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Epithelial Mesenchymal Transition (EMT) -associated S100 proteins are modulators for the progression of bladder cancer

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Abstract

Introduction:

Bladder cancer (BC) is considered to be the tenth in the world. There is much evidence to suggest that the highly metastatic behaviour of BC may be due to the ability of its cells to undergo Epithelial Mesenchymal Transition (EMT).
The S100 family of small Ca-binding proteins are among many genes that have been found to be implicated in BC cancer progression. Activation of some members of the S100 protein family, such as S100A4 and S100A6, have been postulated to be a common mediator of the EMT process and a key driver for cancer cell migration and invasion. To our knowledge, however, there is no conclusive evidence that the presence of the S100 family is causally implicated in EMT-induced BC progression.

Experimental plan:

To link S100 proteins with EMT programs in BC, expression of eleven S100 proteins, a number of epithelial and mesenchymal markers, and several EMT-inducing transcription factors was analysed in a panel of the BC cell lines. Using qPCR and Western blot. Assess the effect of S100 proteins on bladder cell migration *in vivo* was carried out using a Zebrafish xeno-transplant model.

Result:

The results of the present study shows that expression analysis of the S100 family members in a collection of BC cell lines allowed categorization of the S100 genes into two groups, mesenchymal-associated and epithelial-associated. Overexpression of ZEB1 during EMT induction resulted in altered expression of certain S100 protein family members. Of note, only S100A4 and S100A6 were activated, and their expression correlated with expression of EMT-associated proteins, with E-Cadherin being down-regulated whilst ZEB1 and Vimentin were up-regulated. Additionally, it has been found that ZEB1 expression induced EMT prompted BC cells migration via upregulation of S100A4&A6.

Conclusion:

Our study revealed that expression of S100A4 and S100A6 is activated during ZEB1-induced EMT, and plays a vital role in the regulation of BC cell migration.

Abbreviations: BC: Bladder Cancer, hpi: hour post injection, hpf: hour post fertilization

Introduction

Bladder cancer is one of the most frequent cancer worldwide. Despite the divers treatment option for patients with bladder cancer, their survival rate has not significantly improved (Taheri et al., 2017). At the time of the first diagnosis, about 70% to 80% of BCs are non-muscle-invasive BCs (NMIBCs) and the remaining 20% to 30% are muscle-invasive BCs (MIBCs). In the case of MIBC, although only 20% of BC patients are diagnosed with MIBCs, these cancers are responsible for the vast

majority of BC-specific deaths. Moreover, nearly 50% of patients with MIBC already have occult distant metastases at the time of diagnosis (Yun and Kim, 2013). Lymph nodes, bones, lungs, liver, and peritoneum were the most common sites of metastasis from bladder cancer (Jemal et al., 2009).

S100s belong to a family of calcium-binding proteins which consists of more than 20 members with a low molecular weight of around 10-12kDa (Marenholz et al., 2004). They have been involved in the control of many intracellular and extracellular processes including calcium homeostasis, phosphorylation, activation of many signalling pathways and regulation of transcriptional factors, dynamics of cytoskeleton proteins and enzyme regulation (Bresnick et al., 2015). Deregulated expression of several members of the S100 protein family, including S100A2 (Wang et al., 2017), S100A4 (Nasser et al., 2015), S100A6 (Luo et al., 2008), S100A7 (Padilla et al., 2017), S100A11 (Liu et al., 2015), S100A14 (Tanaka et al., 2015) and S100P (Wu et al., 2017), has been reported in association with the progression of various human cancer.

Epithelial mesenchymal transition (EMT) is implicated in tumour progression and is characterised by alterations in cell morphology and formation of highly motile cells. Loss of E- Cadherin is one of the most important hallmarks in EMT and is accomplished by the diminished levels of the epithelial proteins (P-cadherin, ZO-1, claudins and occludins) and upregulated mesenchymal markers (Vimentin, N-cadherin and Fibronectin) (Teng and Li, 2014).

Functional loss of E-Cadherin is regulated by activation of EMT-transcriptional factors including zinc finger proteins (Snail, Slug, ZEB1 and ZEB2) and basic helix loop helix (Twist1 and Twist2) (Smit and Peeper, 2010). These factors exert their effect by repressing the expression of the E-Cadherin via interaction with specific sequences (E-box) of the E-Cadherin promoter while promoting the expression of genes involved with cell motility and migration (Ouyang et al., 2010).

Numerous *in vitro* and *in vivo* studies suggest that the EMT is associated with cancer cell invasion and metastasis in various malignancies, including BC (Yun and Kim, 2013).

Although various studies have explored either the expression of the individual S100 proteins or EMT-TFs in PC progression, up to date there are no investigations linking S100 proteins to the EMT as a mechanism of PC development. However, this study was design to study the role of S100 proteins in association with EMT program in pathogenesis of BC.

Materials and Methods

Tissue culture

BC cell lines including T24, J82, HT1376 and RT112 were obtained from the American Tissue Culture Collection (ATCC) and cultured in the 5% CO_2 and 37 °C incubator in Roswell Park Memorial Institute medium supplemental with 10% FBS and 1% penicillin and streptomycin.

Construction of ZEB1 expression vectors and siRNA transfection

The plasmids construct pEGFP-C1 (control vector) and pEGFP-C1-ZEB1 were amplified in *Escherichia coli* and purified using a Qiagen Midiprep kit (Qiagen, UK). 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 ZEB1 overexpression, cells were transfected with either pEGFP-C1 or pEGFP-C1-ZEB1 plasmid using Ingenio® Electroporation solution (Bio Ingenio, UK) following the manufacturer's instructions. Briefly, 2 x 10^6 cells were transfected with 2ug siRNAs or plasmid suspended with the transfection reagent. The final suspension was transferred into 4mm cuvette and eloctroporated using Gene PulserX cell electroporator which was set at 250V and 250µF.

qRT-PCR 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 PCR was performed using set of primers provided by Sigma, UK and listed in Table 1. Real-time PCR reactions were performed in triplicate in a 20µl reaction volume containing 1· SYBRgreen 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 (GAPDH). Conventional CT method was adopted to analyse the data.

Table 1: List of PCR primers designed using NCBI/Primer-BLAST program

Primer name	Primer sequences	
	Forward Primer	Reverse Primer
ZEB1	GATGACCTGCCAACAGACC	CTTTCACTGCTCCTCCCTGG
P-Cadherin	ACCAACCATCATCCCGACA	GTTAGCCGCCTTCAGGTTC
E-Cadherin	ATGGCTGAAGGTGACAGA	TGCATTCCCGTTGGATGAC
Vimentin	CTCTGGCACGTCTTGACCT	GCCATCAACCTCTTCGTGG
S100A2	CCAGCTTTGTGGGGGAGAA	TGAGTGCCAGGAAAACAG
S100A4	CTAAAGGAGCTGCTGACCC	TGTCCCTGTTGCTGTCCAA
S100A6	GAAGGAGCTCACCATTGGC	CACCTCCTGGTCCTTGTTCC
S100A7	ACCTCGCCGATGTCTTTGA	CCATGGCTCTGCTTGTGGT
S100A8	AAGGGGAATTTCCATGCCG	AGGACACTCGGTCTCTAGC
S100A9	GCTGGTGCGAAAAGATCTG	TGTGTCCAGGTCCTCCATG
S100A10	AAAAGACCCTCTGGCTGTG	AATGGTGAGGCCCGCAATT
S100A11	GGTGTCCTTGACCGCATGA	AGGAAGGAGTCATGGCAA
S100A13	ATCTGCTCAAGGATGTGGG	GCCAGCTCCCCAATCAATC
S00A14	CGCAGAGGATGCTCAGGA	GTAGCTCAGAAGGGGTCAG
S100P	GCTCAAGGTGCTGATGGAG	CAGCCACGAACACGATGA
GAPDH	GTCAAGGCTGAGAACGGG	CAGCCACGAACACGATGA

Western blotting

Cells were washed with phosphate-buffered saline (PBS) and harvested with Loading Buffer lysis1X(2.5ml Tris HCL 1M pH 6.8, 5ml 20%SDS, 5ml 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 analysed by SDS-polyacryamide gel electrophoresis (SDS-PAGE; gel concentration 15%) and blotted onto nitrocellulose membranes. After blocking with 5% bovine serum albumin in a TBS buffer (1.8ml Tween 20, 36ml Tris HCl 1M pH 8 and 49 ml NaCl 5M, topped up with 1L distilled; pH 8.0) with 0.1% Tween-20, the membrane was probed with the primary antibody. Then the membrane was incubated with secondary antiboy and followed by visualization using an ECL detection system (Thermo Scientific, UK). Protein loading was assessed by re-probing 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 10hrs dark, 14hrs light cycles. Fertilised embryos were stored at 28.5°C in egg water ("Instant Ocean" Sea Salts 60µg/ml distilled H_2O) containing methylene blue, to prevent fungal infection, until 48 hours post fertilisation (hpf). To enable visualisation, cells were stained for one hour in RPMI containing CM-Dil (2µg/ml final concentration), washed twice with PBS and detached with 1x trypsin/EDTA. Cells were centrifuged and re suspended in 100µl of fresh RPMI.
Dechorionated zebrafish embryos were anaesthetised in 0.02% tricaine and immobilised for injection by placing in 1% low melting point agarose (in tricaine). Borosilicate glass capillary needles (1.0mm OD, 0.78mm ID) (Harvard apparatus) were pulled using a micropipette puller (Sutter instrument, Novato, USA) and loaded with 10µl 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 secs. Embryos were manually injected in the perivitelline cavity with ~100-150 cells in ~5nl. After 1hr incubation they were screened for successful injection using a Nikon TIRF 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 analysed and generated using the Graph Pad Prism 7 software. $P < 0.05$ was considered to be statistically significant.

Results

The expression profile of EMT-associated genes in bladder cancer cell lines

A series of bladder cancer cell lines with different morphological and adhesion features were selected in this study. We firstly categorised the cell lines as either epithelial or mesenchymal-like according to morphological features or clustering. The cell lines HT1376 and RT112 displayed an epithelial phenotype which comprised of cells that attach tightly together by intracellular adhesion. T24 and J82 cells,

meanwhile, were unambiguously classified as mesenchymal cells, characterised by small protrusions and focal cell-cell adhesion (Figure 1A). This experiment, therefore, aimed to determine the expression of EMT-related proteins as a way of classifying and differentiating these phenotypes.

qPCR and Western blot analysis was consistent with the morphological phenotyping and showed that all the epithelial cells expressed high level of the epithelial markers, E-Cadherin and P-Cadherin. T24 and J82 also appeared to be strongly positive for the mesenchymal protein ZEB1 and Vimentin. Regarding different EMT-TFs, the expression of proteins was inconclusive in BC cells. The expression of Slug was detected in different cell lines but could not be specifically assigned to individual phenotypes. ZEB2 was not detected in any cell line, while Twist1 was expressed only in T24 cells (Figure 1B). ZEB1, however, was the factor that most obviously corresponded to morphological features and the downregulation of E-Cadherin and upregulation of Vimentin.

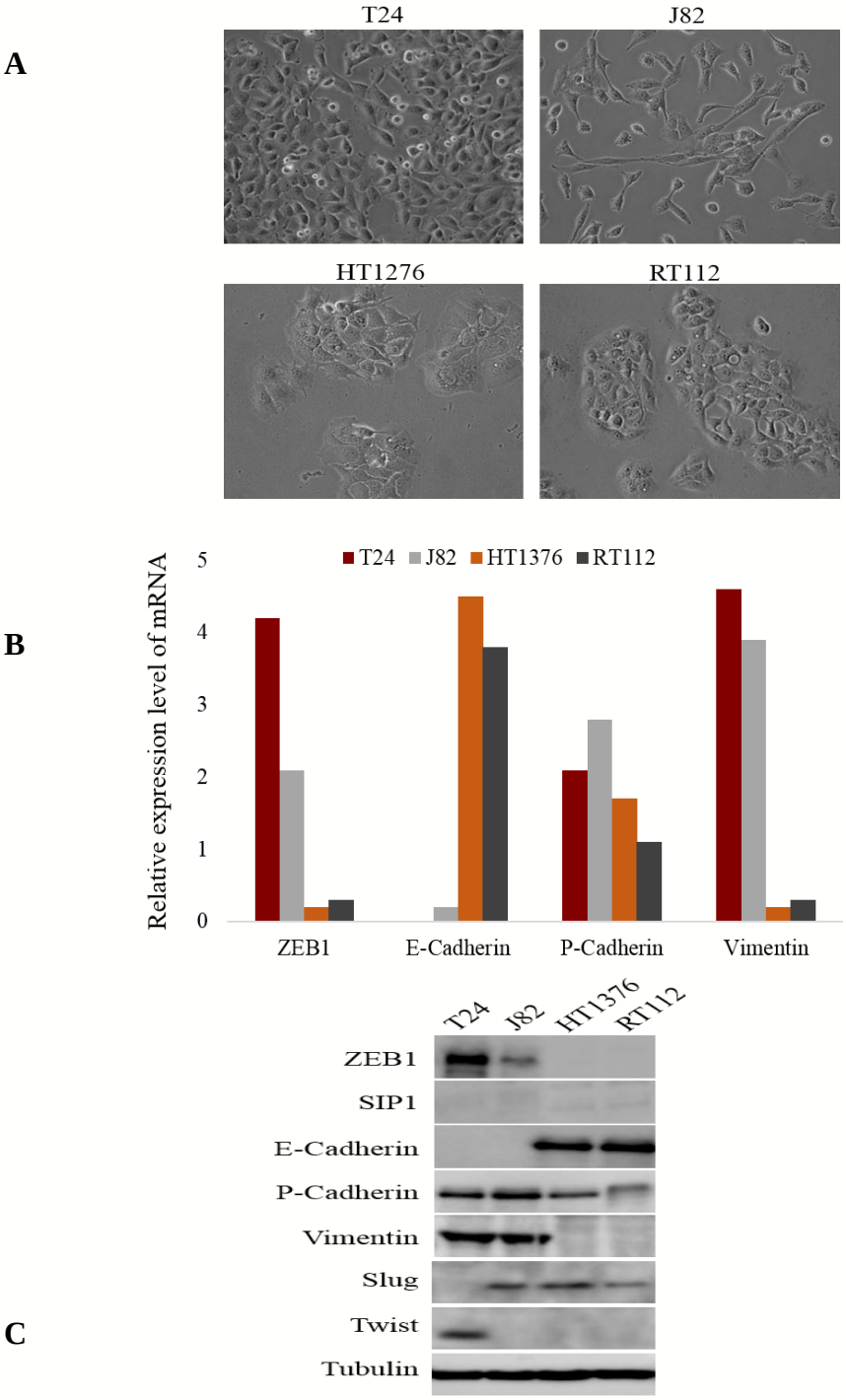


Figure 1: Expression of the EMT-associated proteins in the selected bladder cancer cell lines.

(A). HT1376 and RT112 exhibited epithelial phenotypes. T24 and J82 cells have mesenchymal futures with spindle like extension. Phase-contrast images were taken with 20x objective. **(B).** mRNA expression level of different EMT-related genes was quantified as ΔCt, calculated by normalizing expression level of gene of interest to a reference gene (GAPDH). **(C).** Cell lysates were collected and resolved on polyacrylamide gel. The transferred membranes were stained for EMT-related proteins including ZEB1, ZEB2, Vimentin, E-Cadherin, P-Cadherin, Slug, Twist as well as tubulin as a loading control.

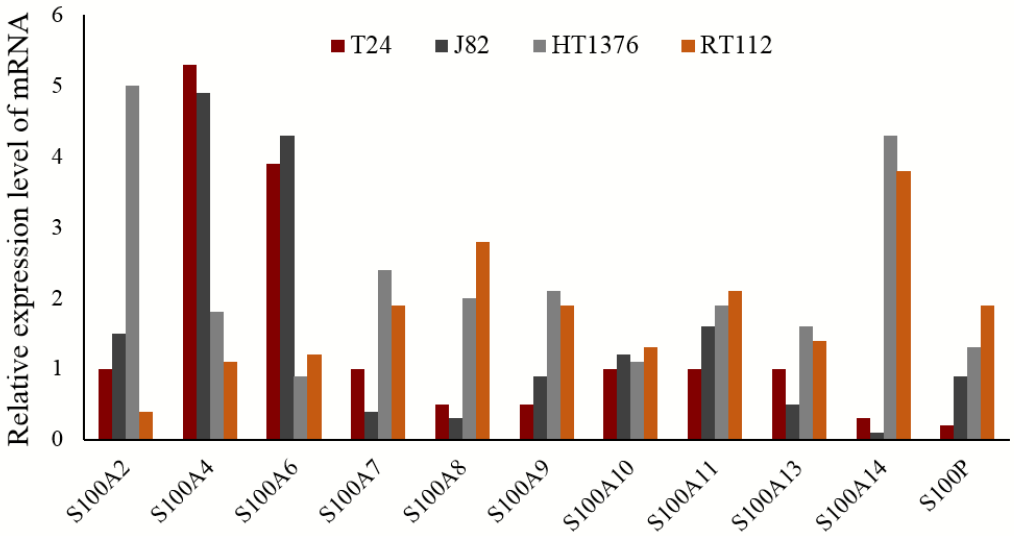
Differential expression of S100 proteins in Bladder cancer cell lines

It has been reported that expression of members of the S100 gene family differs in different human malignancies. The expression of these family members varies between cells and may be specific for certain phenotypes, with some of them upregulated in epithelial cells, while others tend to be mesenchymal markers. qPCR and Western blot analysis were therefore conducted in order to analyse the expression profiles in bladder cell lines of eleven members of the S100 family of genes at the mRNA level (S100A2, S100A4, S100A6, S100A7, S100A8, S100A9, S100A10, S100A11, S100A13, S10014 and S100P) and seven members at protein levels (S100A2, S100A4, S100A6, S100A9, S100A11, S10014 and S100P).

qPCR data showed that mRNA expression levels of S100A2, S100A7, S100A8, S100A9, S100A11, S100A13, S100A14 and S100P genes were upregulated in epithelial phenotype cells compared to mesenchymal cells (Figure 2), and this expression was associated positively with the epithelial related gene, E-Cadherin (See Figure 1). Expression of S100A4 and S100A6 meanwhile, was found to be upregulated in cells with mesenchymal features, T24 and J82, in parallel with the activation of the mesenchymal genes, ZEB1 and Vimentin (See Figure 1). S100A10 genes were inconsistently expressed among all cell lines and could not be specifically assigned to certain phenotypes, since high expression levels were found in both epithelial and mesenchymal cells (Figure 2A).

At the protein level, differential expression of S100 proteins was further verified by Western blot analysis and revealed that protein levels for selected S100 proteins were consistent with the level of mRNA (Figure 2B). In general, therefore, it seems that expression of S100A4 and S100A6 is elevated in cells positive for ZEB1 and Vimentin, while other members of the S100 family are expressed in epithelial BC cells positive for E-Cadherin.

A



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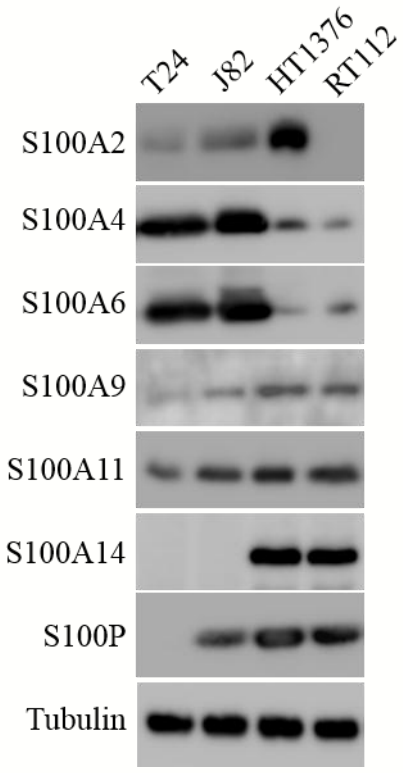

Figure 2: Expression profile of S100 proteins in pancreatic cell lines.
(A). mRNA expression level of different members of S100 was quantified as ΔCt, calculated by normalizing expression level of gene of interest to a reference gene (GAPDH). **(B).** Cells lysate were collected and resolved into polyacrylamide gel. Expression of S100 proteins members were visualised using specific antibodies. Tubulin was used as a loading control.

ZEB1 induced EMT mediates alteration cell morphology and transcriptome of S100 genes in BC cell lines

It is now well established that alteration of the expression of S100 proteins and EMT induction are among many genetic events involved in BC progression. Therefore, it is possible to hypothesise that members of this family may be associated with EMT in the development of BC progression.
EMT was therefore induced in RT112 cell lines by transfecting cells transiently with a GFP-ZEB1 overexpression construct, as well as an empty GFP vector as a control. Phase-contrast images were taken 48h after transfection.

The morphological changes in the cells indicating a transition from epithelial to mesenchymal status can be clearly seen as resulting from ZEB1 induced EMT. The ZEB1 overexpressed cells tend to gain a mesenchymal-like morphology and become more elongated. In contrast, the parental cells maintained epithelial-like features and formed clusters (Figure 3A).
Next, RNA was extracted from transfected cells after 48h and subjected to quantitative PCR in order to investigate the expression profiles of S100 genes as well as EMT-associated genes. The mRNA alterations for these genes are shown in (Figure 3B). Overall, the cells transfected with the GFP-ZEB1 construct exhibited a significant increase in the expression level of ZEB1 mRNA compared to cells transfected with a GFP control vector. Expression profiles of several S100 genes demonstrated that only S100A4 and S100A6 transcripts were activated in ZEB1 overexpression cells in RT112 cell lines. Whereas, other genes (S100A7, S100A8, S100A9, S100A11, S100A13, S100A14, S100P) appeared to be downregulated. For S100A2 and S100A10, a nearly similar gene expression was observed in ZEB1 overexpressed and control cells. The expression levels of a panel of S100 proteins were next confirmed by Western blot analysis (Figure 3C). Comparing the Western blot results with the qPCR data, a concordance of S100 expression at protein and mRNA level was found in all transfected cell lines.

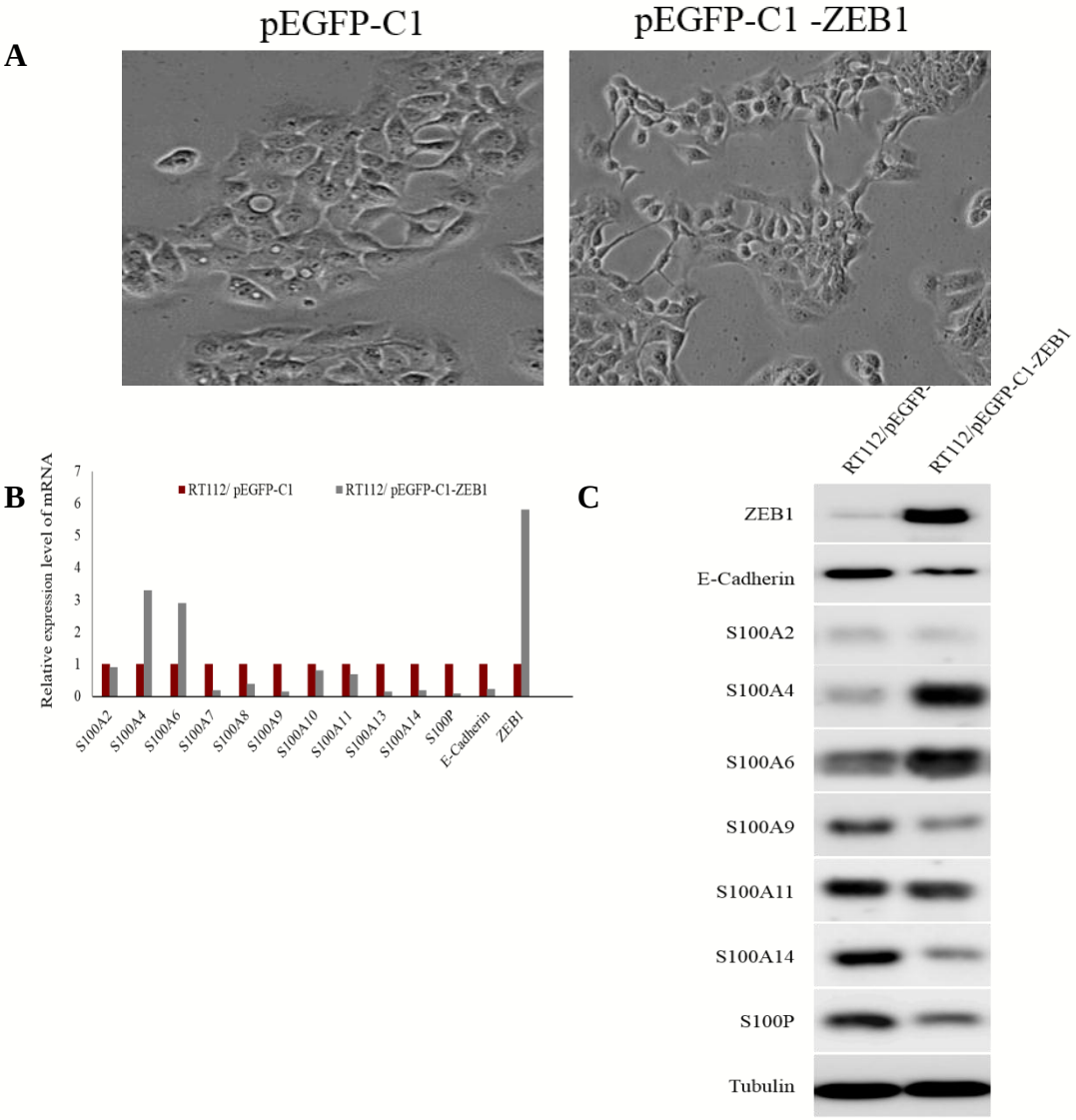

Figure 3: ZEB1 expression alters cell morphology and transcriptome of S100 genes in BC cell lines. (A). Morphological changes induced by ZEB1 expression in RT112 cell line. Cells were transfected with GFP-ZEB1 construct and GFP-control vector, and phase-contrast images were taken with 20x objective 48h post transfection. Under ZEB1expression, cells became more elongated and gain mesenchymal properties compared to control cells. **(B).** qPCR was performed to analyse mRNA expression of selected S100 genes in BxPC-3 and SU.86.86 cell lines 48h post transfection. The relative mRNA level was estimated by the 2−ΔΔCT method, and mRNA level was normalized to housekeeping gene GAPDH. Results from one representative experiment is shown**. (C).** Cell lysates were collected 48h after transfection and loaded into acrylamide gel. The transferred membranes were stained with selected antibodies to confirm level of proteins expression.

ZEB1 expression promotes BC cell migration via upregulation of S100A4&S100A6

EMT is the process whereby polarised epithelial cells convert into the motile mesenchymal cells that underpin cancer cells' invasiveness and metastasis. On the other hand, 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. Since S100A4 and S100A6 activation was observed during ZEB1 induced EMT, it could conceivably be hypothesised that ZEB1 may promote BC migration through activation of S100A4 and S100A6. In order to validate this hypothesis, a series of transfections were performed in RT112 cells.

The cells were transfected as follows: siControl, pEGFP-C1-ZEB1, pEGFP-C1-ZEB1+siS100A4 and pEGFP-C1-ZEB1+siS100A6. The transfected cells were fluorescently labelled with fluorescence dye (Dilc12) and injected into the perivitelline space of 48 hour post fertilization (hpf) zebrafish embryos. The process of invasion and migration of cancer cells in the living animal body were visualised 48 hour post injection (hpi) using fluorescence microscopy. The cell migration rates within fish injected with RT112 cells transfected with siControl, pEGFP-C1-ZEB1+siS100A4 and pEGFP-C1-ZEB1+siS100A6 were 7.3%, 14.3% and 10.6%, respectively, which was lower than from those injected with ZEB1 mediated overexpression of S100A4&A6 cells (Transfected only with pEGFP-C1-ZEB1, 30.1% (Table 2 and Figure 4A).

There were the following significant differences between the control groups (pEGFP-C1-ZEB1) compared with the others: (pEGFP-C1-ZEB1 vs siControl P= 0.0002) (pEGFP-C1-ZEB1 vs siControl vs. pEGFP-C1-ZEB1+siS100A4 P= 0.0007) and (pEGFP-C1-ZEB1 vs siControl vs. pEGFP-C1-ZEB1+siS100A6 P= 0.0005).

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 of the body. Next, the expression level of ZEB1, S100A4 and S100A6 proteins was analysed by Western blot to check the efficiency of transfection.

In summary, these results showed that inducing the activation of ZEB1 led to increased S100A4 and S100A6 expression, and thereby increased cancer cell migration.

Table 2: Migration of RT112 cells transfected with siControl, pEGFP-C1-ZEB1, pEGFP-C1-ZEB1+

Type of cells injected	Migration %	Average migration %
siControl	11	7.3
	0	
	11	
pEGFP-C1-ZEB1	33.3	30.1
	27	
	30	
pEGFP-C1-ZEB1+siS100A4	18	14.3
	11	
	14	
pEGFP-C1-ZEB1+siS100A6	11	10.6
	10	
	11	

Migration ability of the 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

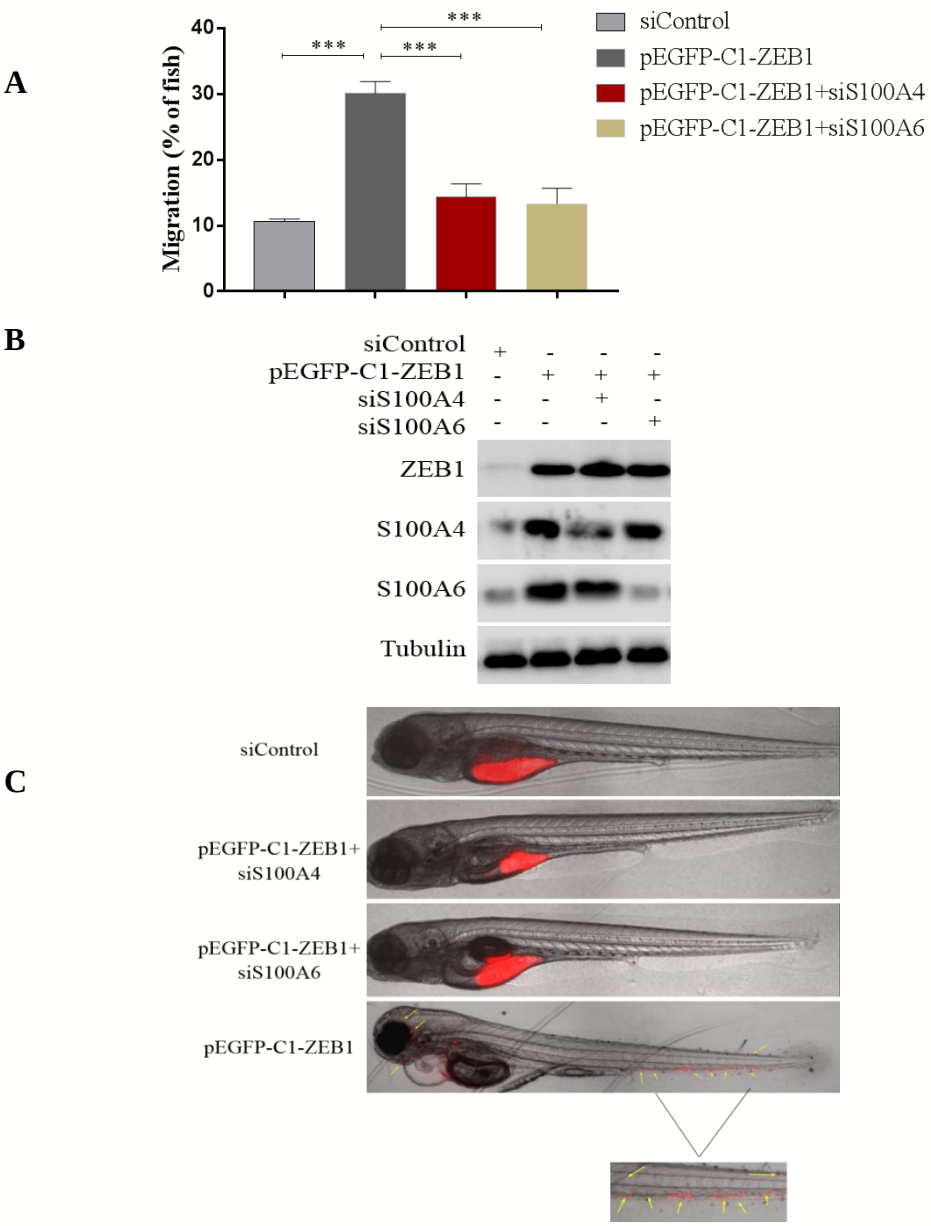

Figure 4: ZEB1 expression induced EMT promotes BC cells migration via upregulation of S100A4&A6. (A). Statistical analysis of ZEB1 overexpression and knockdown of S100A4 or S100A6 on the migration of RT112 cells in zebrafish. Bar charts with standard errors of the mean represent the average migration of control cells or cells with reduced expression of S100 proteins, *** $P \leq 0.0007$. The results of 3 independent experiments are shown. **(B)** Overexpression of ZEB1 and depletion of S100A4 and S100A6 at protein levels were confirmed by Western blotting. **(C).** Merged images of zebrafish embryos 48hpi**.** RT112 cells were transfected and injected into zebrafish. Fluorescence images were taken 48hpi with 4x objective. Arrows indicate migrated tumour cells.

Discussion

BC is one of the most lethal and aggressive of all malignancies, and emerging evidence suggests that EMT is one of the most common factors contributing to metastatic progression and drug resistance in BC (Yun and Kim, 2013, McConkey et al., 2009). Recently, it has been found that the S100 family plays an active role in BC progression (Yao et al., 2007a, Yao et al., 2007b), and the expression pattern of S100 genes is linked to EMT (Ji et al., 2014).

In the present study, ZEB1 is the only one out of all the EMT-TFs which appeared to be strongly positive in cells with a mesenchymal phenotype and its expression correlated negatively with E-Cadherin and positively with Vimentin, thus confirming previous studies (Arumugam et al., 2009, Hanrahan et al., 2017).

EMT is the process whereby polarised epithelial cells convert into the motile mesenchymal cells that underpin cancer cells' invasiveness and metastasis (Bhardwaj et al., 2015). Evidence suggests that ZEB1 is among the most EMT-inducing factors, and exerts its transcriptional activity by binding to E-Cadherin promoter and downregulating the expression of this cell-cell adhesion junction (Ouyang et al., 2010). More recently, expression of ZEB1 has been reported to be implicated in cancer cell invasion and metastasis in a variety of cancer types (Sakata et al., 2017, Caramel et al., 2018).

Next, we systematically evaluated the expression profile of members of the S100 family in a collection of BC cell lines in order to define the S100 protein signature for BC. Additionally, expression of these proteins was determined in EMT-induced BC cells to investigate whether an S100 expression pattern is required for EMT progression.

Differential expression of several members of S100 genes was observed in BC cells. mRNA levels for the majority of the selected members of S100 genes, including S100A2, S100A7, S100A8, S100A9, S100A11, S100A13, S100A14 and S100P genes, were found to be upregulated in cells with an epithelial phenotype, and associated positively with E-Cadherin.

The expression of S100A4 &A6, meanwhile, was found to be upregulated in cells with mesenchymal features; T24 and J82, coinciding with the activation of the ZEB1 and Vimentin.

Additionally, Overexpression of ZEB1 during EMT induction resulted in altered expression of certain S100 protein family members. Of note, only S100A4 and S100A6 were activated, and their expression correlated with expression of EMT-associated proteins, with E-Cadherin being down-regulated whilst ZEB1 and Vimentin were up-regulated. Therefore, these proteins are unambiguously classified as mesenchymal markers and strongly associated with EMT, and the levels observed here confirm previous studies (Chen et al., 2015, Xu et al., 2016).

Generally, much evidence has emerged from previous research to indicate that expression of S100A4 and S100A6 proteins could be a contributing factor to EMT progression, especially since an inverse correlation has been identified between E-Cadherin and S100A4 (Zhai et al., 2014) and S100A6 (Li et al., 2014). In the current study, activation of both S100A4 and S100A6 expression was related to ZEB1 induced EMT in RT112 cells after transfection with pEGFP-C1-ZEB1, which gave rise to speculation that ZEB1 can be a transcriptional regulator for S100 proteins.

The association between S100 proteins and EMT is not exclusive for BC, however. It has been found that S100A4 is a key mediator of EMT in breast cancer (Xu et al., 2016), hepatocellular carcinoma (Zhai et al., 2014), ovarian cancer (Yan et al., 2016), oesophageal squamous cell carcinoma (Jian et al., 2015) and endometrial cancer (Hua et al., 2016). Additionally, S100A6 has also been found to be, to lesser extent, involved in EMT in hepatocellular carcinoma (Li et al., 2014) and prostate cancer (Orr et al., 2012).

Although much is known about the strong correlation between S100A4&A6 and EMT, no study has analysed the effect of these proteins on cell migration during EMT. In this study, therefore, the upregulation of S100A4&A6 in cells transfected with pEGFP-C1-ZEB1 was found to promote cell migration in zebrafish approximately three times more powerfully than cells in which expression of these proteins was not activated (Transfected with empty vector (pEGFP-C1).

In agreement with this, for example, in gastric cancer, overexpression of S100A4 or S100A6 promotes cell migration, while their downregulation inhibits the rate of cell migration (Yuan et al., 2014, Li et al., 2013). In another study regarding the functional role of S100A4 in cancer, (Yuan et al., 2014) overexpressed S100A4 in two gastric cell lines, AGS and SCM-1, and found that this expression significantly increased the invasive activity of these cells. Silencing S100A4 expression in MKN-45 and TMK-1, which displayed high levels of endogenous S100A4, resulted in a decrease in the migration rate but without any effect on cell survival.

Summing up the results, it can be concluded that the expression pattern of S100 proteins is subdivided into two groups, mesenchymal-associated and epithelial-associated. Overexpression of ZEB1 during EMT induction resulted in altered expression of certain S100 protein family members. Of note, only S100A4 and S100A6 were activated, and they play a vital role in cancer cell migration in zebrafish.

Recommendation

In general, therefore, it seems that a strong association presence between EMT implicated factors or/and S100 proteins family members and pancreatic cancer. Therefore, further investigations to reveal signals from the tumour microenvironment that control the activation of S100 proteins family members via regulation of EMT-Transcriptional factors expression in primary tumours and metastatic sites may provide opportunities to develop therapeutic strategies targeting pancreatic cancer progression.

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