Inhibition of Expression of the S100A8 Gene Encoding the S100 Calcium-Binding Protein A8 Promotes Apoptosis by Suppressing the Phosphorylation of Protein Kinase B (Akt) in Endometrial Carcinoma and HEC-1A Cells

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Source of support: The study was supported by the Science Foundation of the Gansu Province, China (Nos. 1606RJZA123 and 1606RJZA133) and the Science Foundation of Chengguan District of Lanzhou City, Gansu, China (No. 054000046)

Background: The aim of this study was to investigate the expression and silencing of the S100A8 gene, which encodes the S100 calcium-binding protein A8 (S100A8), and apoptosis and phosphorylation of protein kinase B (Akt) in tissue samples of endometrial carcinoma and HEC-1A endometrial adenocarcinoma cells in vitro.

Material/Methods: Immunohistochemistry (IHC) was used to detect expression of the S100A8 protein in 74 tissue samples of endometrial cancer and 22 normal endometrial tissue samples. A stable S100A8 gene knockdown cell line was constructed using lentiviral packing short hairpin RNA (shRNA) transfected into HEC-1A cells. S100A8 mRNA and S100A8 protein levels were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting. The effects of expression of the S100A8 gene by endometrial cancer cells was investigated by the MTT assay, cell cycle and apoptotic assays, qRT-PCR, and Western blotting.

Results: IHC showed high levels of expression of S100A8 protein in endometrial carcinoma tissues, and HEC-1A adenocarcinoma cells (in G1 and G2). Increased expression of S100A8 protein was found endometrial cancer tissues compared with normal endometrial tissues (79.7% vs. 4.5%). S100A8 gene knockdown reduced cell proliferation in the HEC-1A cells compared with control cells, induced cell apoptosis, inhibited the phosphorylation of protein kinase B (Akt), and induced the expression of pro-apoptotic genes, including the cytochrome C gene, CYCS, BAD, BAX, FOXO1, FOXO3, CASP9, and CASP3.

Conclusions: In endometrial carcinoma cells, down-regulation of the S100A8 gene induced cell apoptosis via inhibition of the phosphorylated or active form of protein kinase B (Akt).

MeSH Keywords: Apoptosis • Calgranulin A • Endometrial Neoplasms

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/908895
Background

In developed countries, endometrial cancer is the most common form of gynecological cancer [1]. Despite its prevalence, the molecular mechanisms of endometrial carcinogenesis remain poorly understood. Inflammation has a role in endometrial carcinogenesis, as elevated levels of the inflammatory markers C-reactive protein (CRP), interleukin-6 (IL6), and interleukin-1 receptor-α (IL1-Rα) have been shown to be associated with an increased risk of endometrial cancer [2]. Therefore, there may be a relationship between inflammation and the development of endometrial cancer.

The S100 calcium-binding protein A8 (S100A8), which is encoded by the S100A8 gene, has recently attracted research interest because of the association between the expression of S100A8 with human diseases, including cancer, acute and chronic inflammatory conditions, autoimmune diseases, cardiomyopathies, atherosclerosis, and neurodegenerative diseases [3]. S100A8 belongs to the family of low molecular weight (LMW) S100 proteins, with a molecular weight of 10–13 kDa. The known S100 proteins include 22 members and represent the largest subfamily of the EF-hand Ca²⁺-binding proteins (EFCaBPs) [4]. A recently reported study showed that S100A8 and S100A9 could self-assemble into highly heterogeneous amyloid complexes that included oligomeric species and fibrils found in the aging prostate [5]. S100A8 and S100A9 have been shown to be highly expressed in acute and chronic inflammatory conditions, and in several cell types, indicating that one or both proteins may be inflammatory mediators [6]. However, the expression of S100A8 has been shown to be induced by lipopolysaccharide (LPS), fibroblast growth factor-2 (FGF-2), and interleukin-1 beta (IL1β) in murine fibroblasts, or in ultraviolet A (UVA) irradiated skin of mice, but S100A9 was not expressed [6]. Also, increased expression levels of the S100A8 and S100A9 proteins were found in human cancer cells, including breast, lung, gastric, colorectal, pancreatic and prostate cancer [7].

Heterodimers or homodimers of intracellular S100A8 and S100A9 have been shown to have paracrine functions by interacting with the receptor for advanced glycation end products (RAGE) and Toll-like receptor 4 (TLR4) on tumor cells [8]. Extracellular S100A8 and S100A9 have also been shown to contribute to tumor cell invasion, release of tumor necrosis factor-α (TNF-α), vascular endothelial growth factor A (VEGFA) and transforming growth factor-β (TGF-β) and to promote tumor expression of S100A8 and/or S100A9 in lung-associated myeloid cells and in pre-invasive lung lesions [9]. Although the detailed roles of S100A8 in the pathogenesis or progression of human cancer remains a controversial issue, there is increasing published evidence to support the possible role for S100A8 inflammation-associated human neoplasia [10,11].

The S100A8 and S100A9 proteins are considered to be both pro-inflammatory and anti-inflammatory mediators, but their functions might be dependent on the type of cells these proteins interact with and might be context and concentration-dependent [12]. The particular roles for the expression of the S100A8 protein and effects of the S100A8 gene in human cancer, particularly in endometrial cancer, remain poorly understood.

The aim of this study was to investigate the effects of the expression and silencing of the S100A8 gene that encodes the S100 calcium-binding protein A8 (S100A8) on apoptosis and phosphorylation of protein kinase B (Akt) in tissue samples of endometrial carcinoma and in HEC-1A endometrial adenocarcinoma cells in vitro.

Material and Methods

Study samples

There were 74 tissue samples containing endometrial cancer, and 22 normal endometrial tissue samples, which were provided by The First Hospital of Lanzhou University, between December 2015 to November 2016. The age of the patients (mean ±SD) was 55.7±5.9 years (range, 44–65 years). The histopathological diagnosis of the endometrial carcinomas, including the tumor grade, was verified by a pathologist and was according to the current World Health Organization (WHO) system. The 2008 International Federation of Gynecology and Obstetrics (FIGO) staging system was used for confirmed cases of primary endometrial cancer. The study design was approved by the Ethical Committee of the First Hospital of Lanzhou University. All participants signed a written informed consent to participate in the study.

Immunohistochemical (IHC) tissue staining for S100A8 protein expression

Endometrial tissue samples, which had been stored as paraffin wax-embedded tissue blocks, were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by incubating the tissue sections in 0.3% H₂O₂ for 5 min. The sections were incubated with primary antibodies at 4°C in a humid chamber overnight. The primary antibody against the S100 calcium-binding protein A8 (S100A8) (Abcam) (Catalog No: EPR3554) was applied at a dilution of 1: 200. Indirect immunohistochemistry (IHC) was performed using the avidin-biotin-peroxidase complex (ABC) method and using a Vectastain Elite ABC kit (Fukasco Xi. Co., Ltd., Tokyo, Japan). Sections were counterstained with hematoxylin, dehydrated in graded ethanol, dried, mounted, and coverslipped. Confirmation of the diagnosis of primary endometrial
adenocarcinoma was identified using a standard IHC diagnostic panel that included positive immunostaining with antibodies to p53, PR, PTEN and other markers.

Cell culture

The endometrial adenocarcinoma cell line, HEC-1A (BNCC338711), was purchased from BeNa Culture Collection (Beijing, China). HEC-1A cells were cultured in 90% McCoy’s 5a medium and 10% fetal bovine serum (FBS), in an incubator with 5% CO₂ at 37°C and 95% humidity.

Vector construction

Four short hairpin RNA (shRNA) oligonucleotides targeting the S100A8 gene were designed and inserted into the lentiviral vector pGMVL-SC5 at the sites of BamHI and EcoRI. Short hairpin RNA (shRNA) oligonucleotides and lentiviral vector pGMVL-SC5 were obtained from Genomeditech Co., Ltd. (Shanghai, China). The sequences used are shown in Supplementary Table 1.

Cell transfection and selection of stable cells

Several vectors were transfected with the Lipofectamine™ 3000 Reagent (Invitrogen, Foster City, CA, USA) according to the manufacturer’s protocol. Then, 4 hours later, the normal medium was used to culture cells for 48 hours. The HEC-1A cells with pGMVL-SC5 were maintained in a puromycin (0.4 mg/ml) and G418 (1 g/ml) solution. Colonies were selected, expanded, and screened using flow cytometry with the identification of cells containing 5% CO₂ at 37°C and 95% humidity.

Quantitative real-time PCR (qRT-PCR)

Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) was used to extract total RNA, which was then synthesized to cDNA using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The SYBR Green 1 (SG1) detection kit (Applied Biosystems, Foster City, CA, USA) was used to quantify the expression of the S100A8 gene. The sequences of the primers used in the reaction are shown in Supplementary Table 2. The relative expression was measured with the 2⁻ΔΔCT method.

MTT cell viability assay and cell counting kit-8 (CCK-8) cell proliferation assay

A density of 2.0×10⁴ HEC-1A cells were seeded in 96 well plates for 24 hours, 48 hours, and 72 hours, respectively. The cell proliferation assay was performed using the cell counting kit-8 (CCK-8) assay (Dojindo, Japan) according to the manufacturer’s instructions. The absorbance was recorded at 490 nm using a spectrophotometer.

Western blotting

Cells were prepared in a 6-well plate containing 1×10⁶ cells per well, and cultured for 3 days. Then, cells were centrifuged at 4°C for 10min at 2,000 rpm, and the supernatant was collected. After measurement of the protein concentration using bicinchoninic acid (BCA), protein electrophoresis was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The polyvinylidene fluoride (PVDF) membrane was washed three times with Tris-buffered saline (TBS) containing 20% Tween 20 (TBST) for 5 min each time and then blocked with dried skimmed milk powder at 4°C overnight.

The primary antibodies used were to S100A8 (Abcam, EPR3554), β-actin (Abcam, ab8227), AKT1 (phosphor 473) (Abcam, EP2109Y), cleaved caspase-3 (Asp175) (Cell Signaling, #9661), protein kinase B (Akt) (pan)(Cell Signaling, #4691), caspase-3 (Bioss, bs-0081R) and GAPDH (Bioss, bsm-0978M). Following incubation with 4 ml of primary antibody in diluent, at room temperature for 2 hours, the membrane was washed four times with TBST, and then incubated with the secondary antibody for 1 hour, and washed four times with TBST. After between 1–2 minutes, enhanced chemiluminescence (ECL) developing solution (GE Healthcare, Amersham, UK) was added to the PVDF membranes. Samples were stained and photographed.

Cell cycle analysis by fluorescence-activated cell sorting (FACS)

HEC-1A cells were seeded in a 6-well plate at a density of 2×10⁴/well. The cells were then detached using a 0.25% trypsin solution (Gibco, USA), washed twice with PBS, and fixed in 70% ethanol at –20°C overnight. After washing with PBS, cells were incubated with propidium iodide (PI) (50 µg/ml) (Sigma, USA) and RNase for 30 min. Cellular DNA content was analyzed using a Becton Dickinson FACS Calibur™ analyzer. The cell number in each phase of the cell cycle was verified using FlowJo software (Treestar Inc., USA).

Detection of apoptotic cells using flow cytometry

After incubation for 72 h, HEC-1A cell apoptosis was measured in the parent cells (Control) empty vector (NC), and the short hairpin sh–S100A8 transfected, or S100A8 knocked down cells. Firstly, the cells were stained with Annexin V- fluorescein isothiocyanate (FITC) and PI for 20 min at room temperature. The cells were analyzed using a Becton Dickinson FACS Calibur™ analyzer, according to the instruction of the Annexin V-FITC apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China).
Results

The S100 calcium-binding protein A8 (S100A8) was highly expressed in endometrial cancer tissues

Expression of the S100 calcium-binding protein A8 (S100A8) in tissues containing endometrial cancer was analyzed using immunohistochemistry (IHC) on normal endometrial tissue and tissue containing endometrial adenocarcinoma. No positive immunostaining for S100A8 was found in normal endometrial tissues (proliferative phase and secretory phase) (Figure 1A, 1B). However, strong positive S100A8 immunostaining was seen in well differentiated (Grade 1) endometrial adenocarcinoma (Figure 1C), in moderately differentiated (Grade 2) endometrial adenocarcinoma (Figure 1D), and poorly differentiated (Grade 3) endometrial adenocarcinoma (Figure 1E). Diffuse positive S100A8 immunostaining was observed throughout the plasma membrane and the cytoplasm in glandular cells of endometrial adenocarcinoma Grade 1 and Grade 2. Extracellular localization of S100A8 protein expression was also noted. However, in endometrial adenocarcinoma Grade 3, S100A8 immunostaining was also found in cells other than endometrial cancer cells.

Of the 74 tissue samples of endometrial cancer studied, S100A8 expression was seen in the carcinoma cell cytoplasmic or in the cell membrane in 59 tumors (IHC score ≥4). In all cases, this was significantly greater immunostaining compared with that in the glands of the normal endometrium (Figure 1F). As shown in Table 1, expression of the S100A8 protein was significantly increased in endometrial cancer when compared with normal endometrial tissues (79.7% vs. 4.5%, respectively). Normal endometrial tissue samples did not show expression of the S100A8 protein.

There were some endometrial tissue samples that contained malignant tumors that could not be confirmed as primary endometrial carcinoma. In tissues containing non-endometrial cancer, expression levels of the S100A8 protein were significantly lower compared with confirmed primary endometrial adenocarcinoma (66.7% vs. 80.2%, respectively) (P=0.499). In Grade 3, or poorly differentiated primary endometrial adenocarcinoma, expression of the S100A8 protein was significantly increased, compared with Grade 1 and Grade 2 endometrial adenocarcinoma (95.5% vs. 73.5%, respectively) (P=0.049). However, surgical stage, according to the 2008 International Federation of Gynecology and Obstetrics (FIGO) staging system, was not significantly associated with increased expression...
of the S100A8 protein in endometrial cancer (Stage I and II vs. Stage III and IV: 80.0% vs. 86.2%) (P=0.562).

Knockdown of the S100A8 gene in HEC-1A cells induced cell apoptosis

To investigate the role of the expression of the S100A8 gene in endometrial adenocarcinoma, a stable transfected HEC-1A cell line was established that expressed shRNA against S100A8 (Figure 2). Firstly, the efficiency of the lentivirus transfection method was evaluated, and >90% of the HEC-1A cells expressed green fluorescent protein (GFP), 72 hours following infection (Figure 2A, 2B). The protein level of S100A8 and the RNA level of S100A8 were confirmed to be down-regulated in the HEC-1A transfected cells when compared with the parent HEC-1A cells (Control) and empty vector HEC-1A cells (NC) in S100A8-sh2 (Figure 2C, 2D). Parent, or non-transfected, HEC-1A as the negative control was named as the Control, and the empty vector-infected HEC-1A cell line was named as NC. The transfection of S100A8-sh2 significantly and specifically inhibited the endogenous expression of the S100A8 gene in HEC-1A endometrial adenocarcinoma cells, and so the S100A8-sh2 line, named as the sh-S100A8, was used in subsequent experiments.

The results of the MTT assay showed that sh-S100A8 cell viability was less than for the NC and Control HEC-1A cells (Figure 3A). Flow cytometry evaluated the cell cycle of the Control, NC, and sh-S100A8 cells and showed that the knocked down expression of S100A8 did not affect G1-phase cell cycle arrest in the HEC-1A cell line (Figure 3B). Control, NC, and sh-S100A8 cells were examined by flow cytometry using a conjugated Annexin-V antibody and propidium iodide (PI) staining which could differentiate live, necrotic, early apoptotic, and late apoptotic cells (Figure 3C–3E). The sh-S100A8 cell samples showed no significant increase in the number of necrotic cells (P>0.05), but showed a significant increase in early apoptotic cells, but not late or secondary apoptotic cells, when compared with the Control and NC cells (P<0.05), suggesting that knockdown of the S100A8 gene induced cell death only by apoptosis (Figure 3E). Figure 3F shows that 44.5% of the sh-S100A8 cells were apoptotic cells, but only 3.2% of the Control cells and 5.5% of NC cells were apoptotic cells.

| Overexpression of S100A8 | Negative (n=36) | Positive (n=60) | P* |
|-------------------------|----------------|----------------|----|
| Endometrial tissues     |                |                | 0.000 |
| (NEM vs. EC)            |                |                |    |
| NEM                     |                |                |    |
| Proliferative phase     | 9              | 0              |    |
| Secretory phase         | 12             | 1              |    |
| EC                      | 15             | 59             |    |
| FIGO surgical stage     |                |                | 0.562 |
| (I, II vs. III, IV)     |                |                |    |
| I                       | 7              | 29             |    |
| II                      | 4              | 15             |    |
| III                     | 4              | 12             |    |
| IV                      | 0              | 13             |    |
| Histological type       |                |                | 0.499 |
| (Non-EA vs. EA)         |                |                |    |
| Serous adenocarcinoma   | 1              | 1              |    |
| Clear cell adenocarcinoma | 0            | 1              |    |
| EA                      | 14             | 57             |    |
| Grade (G1 and 2 vs. G3) |                |                | 0.049 |
| G1                      | 7              | 21             |    |
| G2                      | 6              | 15             |    |
| G3                      | 1              | 21             |    |

* χ² test. NEM – normal endometrium; EC – endometrial cancer; Non-EA – non-endometrioid adenocarcinoma; EA – endometrioid adenocarcinoma.
Down-regulation of the \textit{S100A8} gene induced cell apoptosis via inhibition of the phosphorylated active form of anti-apoptotic protein kinase B (Akt)

Down-regulation of the \textit{S100A8} gene did not significantly alter the expression level of protein kinase B (Akt), compared with the NC and the Control cells (Figure 4A–4C). However, the phosphorylation of protein kinase B (Akt) at Ser473 in sh-\textit{S100A8} cells was significantly decreased compared with the NC and the Control cells (Figure 4B, 4D). The role of protein kinase B (Akt) signaling was to transduce growth factor-mediated cell survival and block apoptosis, which was activated by phosphorylation.

\textit{S100A8} gene knockdown reduced cell proliferation in the HEC-1A cells compared with control cells, induced cell apoptosis, inhibited the phosphorylation of protein kinase B (Akt), and induced the expression of pro-apoptotic genes, including the cytochrome C gene, CYCS, BAD, BAX, FOXO1, FOXO3, CASP9, and CASP3. For the expression of the cytochrome C gene, CYCS, suppression of the \textit{S100A8} gene resulted in a significant increase in apoptosis compared with the NC and the Control cells (Figure 5A). Gene expression levels of \textit{BAD}, \textit{BAX}, \textit{FOXO1}, \textit{FOXO3}, \textit{CASP9}, and \textit{CASP3} were all increased in the sh-\textit{S100A8} cells (Figure 5B–5G). This finding indicated that down-regulated \textit{S100A8} could induce mRNA of these pro-apoptotic genes in HEC-1A cells. A vulnerability factor and final effector in the apoptotic signaling gene for caspase-3, \textit{CASP3}, showed increased mRNA expression (Figure 5G), which was supported by the finding that Western blotting showed that cleaved caspase-3 was increased in sh-\textit{S100A8} cells (Figure 5H).

\textbf{Discussion}

The \textit{S100} calcium-binding protein A8 (\textit{S100A8}) has been shown to be expressed and released during tissue damage or...
cellular stress reactions and may be involved in the early stages of inflammatory processes [13]. However, the mechanism of the effects of activation of the S100A8 gene that encodes the S100A8 protein, and the effects of the levels of expression on the development and progression of human cancer remain unknown, including in endometrial cancer.

The aim of this study was to investigate the effects of the expression and silencing of the S100A8 gene on apoptosis and phosphorylation of protein kinase B (Akt) in tissue samples of endometrial carcinoma and in HEC-1A endometrial adenocarcinoma cells in vitro. The findings of this study showed that the S100A8 protein was expressed in tissues containing endometrial cancer, and that down-regulation of the S100A8 gene induced significant cell apoptosis in the endometrial adenocarcinoma HEC-1A cell line.

Previously published studies have shown that the effects of S100A8 gene expression in cancer might be dependent on cell type and context, as shown by an in vivo study in mice using anaplastic thyroid carcinoma cells with S100A8 gene knockdown resulting in reduction in tumor growth and lung metastasis, and increased survival in the animal model [14]. In a previously published study, recombinant S100A8 and S100A9 proteins have been shown to promote the viability and migration of stromal cells and colorectal carcinoma cells [15]. The findings of the present study showed that inhibition of expression of the S100A8 gene reduced the growth of endometrial adenocarcinoma cells in vitro.

In a previously published study on breast cancer, the expression of intracellular S100A8 and S100A9 protein suppressed breast cancer cell growth [16]. In 2016, a study using an S100A8/A9-negative human carcinoma cell line (KB) and transfection to express S100A8 and S100A9 resulted in down-regulation of the MMP-2 gene and inhibited cell migration, while silencing of S100A8 and S100A9 gene expression in the head and neck squamous cell carcinoma (HNSCC) cell line, TR146, increased the activity of MMP-2 and cell migration in vitro [17]. In 2014, a study on inflammation and pancreatic cancer demonstrated...
both functional and molecular interactions between S100A8, S100A9, TGF-β1 and stromal cells, supporting the view that the effects of S100A8 and S100A9 on cell signaling in human cancer were dependent on cell-type and context [18].

In the present study, low grade (Grade 1) and moderate grade (Grade 2) endometrial cancer expressed the S100A8 protein mainly on the plasma membrane and in the cytoplasm of the adenocarcinoma cells and also found in the extracellular space. However, in poorly differentiated (Grade 3) endometrial adenocarcinoma, all tumor cells showed strong positive expression of the S100A8 protein (Figure 1). The expression of the S100A8 protein might reflect de-differentiation in endometrial cancer, which suggests that the protein levels might be studied further as both a diagnostic and prognostic marker. Previously published studies have supported a further finding of the present study, that the S100A8/A9 proteins can be passively released into the extracellular space, where they may have biological functions, including cellular damage, stromal necrosis or recruitment of inflammatory cells [19]. Further studies are required to determine the role of extracellular S100A8 in endometrial adenocarcinoma.

There have been some previously published studies on the role of measurement of S100A8/A9 concentrations as serum biomarkers of certain diseases, including arthritis, and in some malignant tumors, including lung, breast, gastric, colorectal, pancreatic and prostate cancers [19]. However, it remains unclear whether serum S100A8 concentrations reflect the degree of inflammation or the presence and progression of malignancy. Further studies are required to determine whether measurement of serum S100A8 might be used as a biomarker for the early diagnosis of endometrial adenocarcinoma.

An important finding of the present study was that S100A8 gene knockdown not only reduced cell proliferation in the HEC-1A cells infected with lentiviral short hairpin RNA, sh-S100A8 were analyzed by: (A) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (B) Western blotting. (C) The relative density of protein kinase B (Akt) and p-Akt are expressed as the ratio of Akt/GAPDH. (D) The ratio of p-Akt/Akt. Data represent the mean ± standard deviation (SD) of independent experiments performed in triplicate. *A significant difference with respect to NC (p<0.05).
cells compared with control cells, but also induced the expression of pro-apoptotic genes, including the cytochrome C gene, CYCS, BAD, BAX, FOXO1, FOXO3, CASP9, and CASP3. This finding is supported by a previously published study that showed that inhibition of protein kinase B (Akt) promoted FOXO3a-dependent apoptosis in prostate cancer [20]. It has previously been reported that S100A8 and S100A9 transduce the anti-apoptotic signal via Toll-like receptor 4 (TLR4), which binds to S100A8 and S100A9, in normal neutrophils and in neutrophils from patients with asthma [21]. Liu et al. showed that loss of TLR4 increased phosphorylation of protein kinase B (Akt), while TLR4 overexpression reduced phosphorylation of protein kinase B (Akt) in the mouse retina [22]. S100A8 and S100A9 have been shown to bind to the receptor for advanced glycation end products (RAGE) and TLR4 on tumor cells [8]. It has also previously been shown that the complex of S100A8 and S00A9 (S100A8/A9) binds to RAGE, with S100A8/A9 induced phosphorylation of RAGE and which resulted in the activation of diverse signal effectors including protein kinase B (Akt), p38, JNK, and NFκB [23].

In this study, down-regulation of the expression of the S100A8 gene was shown to significantly alter the phosphorylation level of protein kinase B (Akt) (Figure 4). Therefore, S100A8 expression and increased levels of S100A8 protein were positively correlated with the phosphorylation level of protein kinase B (Akt) in endometrial adenocarcinoma. However, a limitation of this study was that in the tissue samples containing endometrial cancer, these were formalin-fixed tissues and little material was left at the end of the study, which meant that the use of tissue immunohistochemistry for detection of S100A8

Figure 5. Suppression of the S100A8 gene in HEC-1A endometrial adenocarcinoma cells induced the expression of pro-apoptotic genes. Expression levels of pro-apoptotic genes induced by lentiviral short hairpin RNA (shRNA) transfected into HEC-1A cells, induced in sh-S100A8, were analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (A) Expression of the pro-apoptotic cytochrome C gene, CYCS. (B) Expression of the pro-apoptotic gene BAD. (C) Expression of the pro-apoptotic gene BAX. (D) Expression of the pro-apoptotic gene, FOXO1. (E) Expression of the pro-apoptotic gene, FOXO3. (F) Expression of the pro-apoptotic gene for caspase-9, CASP9. (G) Expression of the pro-apoptotic gene for caspase-3, CASP3, which is also and final effector in apoptotic signaling. (H) Western blotting analysis for the apoptotic factor and final effector in apoptotic signaling, caspase-3. Data represent the mean ± standard deviation (SD) of independent experiments performed in triplicate. * A significant difference with respect to NC (p<0.05).
protein levels in tissues did not also include the evaluation of levels of phosphorylated protein kinase B (Akt) in endometrial cancer tissue. The evaluation of tumor levels of phosphorylated protein kinase B (Akt) in endometrial carcinoma tissue sample requires future study. Of relevance to the findings of this study, a group of anti-inflammatory drugs, quinoline-3-carboxamides (Q-compounds), have been shown to block the interaction specifically between S100A8/A9 with TLR4 [24].

Conclusions

The findings of this study showed that the S100 calcium-binding protein A8 (S100A8) was highly expressed in tissues containing endometrial cancer and showed a correlation between expression of the S100A8 protein and tumor grade. Also, down-regulation of the S100A8 gene induced significant cell apoptosis via inhibition of the phosphorylated or active form of the anti-apoptotic protein kinase B (Akt). The findings of this study indicated that the inhibition of the S100A8 gene could present a relevant therapeutic target, with the potential of enabling a more effective treatment path for patients with endometrial adenocarcinoma.

Conflict of interest

None.

Supplementary Tables

Supplementary Table 1. shRNA oligo sequences.

| Oligonucleotide | Sequences |
|-----------------|-----------|
| S100A8-sh1      | GATCCCGGATACCTGAAGAAATTGCTTCAAGAGAGCATCCCTGATCCCTTTTTT |
| S100A8-sh1      | AATTCAAAAAAGGGATACCTGAAAGAATTGCTTCAAGAGAGCATCCCTGATCCCTTTTTT |
| S100A8-sh2      | GATCCGTCAACACTGTTGGTACGTCTACCTGAGTAACTGCACCATCAGTGTTGATTTTTT |
| S100A8-sh3      | GATCCGAATCTATCATCGACCTCCTACTGAGTAACTGCACCATCAGTGTTGATTTTTT |
| S100A8-sh3      | AATTCAAAAAAGGGATACCTGAAAGAATTGCTTCAAGAGAGCATCCCTGATCCCTTTTTT |
| S100A8-sh4      | GATCCGTTCCTCAGTTATACGGAACCTCAGTCTGCTGATGAGATCAGACCTAGATAGCTGAGACCTT |

F – forward; R – reverse.

Supplementary Table 2. Primers for reverse transcription-polymerase chain reaction.

| Gene     | Primer sequence (5’-3’)         |
|----------|---------------------------------|
| AKT      | CACTTCATCGAGGACGACCT            |
| FOXO1    | AGCCACAGGACGACGAC               |
| Cytochrome C | TCCATCTCCTCCTCCTCCTG             |
| Bad      | ATGCGGAGAAGACAGAACGAC           |
| Reverse  | TGCCTGAGAAGACGAC                |
| Bax      | GATGCGGCTGCTGCTGCTGCTG          |
| Reverse  | TGCCTGAGAAGACGAC                |
| FOXO1    | AGCCACAGGACGACGAC               |
| FOXO1    | ACGCCCGGCTGCTGCTGCTG           |
| FOXO1    | GCCATGCGGCTGCTGCTGCTG          |
| Caspase-9| AGCACAGGACGACGAC                |
| Caspase-3| CAGCCACAGGACGAC                 |
| GAPDH    | GAGACGACGACGAC                  |
| Reverse  | GAGACGACGACGAC                  |

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]
References:

1. Allard J, Maxwell G: Race disparities between black and white women in the incidence, treatment, and prognosis of endometrial cancer. Cancer Control, 2009; 16: 53–56

2. Fortner R, Hüsing A, Kühn T et al: Endometrial cancer risk prediction including serum-based biomarkers: results from the EPIC cohort. Int J Cancer, 2017; 140: 1317–23

3. Vogl T, Gharibyan AL, Morozova-Roche LA: Pro-inflammatory S100A8 and S100A9 proteins: self-assembly into multifunctional native and amyloid complexes. Int J Mol Sci, 2012; 13: 2893–917

4. Ravasi T, Hsu K, Goyette J et al: Probing the S100 protein family through genomic and functional analysis. Genomics, 2004; 84: 10

5. Yanamandra K, Alexeyev O, Zamotin V et al: Amyloid formation by the pro-inflammatory S100A8/A9 proteins in the aging prostate. PloS One, 2009; 4: e5562

6. Goyette J, Geczy CL: Inflammation-associated S100 proteins: new mechanisms that regulate function. Amino Acids, 2011; 41: 821–42

7. Srikrishna G: S100A8 and S100A9: New insights into their roles in malignancy. J Innate Immun, 2012; 4: 31–40

8. Bresnick AR, Weber DJ, Zimmer DB: S100 proteins in cancer. Nat Rev Cancer, 2015; 15: 96–109

9. Hiratsuka S, Watanabe A, Sakurai Y et al: The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. Nat Cell Biol, 2008; 10: 1349–55

10. Gebhardt C, Németh J, Angel P, Hess J: S100A8 and S100A9 in inflammation and cancer. Biochem Pharmacol, 2006; 72: 1622–31

11. Zhang X, Al F, Li X et al: Inflammation-induced S100A8 activates IκBα and promotes colorectal tumorigenesis. Int J Cancer, 2015; 137: 2803–14

12. Hiroshima Y, Hsu K, Tedla N et al: S100A8/A9 and S100A9 reduce acute lung injury. Immunol Cell Biol, 2017; 95: 461–72

13. Vogl T, Eisenblätter M, Völler T et al: Alarmin S100A8/A9 regulates MMP-2 expression and invasion and migration by carcinoma cells. Int J Biochem Cell Biol, 2014; 55: 279–87

14. Basso D, Bozzato D, Padoan A et al: Inflammation and pancreatic cancer: Molecular and functional interactions between S100A8, S100A9, NF-S100A8 and TGFβ1. Cell Commun Signal, 2014; 12: 20

15. Prenster M, Vogl T, Roth J, Sperandio M: S100A8/A9: From basic science to clinical application. Pharmacol Ther, 2016; 167: 120–31

16. Kim D, Choi E, Lee J et al: House dust mite allergen regulates constitutive apoptosis of normal and asthmatic neutrophils via toll-like receptor 4. PloS One, 2015; 10: e0125983

17. Liu L, Xiang Y, Curtis E et al: TLR4 regulates insulin-resistant proteins to increase apoptosis in the mouse retina. Inflamm Res, 2017; 66: 993–97

18. Sakaguchi M, Murata H, Yamamoto K et al: TIRAP, an adaptor protein for TLR2/4, transduces a signal from RAGE phosphorylated upon ligand binding. PloS One, 2011; 6: e23132

19. Björk P, Björk A, Vogl T et al: Identification of human S100A9 as a novel target for treatment of autoimmune disease via binding to quinoline-3-carboxamides. PloS Biol, 2009; 7: e97