent the average migration rate of control cells or cells with transit expression of S100A4, $*P \leq 0.05$. The results of three representative experiments are shown

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Nil.

Conflicts of interest

There are no conflicts of interest.

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