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The emerging role of S100A4 and S100A14 proteins in colorectal cancer progression

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Abstract

Colorectal cancer (CRC) is the third most frequent type of cancer and the second leading cause of cancer-related deaths globally. Despite a thorough understanding of its biology, etiology, and epidemiology, an estimated 1.8 million new cases are diagnosed each year, and 900000 people die as a result of malignancy. The current study aims to investigate the expression pattern of S100A4 and S100A14 proteins in CRC tissue specimens and a panel of cell lines. Furthermore, to explore the metastatic potential of the aforementioned proteins in relation to the epithelial-mesenchymal transition and their possible association with the clinical features of CRC. The present study involved 80 patients diagnosed with CRC. Upon identification of the sociodemographic and clinicopathological features of the participants, immunohistochemical studies were conducted to measure the expression pattern of the S100 proteins in CRC tissues. In addition to qPCR and western blot studies, a series of *in vitro* experiments were conducted in a panel of CRC cell lines to assess the effects of S100 protein expression in cell migration, invasion, and proliferation. The number of CRC patients with high S100A4 expression levels was considerably higher than those with low expression ($p < 0.0001$). S100A4 is positively correlated with TNM staging, nodal metastasis, distant metastasis, and perineural invasion and was statistically significant ($p = 0.02$, 0.01, 0.0001, and 0.02, respectively). *In vitro* studies demonstrated that S100A14 knockdown induced EMT and resulted in a substantial increase in cell proliferation, migration, and invasion in HT29 cells. Moreover, S100A4 knockdown substantially inhibited migration, invasion, and proliferation in LoVo cells. The findings collectively suggest that both S100A4 and S100A14 play a pivotal role in colorectal cancer progression. Overexpression of S100A4 consistently with S100A14 downregulation is associated with the activation of epithelial-mesenchymal transition, which in turn enhances cell proliferation, migration, and invasion.

Keywords: Colorectal cancer, S100 proteins, Epithelial-mesenchymal transition, Migration, Invasion, Proliferation, Metastasis

1. Introduction

The International Agency for Research utilized the GLOBOCAN 2020 model to estimate the global cancer incidence and mortality rates. In 2020 alone, there were an anticipated 19.3 million cases and 10 million cancer-related deaths. The morbidity rate is predicted to reach 28.4 million cases in 2040, a 47% increase from 2020 [1]. Colorectal cancer (CRC) is the third most frequently diagnosed cancer and the second leading cause of cancer-related deaths globally [2]. Despite a thorough understanding of its biology, etiology, and epidemiology, an estimated 1.8 million new cases are diagnosed each year, and 900000 people die as a result of malignancy. By 2030, the CRC burden is anticipated to increase by 60%, reaching 2.2 million new cases and 1.1 million deaths [3]. The majority of CRC cases (about 60%) occur sporadically and are linked to environmental risk factors such as obesity, poor dietary habits, smoking, drinking, and physical inactivity. The remaining percentage is attributed to genetic abnormalities in certain genes, including adenomatous polyposis coli (APC) mutations and BRAF oncogenes [4]. It should be highlighted that the incidence of CRC in populations under the age of 50 appears to be increasing, with an age-standardized rate of 30.8 per 100,000 individuals, particularly in industrialized countries. Furthermore, the burden of the disease is shifting to low- and middle-income countries as they are modernized [5].

Adenocarcinoma, the malignant form of CRC, accounts for the largest percentage (more than 90%) among the other forms. Most tumors develop from two distinct types of precancerous polyps: tubular adenomas and serrated polyps. Adenomas are formed as a result of impaired DNA repair synthesis and changes in the cell proliferation process [6]. The carcinogenesis pathways include chro-

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mosome instability (CIN), microsatellite instability, and serrated neoplasia, although they may overlap at certain points. One of the distinct molecular alterations that is associated with the transformation of normal colonic cells into adenomatous and subsequently malignant cells is the epithelial-mesenchymal transition (EMT) [7]. It involves trans-differentiation of normal non-motile epithelial cells into motile mesenchymal phenotypes that contribute pathologically to fibrosis and cancer progression [8]. The motility of epithelial cells is increased upon losing their junctions and reorganization of their cytoskeleton, eventually changing to the invasive mesenchymal phenotype. Alterations in cell behavior during EMT are orchestrated by various transcription factors, mainly zinc-finger E-box-binding (ZEB) and SNAIL. Moreover, several signaling pathways regulate the gene expression and non-transcriptional changes during EMT, mainly transcription growth factor beta (TGF-β) [9].

One of the important players that contribute to various biological processes in the extracellular matrix is the S100 family of proteins. The 21 members of this family are structurally similar but different in their biological activities. They modulate cellular responses by acting as extracellular factors through binding to a variety of cell surface receptors such as Toll-like receptor 4 (TLR-4), G protein-coupled receptor, and fibroblast growth factor receptor 1 (FGFR1). They also act as Ca2+ sensors, responding to changes in the Ca2+ intracellular levels and extracellular environment [10]. Despite their complex biology and multifunctional signature, their involvement in cancer has been so far well documented. Evidence suggests that altered expression of approximately 10 members of the S100 family substantially contributes to cancer growth, angiogenesis, and metastasis [11, 12].

The present study aimed to investigate the expression pattern of 2 family members of S100 proteins, S100A4 and S100A14, in CRC tissue specimens and cell lines. Moreover, to explore the metastatic potential of the aforementioned proteins in relation to the epithelial-mesenchymal transition and their possible association with the clinical features of CRC.

2. Materials and methods

2.1. Patients and spicemens

With informed consent obtained and approval from the Research Ethics Committee of the College of Medicine at the University of Duhok, a study was conducted involving 80 patients diagnosed with CRC. For this study, patients were selected based on specific inclusion and exclusion criteria to ensure the homogeneity of the study population. The inclusion criteria required that all participants have a confirmed histopathological diagnosis of CRC, be aged between [18-80], and have no prior history of chemotherapy or radiotherapy for CRC. Exclusion criteria included patients with a history of other malignancies, if they refused participation, had incomplete examination data, had unreadable immunohistochemical results, or had autoimmune and inflammatory diseases, and patients who had already undergone surgical or therapeutic interventions for CRC. After surgical resection, tissue specimens were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Four-micrometer-thick tissue sections were stained with hematoxylin and eosin (H&E) for histopathological examination.

2.2. Immunohistochemistry (IHC)

Deparaffinized tissue slides were treated with mouse monoclonal primary antibodies targeting S100A4 and S100A14 proteins (Santa Cruz, USA). A secondary detection system (DAKO, Denmark) using a conjugated polymer was employed to bind the primary antibodies. DAB chromogen was used for permanent color development and microscopic detection. The slides were heated on a hot plate at 60°C for 30 minutes, then immersed in two changes of xylene, followed by three ethanol concentrations. They were rinsed with distilled water to remove ethanol residue. Next, the slides were placed in EDTA buffer pH 9.0 (DAKO) for antigen retrieval and heated in a microwave. After cooling to room temperature, they were rinsed with Tris Buffered Saline (TBS) with Tween 20. The slides were then covered with a peroxidase-blocking solution (DAKO, Denmark) and rinsed again with TBS with Tween 20. Subsequently, 200 µl of the primary antibody (S100A4 at 1:200 and S100A14 at 1:100) was applied, followed by another rinse with TBS with Tween 20. Two drops of the mouse secondary antibody (DAKO, Denmark) were added, and the slides were rinsed once more with TBS with Tween 20. DAB substrate (DAKO) was then applied, and the slides were rinsed, immersed in hematoxylin, and passed through four ethanol concentrations. Finally, the slides were immersed in two changes of xylene and then cover slipped.

2.3. IHC evaluation

S100A14 expression was primarily observed on the cell membrane, while S100A4 expression was found in the nucleus and cytoplasm. Two independent pathologists assessed the protein expression using a semi-quantitative scoring system, which considered both the staining intensity and the proportion of positive cells. Staining intensity was classified as negative (0), weak (1), moderate (2), and strong (3). The percentage of cells with positive staining was categorized into five groups: 0-5% (0), 6-25% (1), 26-50% (2), 51-75% (3), and 76-100% (4). A staining index score, ranging from 0 to 12, was calculated by multiplying the intensity score by the proportion score. For statistical analysis, a final score of 0-6 was considered low protein expression, while a score of 7-12 was considered high expression [13, 14].

2.4. Western blot

Cells were lysed with Laemmli buffer (Thermo Fisher Scientific, USA) and centrifuged at 12,000 g for 10 minutes to collect the protein-containing supernatant. Equal protein amounts from each experimental group were loaded onto SDS-PAGE, transferred to a PVDF membrane (Millipore, MA, USA), and incubated overnight at 4°C with the primary antibodies listed in supplementary Table 1. After washing with PBS, the membranes were incubated with species-specific secondary antibodies conjugated to horseradish peroxidase for 2 hours at room temperature. The membranes were then visualized using an enhanced chemiluminescence kit (Millipore, MA, USA).

2.5. Cell culture

Human colorectal cancer cell lines LoVo, SW620, HT29, and HCT116 were obtained from the American Type Culture Collection (ATCC). These cells were grown in RPMI 1640 medium, which was supplemented

with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cultures were kept in an incubator with saturated humidity at 37°C and 5% CO2. For cell treatment, HT29 cells were initially grown in 60 mm dishes with complete media until they reached 50% confluence. After this, the media was replaced with serum-free media, and the cells were incubated for 24 hours. Subsequently, the dishes were treated with 2 µg/ml of the recombinant S100A4 protein (Biotechn, UK) dissolved in dimethyl sulfoxide (DMSO) at the required concentration for the experiments.

2.6. Transient transfection

To silence the expression of S100A4 and S100A14 in SW640 and HT29 cells, we utilized S100A4 siRNA, S100A14 siRNA, and non-targeting siRNA (Thermo Fisher Scientific, USA). All transfections were conducted using Ingenio® Electroporation solution (MirusBio, USA). Briefly, cells were cultured to 80% confluence, harvested in fresh media, and centrifuged at 1000 rpm for 5 minutes. The cell pellet was then mixed with 60 µl of Ingenio® Electroporation solution. Subsequently, 1 µg/ml of either plasmid DNA or siRNA was added to the mixture. This cell suspension was transferred into a 4 mm cuvette, and electroporation was performed using the Gene Pulser Xcell electroporator (Bio-Rad, USA) set at 250 V and 250 µF. Following electroporation, the transfected cells were resuspended in 1 ml of fresh growth media, maintained under normal growth conditions, and prepared for subsequent experimental procedures.

2.7. MMT assay

To assess the effect of S100A4 and S100A14 expression on CRC cell viability, an MTT colorimetric assay was conducted with SW620 and HT29 cells three days after transfection. The cells were plated in 96-well plates at a density of 2000 cells per well and incubated at 37°C for 1-3 days. Each day, 20 µl of a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Thermo Fisher Scientific, USA) was added to each well and incubated for 4 hours. After incubation, an acidic isopropanol solution (containing 10% SDS, 5% isopropanol, and 0.01 M HCl) was added (100 µl per well) and left overnight at 37°C. The optical density (OD) was measured at 570 nm using a microplate reader (Bio-Rad, USA).

2.8. Wound-healing assay

The migration of SW6200 and HT29 cells, after transfection with siRNA, was assessed using a wound-healing assay. Cells were plated at a density of 1.5×10^6 cells per well in 6-well plates and allowed to grow overnight until they reached 90% confluence. A straight scratch was made using a sterile pipette tip to create a wound. After gently washing away detached cells with PBS three times, the cells were cultured in medium for an additional 24 hours. The migration of cells into the wound area was observed and documented using a digital camera (Leica, Germany) at both the beginning (0 hours) and after 24 hours.

2.9. Transwell migration and invasion assay

We performed transwell migration and invasion assays using a 24-well transwell system with 8 µm pore size (Corning, USA). In the migration assay, 5×10^4 cells were suspended in 300 µl of serum-free medium and added to the upper chamber. The lower chamber was filled with 800 µl of medium containing 10% FBS. After incubating for 48 hours, the chambers were fixed with 4% paraformaldehyde, and stained with 0.5% crystal violet, and cells from the upper chamber were removed with a cotton swab. Cells in four randomly selected microscopic fields were counted and photographed. For the invasion assay, the protocol was similar to the migration assay, except that 1×10^5 cells were seeded into the upper chamber pre-coated with matrigel (BD Biosciences, USA). Each experiment was conducted in triplicate.

2.10. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells were subjected to RNA extraction using an RNeasy RNA isolation kit (Germantown, MD, USA), followed by reverse transcription into cDNA with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Quantitative PCR was performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) for 40 cycles at an annealing/extension temperature of 60 °C. GAPDH served as an internal control for normalization, and the relative expression of mRNA levels was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences used in this study are provided in Supplementary Table 2.

2.11. Kaplan-Meier plotter analysis

We utilized the Kaplan-Meier plotter (https://kmplot.com/) to validate the prognostic role of S100A4 and S100A14 in CRC. By analyzing the correlation between S100A4 and S100A14 expression levels and key survival metrics— progression-free survival (PFS), overall survival (OS) and post-progression survival (PPS).

2.12. Statistical analysis

All in vitro experiments were conducted at least twice to ensure consistency. Data comparisons between study groups were performed using one-way ANOVA and t-test with GraphPad Prism 7.1 software. For clinical data interpretation, all statistical analyses were done using Statistical package for Social Sciences version 26.0 (SPSS, Chicago, Illinois, USA). Spearman's correlation coefficient was used to determine the association between S100 protein expression and the clinicopathological features of CRC patients. The p values < 0.05 were considered significant and highly significant respectively.

3. Results

3.1. Clinicopathological features of CRC patients

The demographic parameters and the clinicopathological features are illustrated in Table 1. The majority of the 80 obtained specimens were over 50 years old and male (No 48, 55%). A higher number of patients had tumor grade 2 and TNM stages 2 and 3. Precisely, 40% tested positive for nodal metastasis, 61.25% for vascular invasion, and 48.75% for perineural infiltration.

3.2. Expression of S100A4 and S100A14 in CRC tissues

Immunohistochemistry was used to assess the expression pattern of S100A4 and S100A14 in CRC tissue specimens. The proteins were classified as low or high expres-

Table 1. Demographic data and the clinicopathological features of CRC patients.

Clinicopathological features		Number	Percent (%)
Age (Years)	=<50	32	60
	>50	48	40
Sex	Male	44	55
	Female	36	45
Histological type	Adenocarcinoma	66	82.5
	Mucinous Adenocarcinoma	14	17.5
Tumor grade	1	7	8.75
	2	56	70
	3	17	21.25
TNM stage	1	13	16.25
	2	30	37.5
	3	31	38.75
	4	6	7.5
Nodal metastasis (N)	Negative	44	55
	One LN involved	19	23.75
	Two or more LNs involved	17	21.25
Distant metastasis (M)	Negative	72	90
	Positive	8	10
Vascular invasion	Negative	31	38.75
	Positive	49	61.25
Perineural invasion	Negative	41	51.25
	Positive	39	48.75

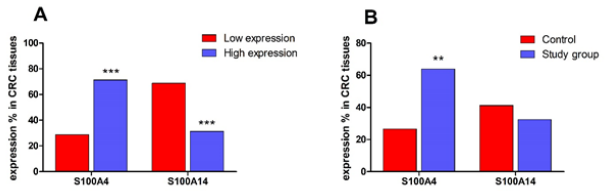

Fig. 1. The expression pattern of S100A4 and S100A14 in CRC tissues. A: the number of percentages of CRC tissue specimens with high (blue) and low (red) expression. B: the number and percentages of specimens between the control (negative for angiolymphatic and perineural invasion) and the study group (positive for angiolymphatic and perineural invasion). Results are expressed as percentages. ** p value ≤ 0.01, *** p-value ≤ 0.001.

sion. The number of patients with high S100A4 expression levels (n=57, 71.25%) was considerably higher than those with low expression (n=23, 28.75%) and was statistically significant ($p < 0.0001$). Contrariwise, the S100A14's high expression number and percentage were significantly lower compared to the number of samples with low expression ($p = 0.001$) (Figure 1A, and Figure 2).

To further illustrate the expression pattern of S100 proteins in metastatic specimens, patients were divided into two groups; the first control category includes specimens negative for angiolymphatic and perineural invasion (n=34), and the second study group includes positive for both parameters (n=46). Data showed that S100A4 was statistically substantially higher in the study group (n=23, 63.88%) compared to control (n=9, 26.47%) ($p = 0.01$). In contrast, the number and percentage of samples with high S100A14 expression was lower in the study group (n=14, 41.17%) compared to control (n=11, 32.36%) but didn't show any significant difference (Figure 1B).

3.3. S100A4 expression is associated with the clinical outcome of CRC

Following the confirmation of its expression in CRC tissues, it was crucial to study the association between S100 proteins and CRC-specific clinical characteristics. S100A4 was found to be positively correlated with TNM staging, nodal metastasis, distant metastasis, and perineural invasion and was statistically significant (p= 0.02, 0.01, 0.0001, and 0.02 respectively) (Table 2). With the exception of nodal metastasis, no significant association between S100A14 and the aforementioned characteristics was detected.

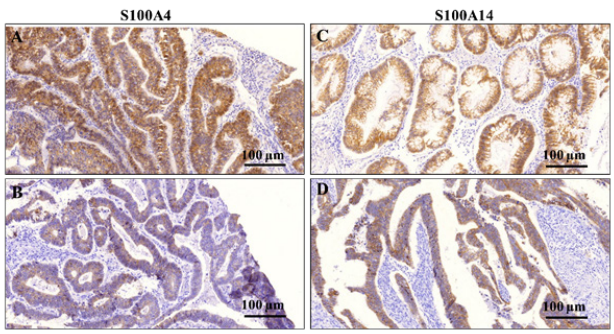

Fig. 2. The expression of S100A4 and S100A14 in CRC tissues. **(A).** High nuclear and cytoplasmic expression of S100A4 detected in advanced stages of CRC. **(B).** Low expression of S100A4 in the early stages of the disease. In contrast, **(C).** High expression of S100A14 was observed in the early stages, whereas **(D).** Low expression was noted in advanced stages of CRC. Scale bar is 100 µm.

Table 2. Correlation between the clinicopathological features of the MCPs expression.

Parameters	S100A4 expression	S100A14 expression
TNM stage	0.02	0.06
Nodal metastasis (N)	0.01	0.04
Distant metastasis (M)	0.0001	0.1
Vascular invasion	0.33	0.72
Perineural infiltration	0.02	0.08

3.4. Expression of S100 family proteins in CRC cell lines

The expression of S100 proteins at mRNA and protein levels was determined using qPCR and western blot in a series of CRC cell lines ranging from more aggressive to less (LoVo, SW620, HCT116, and HT29). The mRNA levels of mesenchymal markers S100A4 and S100A6 were significantly higher in aggressive cell lines (LoVo and SW620) than in less aggressive cells (HCT116 and HT29). In contrast, HCT116 and HT29 cells showed high levels of epithelial S100A14. Confirmed by the protein levels, western blot analysis revealed the same trend as qPCR with up-regulated expression levels of mesenchymal proteins in more aggressive cell lines and elevated levels of epithelial proteins in less aggressive cells (Figure 3).

3.5. S100A14 knockdown increases HT29 cells migration, invasion, and proliferation in vitro

To investigate the in vitro effects of S10014 on proliferation, migration, and invasion, S100A14 was silenced in HT29 cells. For that purpose, HT29 cells were transfected with S100A14 siRNA, while non-targeting siRNA was used as a control. The silencing effect was successful and was confirmed by the western blot compared to the tubulin control (Figure 4E). The obtained data demonstrated that S100A14 knockdown resulted in a substantial increase in cell migration (Figure 4A and 4C), invasion (Figure 4B) and proliferation (Figure 4D) in HT29 cells when compared to non-targeting siRNA control.

3.6. S100A14 knockdown induced EMT in HT29 cells

To further validate the outcomes of S100A14 knockdown and its association with EMT induction, the expression of various epithelial and mesenchymal markers was evaluated using western blot. Based on the morphological examination, knocking down S100A14 caused HT29 cells to transition from epithelial to mesenchymal phenotype (Figure 5A). Consistently, the expression levels of mesenchymal markers (S100A4, S100A6, Vimentin, and N-cadherin) were significantly up-regulated in the S100A14 siRNA group. However, the epithelial markers (S100A14 and E-cadherin), however, were considerably down-regulated compared to non-targeting control (Figure 5B).

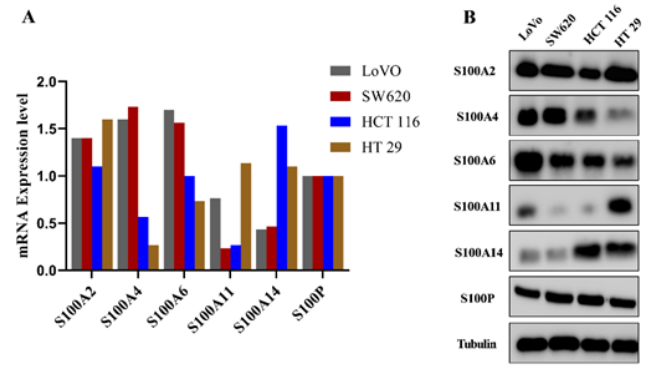

Fig. 3. Expression pattern of S100 proteins in CRC cell lines. (A) The bar graph represents the relative mRNA expression levels of S100 family members across various CRC cell lines, as determined by real-time PCR. The mRNA levels were normalized to the control gene GAPDH. **(B)** Western blot analysis was performed to validate the expression of S100 family members in CRC cell lines. The results shown are from three representative experiments.

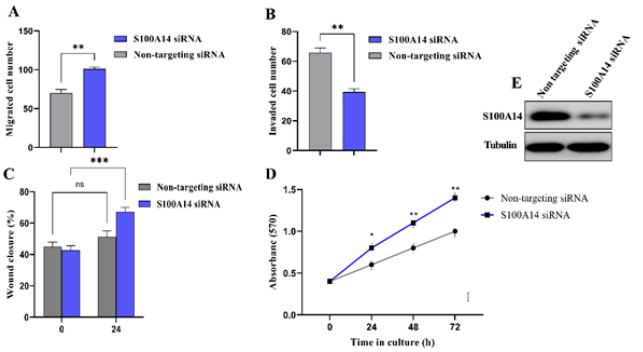

Fig. 4. Downregulation of S100A14 enhances HT29 cell migration, invasion and proliferation in HT29 cell lines. HT29 cells were transiently transfected with either S100A14 siRNA or Non-targeting siRNA as a control for migration, invasion, and proliferation experiments. **(A-C)** The transwell cell migration and invasion assays as well as Wound-healing assay demonstrated that knockdown of S100A14 significantly increased HT29 cell migration and invasion. **(D)**. Proliferation of transfected cells was measured using the MTT assay, with absorbance read at 570 nm. **(E).** Knockdown of S100A14 was confirmed by Western blot analysis, showing decreased protein expression in cells transfected with S100A14-targeted siRNA. Data are presented as the standard error of the mean (*P < 0.03, **P < 0.01, ***P < 0.001). Results from three representative experiments are shown.

3.7. Knockdown of S100A4 inhibits LoVo cells migration, invasion and proliferation

To further understand its significance as a mesenchymal marker, S100A4 was knocked down using the most aggressive CRC cell line (LoVo). The cells were transfected with the S100A4 siRNA and a non-targeting siRNA for control. Western blot analysis was performed to confirm the findings (Figure 6E), which showed significant down-regulation in S100A4 expression levels following the knockdown. After silencing, the migration (6A and C) and invasive (6B) abilities of LoVo cells were substantially inhibited compared to the non-targeting siRNA control. The same pattern was detected for cell priliferation after 72 hours of incubation (6D).

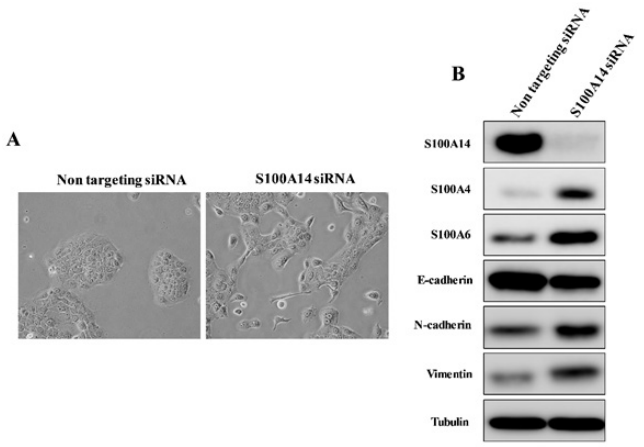

Fig. 5. Knockdown of S100A14 induces EMT and alters cell morphology from epithelial to mesenchymal phenotype. (**A).** Phase-contrast images of HT29 epithelial cells transfected with either S100A14-targeting or control siRNAs. Knockdown of S100A14 results in cell scattering. **(B).** S100A14 knockdown increases the expression levels of mesenchymal S100 proteins (S100A4 and S100A6) and canonical EMT markers, including N-cadherin and vimentin.

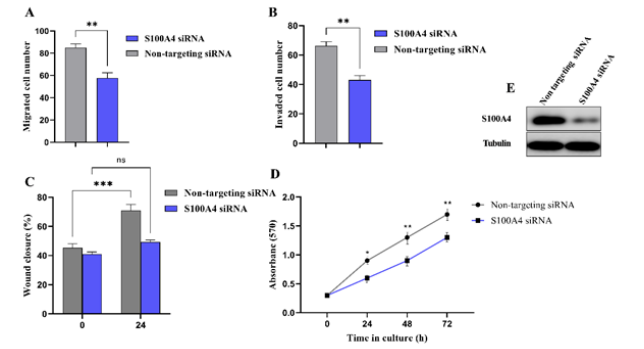

Fig. 6. Downregulation of S100A4 decreased cell migration, invasion and proliferation in LoVo cell lines. LoVo cells were transiently transfected with either S100A4 siRNA or Non-targeting siRNA as a control for migration, invasion, and proliferation experiments. **(A-C)** The transwell cell migration and invasion assays as well as Wound-healing assay demonstrated that knockdown of S100A4 significantly decreased **LoVo** cell migration and invasion. (**D**). Proliferation of transfected cells was measured using the MTT assay, with absorbance read at 570 nm. (**E).** Knockdown of S100A4 was confirmed by Western blot analysis, showing decreased protein expression in cells transfected with S100A4-targeted siRNA. Data are presented as the standard error of the mean (*P < 0.03, **P < 0.01, ***P < 0.001). Results from three representative experiments are shown.

3.8. S100A4 expression activates various signaling pathways

We further investigated the role of S100A4 in cancer progression by examining its effect on the activation of several signaling pathways involved in this process. For this purpose, cells were treated with recombinant human S100A4 protein (2 µg/ml) for 12 hours. The activation of signaling pathways, including p-ERK (MAPK), p-SMAD (TGF-β), p-P65 (NF-kB), p-STAT3 (STAT3/JAK), Cleaved Notch1 (Notch), and p-LRP6 (Wnt/β-catenin), was assessed by Western blotting. We observed that S100A4-treated cells exhibited activation of the MAPK, NF-kB, and STAT3 signaling pathways, as indicated by the increased expression levels of phosphorylated proteins corresponding to each pathway (Figure 7).

3.9. S100A4 expression is associated with unfavorable outcomes

The Kaplan–Meier analysis of PFS, OS, PPS in all CRC patients revealed that those with high S100A4 expression experienced significantly worse outcomes compared to those with low S100A4 expression (Figures 3A–C). In contrast, PPS analysis indicated that patients with low S100A14 expression had a more favorable prognosis than those with high S100A14 expression (Figure 3F). These findings suggest that S100A4 is upregulated, while S100A14 is downregulated in CRC tumor tissues, and the differential expression of S100A4 and S100A14 may be

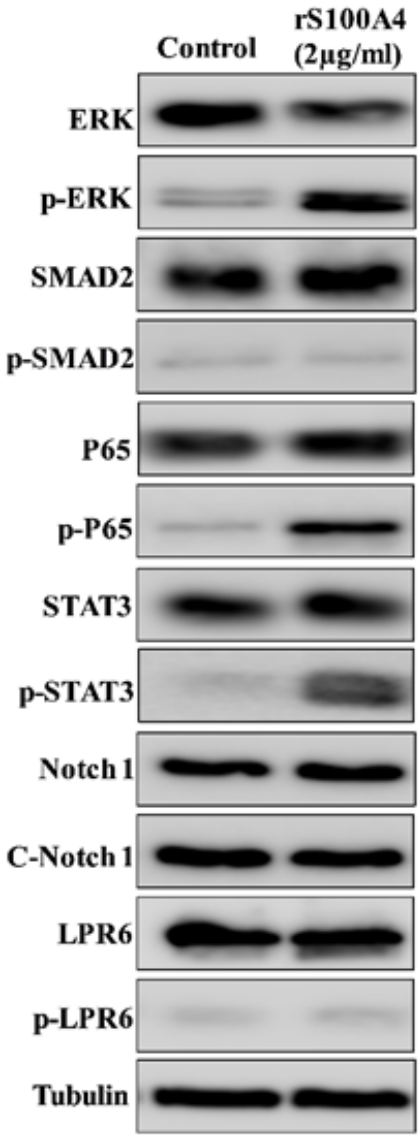

Fig. 7. Western Blot analysis of various signaling proteins in response to rS100A4 treatment. Proteins were extracted from cells treated with rS100A4 (2 µg/ml) for 12h and compared with untreated control cells. The figure shows the expression levels of total and phosphorylated forms of the following proteins: ERK, p-ERK, SMAD2, p-SMAD2, P65, p-P65, STAT3, p-STAT3, Notch1, cleaved Notch1 (C-Notch1), LPR6, p-LPR6, and Tubulin (loading control). Treatment with rS100A4 resulted in differential expression and phosphorylation of these proteins, indicating activation or repression of specific signaling pathways.

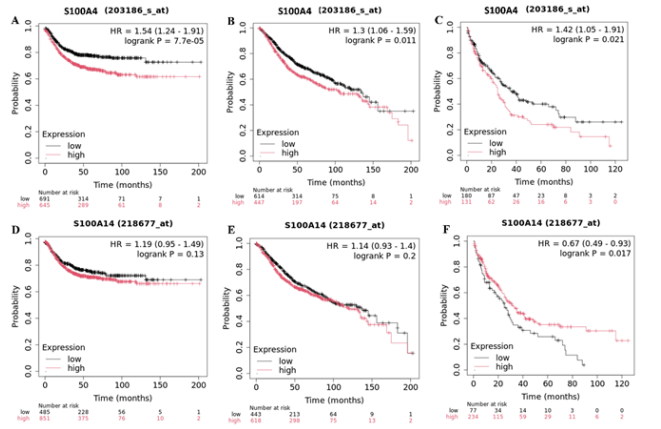

Fig. 8. The prognostic role of the S100A4 and S100A14 gene (Kaplan-Meier plotter). (A, B, C) High S100A4 expression was correlated with poor prognosis of PFS, OS and PPS. **(D, E, F)** represent curve analyses illustrating the correlation between S100A14 expression and key survival metrics.

associated with poor CRC prognosis (Figure 8).

4. Discussion

The superfamily of S100 proteins is implicated in various intracellular and extracellular pathway activities. The 22 currently known members are involved in regulating a diverse range of cellular processes, most notably muscle contraction, local inflammation, cell differentiation, and cell death. Despite their exclusive expression in vertebrates, each member has a unique expression pattern in different cells and tissue types [15]. In addition to their role in maintaining homeostasis, these proteins play a pivotal role in the pathogenesis of many inflammatory diseases, such as rheumatoid arthritis and atherosclerosis. The proteins already expressed in immune cells, such as neutrophils and macrophages, are released into extracellular space upon cell damage and act as DAMPs (pathogen associated molecular patterns), thereby activating the inflammatory immune response by binding to toll-like receptors. This leads to activation of a variety of inflammatory signaling pathways, including TNF-α, IL-1β, and IL-6 [16]. However, the present study aims to investigate the expression pattern of certain S100 proteins (S100A4 and S100A14) in CRC tissues and to evaluate their emerging role in CRC cancer progression in both CRC tissues and cell lines by exploring their association with cell migration, invasion, and proliferation.

Firstly, we investigated the expression pattern of S100A4 and S100A14 in a sample of 80 CRC tissue specimens. Results indicated that the high expression percentage of the mesenchymal S100A4 was statistically significantly higher compared to low expression. Furthermore, the expression percentage of the epithelial S100A14 was substantially lower (Table 2). Consistently, the percentage of samples with S100A4 high expression was significantly higher in CRC tissues with angiolymphatic and perineural infiltration, whereas the situation was opposite for S100A14. Moreover, S100A4 expression was positively correlated with the TNM staging, grade, nodal metastasis, distant metastasis, and perineural invasion. Our findings are consistent with previous literature indicating that the S100A4 overexpression appears to correlate with CRC progression and poor prognosis [17]. Other studies reported that the S100A4 overexpression is correlated with the clinicopathological features of CRC (invasion and nodal metastasis), disease-free survival, and overall survival rate, which might be utilized as a valuable biomarker, especially for predicting poor prognosis [18-20].

The second part of the present study included a series of in vitro experiments against a panel of CRC cell lines. The mRNA levels of mesenchymal S100A4 and S100A6 were overexpressed in aggressive LoVo and SW620 cell lines compared to less aggressive HCT116 and HT29 cells. Contrariwise, the epithelial S100A14 was highly expressed in HCT116 and HT29 cells compared to controls. The findings were further confirmed using western blot, where the protein levels displayed the same expression pattern as mRNA levels. Accordingly, both LoVo and HT29 cell lines were considered for further functional experiments.

To explore the precise contribution of S100A4 and S100A14 in cancer progression, a set of knockdown experiments were conducted. Based on the obtained data, S100A14 knockdown increased HT29 cell migration and proliferation. Furthermore, the siRNA-mediated S100A14 knockdown resulted in morphological EMT change in which cells differentiated from epithelial to mesenchymal phenotype, accompanied by increased expression levels of mesenchymal S100 proteins. Concerning S100A4, its knockdown inhibited migration, invasion, and proliferation in LoVo cells. These changes were consistent with activation of various signaling pathways confirmed by western blot, including p-ERK, P65, p-STAT3, STAT3, and SMAD2. Taking findings collectively suggests that the S100A4 protein is greatly involved in the metastatic cascade of CRC, particularly proliferation, migration, and invasion via activating epithelial-mesenchymal transition (EMT). A previously published study by our group highlighted the role of EMT in CRC cancer progression [21]. It was shown that the EMT is activated by matricellular proteins, mainly CYR61, which activates mesenchymal markers ZEB1 and N-cadherin, subsequently enhancing metastasis. A study exploring the correlation between S100A4 overexpression and the clinicopathological features of hepatocellular carcinoma (HCC) demonstrated that the expression of EMT-inducers (S100A4 and vimentin) and low E-cadherin levels correlate with the malignant phenotype of HCC [22]. S100A4 was found to mediate metastasis in gastric cancer. It was concluded that overexpression in the metastatic form correlated with a poor prognosis. It induced EMT through TGF-β stimulation [23]. Moreover, elevated S100A4 expression and loss of E-cadherin are associated with EMT induction and regarded as unfavorable prognostic features in patients with uterine cervical carcinoma [24].

Numerous studies have linked S100A4 upregulation to cancer progression and metastasis owing to its diverse biological functions such as regulation of angiogenesis, cell motility, survival, and invasion. Therefore, it is important to highlight the precise molecular mechanism underlying the metastatic potential of this protein. A large number of experimental studies have linked the S100A4 gene to developing of the metastatic phenotype of cancer cells [25]. A study aimed to identify the region in S100A4 responsible for metastasis-inducing properties concluded that deletion in the last 15 amino acid residues substantially reduced cell motility and invasion [26]. Once released into the extracellular space, S100A4 has the potential to

interact with the cytoskeletal components of the motility, mainly the nonmuscle myosin heavy chain AII (MHC-IIA), disrupting myosin self-assembly and inhibiting protein kinase C, thus promoting cell motility [27]. Additional S100A4-interacting proteins were reported by other studies, including tropomyosin, CCN3 (cysteine-rich 61/connective tissue growth factor), and methionine amino peptidase [28]. A retrospective study investigated the association between S100A4 gene expression and the clinical features; mutational profiles of 227 acute myeloid leukemia (AML) patients revealed that S100A4 overexpression is associated with poor clinical outcomes. Moreover, S100A4 overexpressed cells were enriched with interferon and epithelial-mesenchymal transition pathways [29].

After validating the critical role of S100A4 in metastasis and inducing the EMT, the last part of the current project explored the therapeutic potential of S100A4. As indicated in our findings, the S100A4 knockdown substantially inhibited migration, invasion, and proliferation in the aggressive LoVo cells. These findings are consistent with previous literature indicating that blocking S100A4 might be considered as a therapeutic target. A study examined the effects of microRNA-323-3p (miR-323-3p) on bone metastasis in CRC and revealed that targeting S100A4 by the aforementioned miR inhibited the osteoclastogenesis in osteoclast precursors, which substantially attenuated bone resorption in bone metastasis in CRC [30]. Single-cell analysis of 201,986 glioma, immune, and other stromal cells was conducted to identify prognostic indicators of glioma patient survival. It was shown that targeting S100A4 in non-cancer cells is sufficient to reprogram the immune landscape and improve overall survival [31]. An experimental study used a novel blocker (proteolytic targeting chimera) to selectively eliminate S100A4 in triple-negative breast cancer cells (TNBC) in rats and concluded that destructive targeting of S100A4 significantly inhibited metastasis [32]. Furthermore, antibodies blocking S100A4 can suppress metastasis in a breast cancer model, indicating the paracrine mechanism of the aforementioned protein that involves modulation of T-cell infiltration in the primary tumor and the pre-metastatic niche [33].

While this study provides valuable insights into the roles of S100A4 and S100A14 in colorectal cancer, several limitations should be acknowledged. First, the relatively small sample size may limit the generalizability of our findings to a broader population. Additionally, the study's cross-sectional design restricts our ability to draw causal inferences regarding the relationship between S100A4 and S100A14 expression and clinical outcomes. Furthermore, the focus on specific stages of colorectal cancer may overlook potential variations in these biomarkers in earlier or later stages of the disease. Future studies with larger, more diverse cohorts and longitudinal designs are necessary to validate our results and explore the full clinical implications of targeting S100A4 and S100A14 in CRC treatment.

Our findings highlight the potential clinical relevance of S100A4 and S100A14 as biomarkers and therapeutic targets in CRC. The overexpression of these proteins may be linked to tumor progression, migration, and invasion, suggesting that targeting S100A4 and S100A14 could hinder CRC metastasis and improve patient outcomes. Future therapies might focus on inhibiting the activity or expression of these proteins, possibly in combination with existing treatments, to enhance therapeutic efficacy. Additionally, to strengthen the clinical applicability of these findings, further validation in a larger, more diverse cohort is essential. Such studies would help determine the generalizability of S100A4 and S100A14 as universal markers for CRC progression and therapeutic intervention points.

5. Conclusion

In conclusion, the findings gained by the present study collectively suggest that both S100A4 and S100A14 play a pivotal role in colorectal cancer progression. Overexpression of the mesenchymal S100A4 consistently with downregulation of the epithelial S100A14 in both CRC tissue specimens and cell lines is associated with the activation of epithelial-mesenchymal transition, which in turn enhances cell proliferation, migration, and invasion. Furthermore, substantial inhibition of the metastatic cascade upon S100A4 silencing provides clear evidence concerning the therapeutic potential of the aforementioned protein, particularly in metastatic forms of CRC. Future directions should focus on targeting such proteins that are highly involved in the metastatic form of CRC to minimize the mortality rate and improve the overall survival rates and patient outcomes.

Conflict of Interests

The author have no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

Data, specimens, and CRC patients were used in the curret study which was approved by the Research Ethics Committee of the College of Medicine at the University of Duhok (Reference number: 13072021-7-18 on 13th of July 2021).

Informed Consent

Patients were recruited for the current study upon their consent.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Hazhmat Ali and Qais Al Ismaeel: Research design and supervision

Shelan Rasool, Hanaa AL-Mahmoodi, Mayada Yalda, Hishyar Najeeb and Zihel Hussein: performed laboratory experiments

Shelan Rasool, Hazhmat Ali, and Qais Al Ismaeel 1: wrote the draft

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