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# 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

Authors' Contribution:
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Data Collection B
Statistical Analysis C
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Manuscript Preparation E
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**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**

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## 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 $Ca^{2+}$-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 (IL-1β) 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% $H_2O_2$ 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 (Funakoshi Co., Ltd., Tokyo, Japan). Sections were counterstained with hematoxylin, dehydrated in graded ethanol, dried, mounted, and coverslipped. Confirmation of the diagnosis of primary endometrial

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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% $CO_2$ 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 pGMLV-SC5 at the sites of BamHI and EcoRI. Short hairpin RNA (shRNA) oligonucleotides and lentiviral vector pGMLV-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 pGMLV-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 with uniformly high green fluorescent protein (GFP) expression, which is a protein that showed bright green fluorescence on exposure to light in the ultraviolet range.

**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 confirm 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 $2^{-\Delta\Delta Ct}$ method.

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

A density of $2.0\times10^4$ 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 $1\times10^6$ 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 measuring 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 $2\times10^5$/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).

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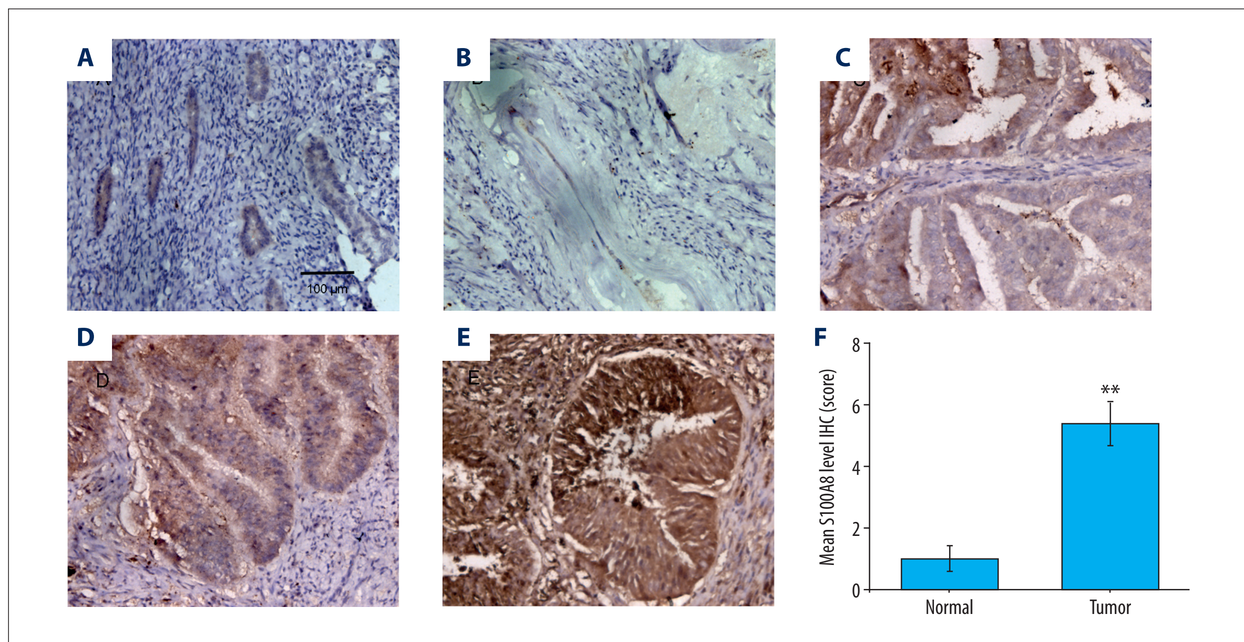


**Figure 1.** Photomicrographs of the immunohistochemistry (IHC) findings and scoring of the expression of the S100A8 protein in normal endometrium and endometrial carcinoma tissues (**A, B**). Photomicrographs show very weak expression of the S100A8 protein in the endometrial glandular cells of the proliferative and secretory phase of normal endometrium. (**C**) Photomicrograph shows strong expression of the S100A8 protein in the cytoplasm and cell membrane of glandular cells in endometrial adenocarcinoma, Grade 1. (**D**) Photomicrograph shows diffuse positive staining for the S100A8 protein in the extracellular space in endometrial adenocarcinoma, Grade 2. (**E**) Photomicrograph shows strong positive staining for the S100A8 protein in many cells of endometrial adenocarcinoma, Grade 3. Scale bars=100 µm. (**F**) Statistical analysis of the immunostaining scores for S100A8 protein in normal endometrium (P<0.01).

## 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

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**Table 1.** Relationship between overexpression of S100A8 and clinicopathological facts.

| Overexpression of S100A8 | Negative (n=36) | Positive (n=60) | P* |
|---|---|---|---|
| Endometrial tissues | | | |
| (NEM *vs.* EC) | | | 0.000 |
| 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 | | | |
| (Non-EA *vs.* EA) | | | 0.499 |
| Non-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 | |

* $\chi^2$ test. NEM – normal endometrium; EC – endometrial cancer; Non-EA – non-endometrioid adenocarcinoma; EA – endometrioid adenocarcinoma.

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.

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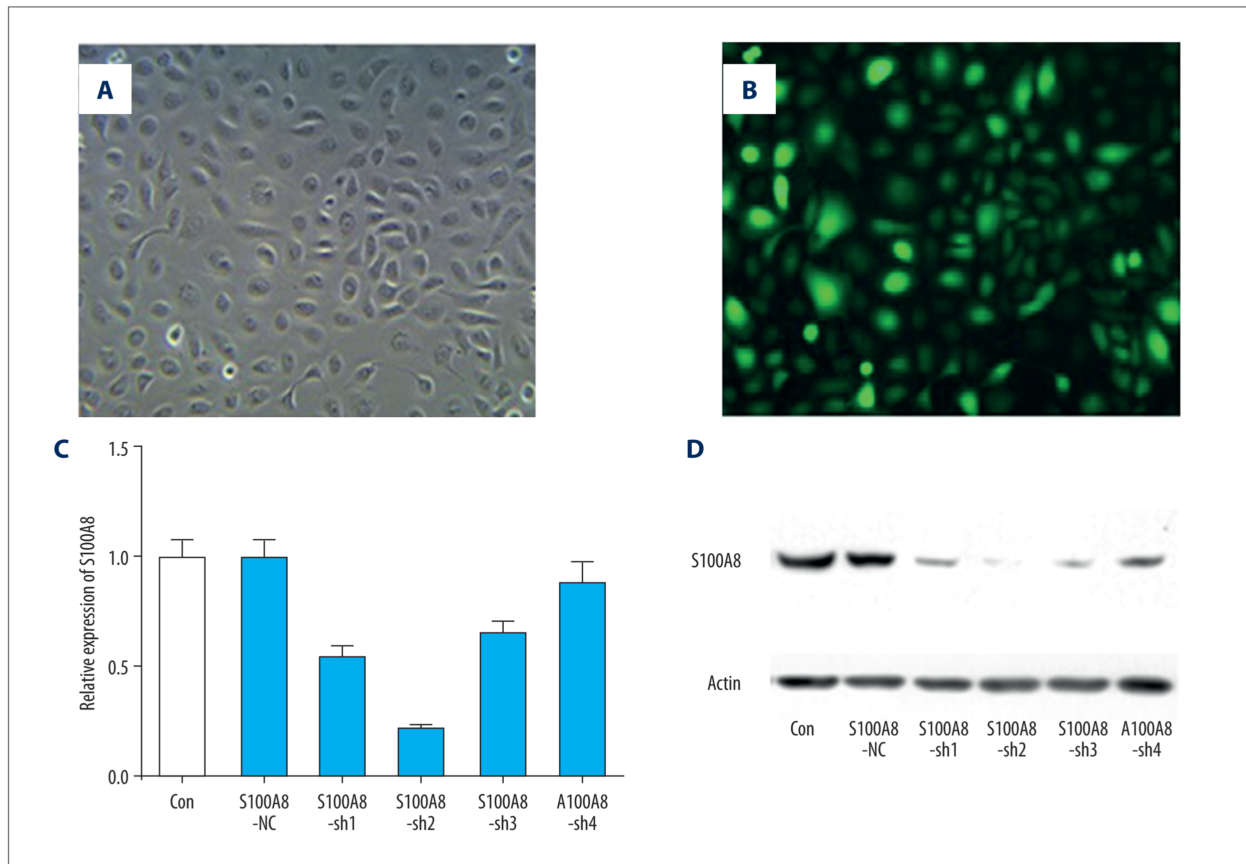


**Figure 2.** Knockdown of S100A8 protein expression in HEC-1A endometrial adenocarcinoma cells using lentivirus-mediated short hairpin RNA (shRNA) (**A, B**). Expression of the green fluorescent protein (GFP) in HEC-1A endometrial adenocarcinoma cells, 72 hrs after lentiviral short hairpin RNA (shRNA) infection. Original magnification ×200. (**C**) The expression of the *S100A8* gene was analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in HEC-1A cells infected by different *S100A8* shRNA oligonucleotides. *S100A8*-sh2 resulted in maximum inhibition compared with both parent cells (Control) and empty vector (NC) (** P<0.01). In these experiments, *GAPDH* was used as an internal control. (**D**) Protein levels of S100A8 were analyzed by Western blot, with actin as an internal control.

### Down-regulation of the *S100A8* gene induced cell apoptosis via inhibition of the phosphorylated active form of anti-apoptotic protein kinase B (Akt)

Down-regulation of the *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-*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.

*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 *S100A8* gene resulted in a significant increase in apoptosis compared with the NC and the Control cells (Figure 5A). Gene expression levels of *BAD*, *BAX*, *FOXO1*, *FOXO3*, *CASP9*, and *CASP3* were all increased in the sh-*S100A8* cells (Figure 5B–5G). This finding indicated that down-regulated *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, *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-*S100A8* cells (Figure 5H).

## Discussion

The S100 calcium-binding protein A8 (S100A8) has been shown to be expressed and released during tissue damage or

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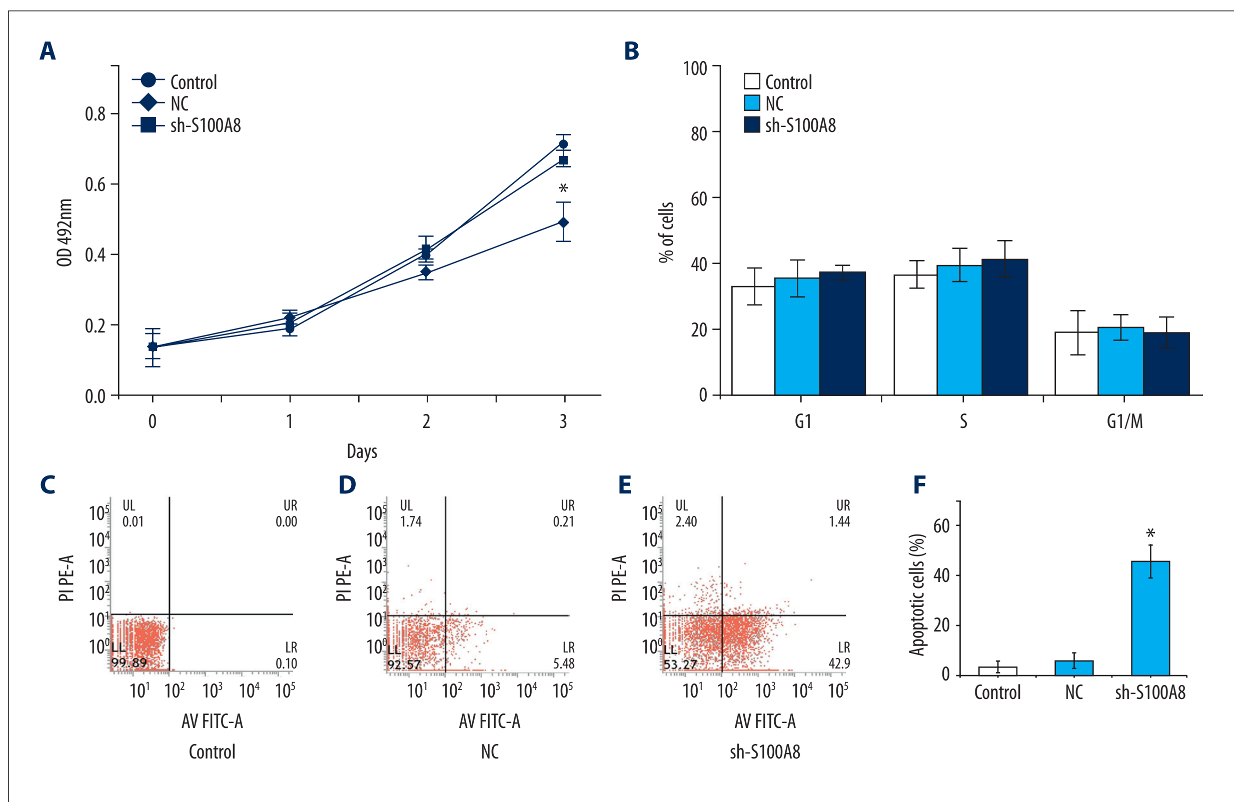


**Figure 3.** The effect of suppression of the *S100A8* gene in HEC-1A endometrial adenocarcinoma cells. (**A**) The HEC-1A cell viability was observed following treatment with the MTT reagent in the indicated cell lines on days 1, 2, and 3. (**B**) The percentage of HEC-1A cells in the G1, S, and G2/M phases of the cell cycle were counted. Data represent the mean ± standard deviation (SD) of independent experiments performed in triplicate. (**C–E**) Flow cytometry analysis of HEC-1A cell apoptosis using Annexin V and propidium iodide (PI) staining. (**C**) Control (parent cell). (**D**) NC (empty vector). (**E**) Infected with lentiviral short hairpin RNA, sh-S100A8. (**F**) The percentage of apoptotic HEC-1A cells (Annexin-V-positive; PI-positive; Annexin-V and PI double positive cells). * P<0.05.

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

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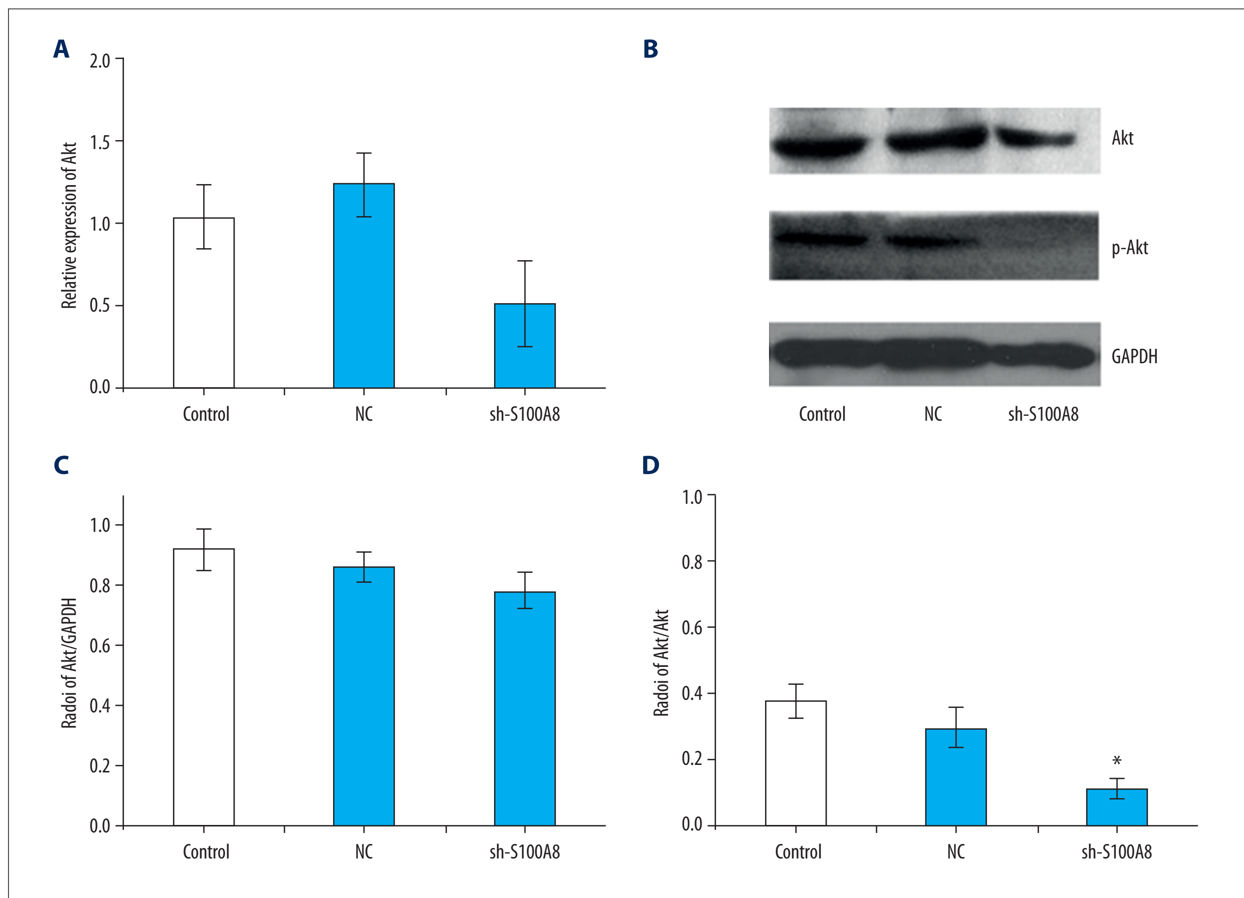


**Figure 4.** Suppression of the *S100A8* gene in HEC-1A endometrial adenocarcinoma cells inhibited the phosphorylation of protein kinase B (Akt). Expression levels of protein kinase B (Akt) in the indicated parent cells (Control), empty vector (NC) and HEC-1A cells infected with lentiviral short hairpin 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 ($p<0.05$).

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

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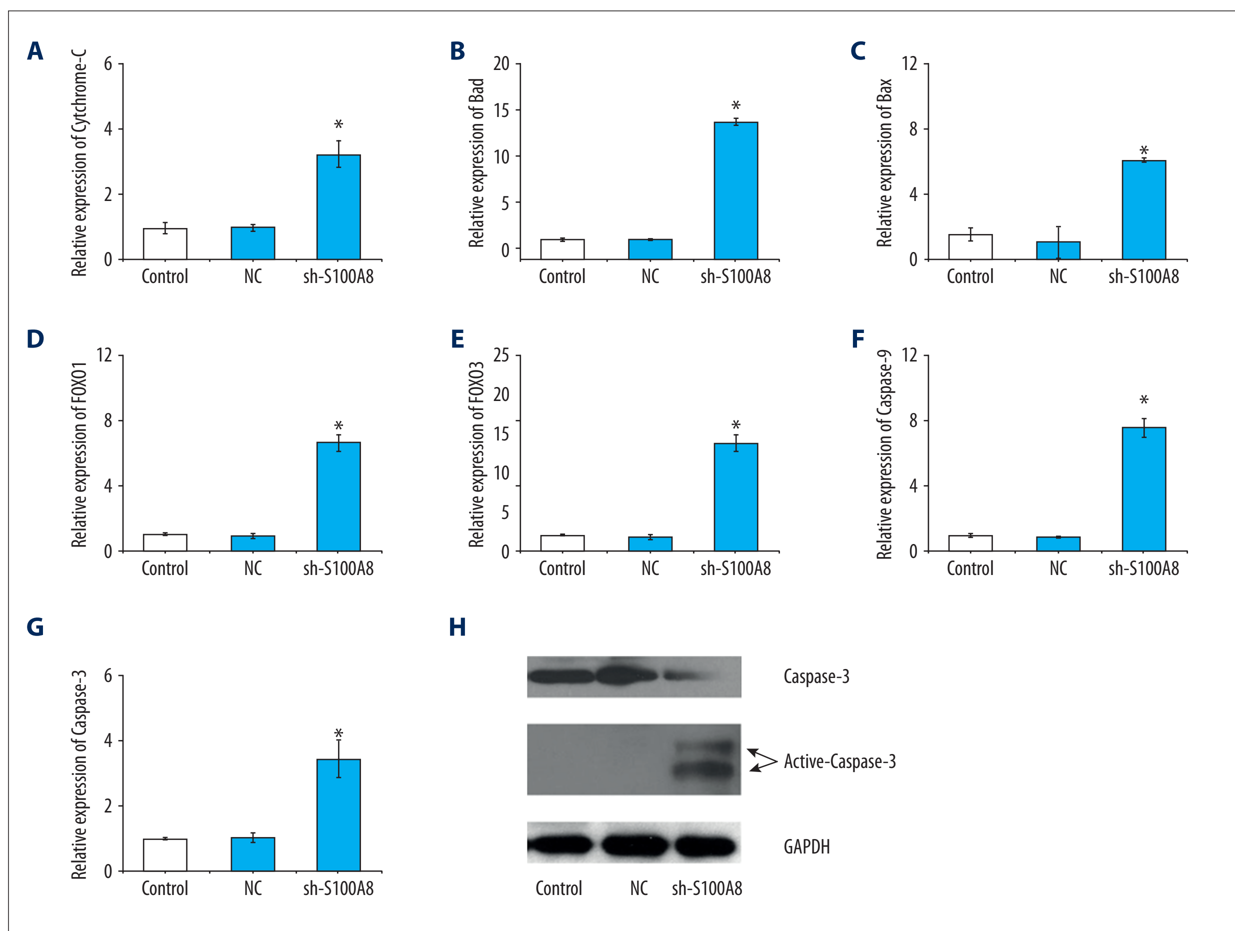


**Figure 5.** Suppression of the *S100A8* gene in HEC-1A endometrial adenocarcinoma cells induced the expression of pro-apoptotic genes. Expression levels of pro-apoptotic genes induced by lentiviral short hairpin RNA (shRNA) transfected into HEC-1A cells, induced in sh-S100A8, were analyzed by quantitative 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).

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

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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 | GATCCGGGATGACCTGAAGAAATTGCTTCAAGAGAGCAATTTCTTCAGGTCATCCCTTTTTT |
| S100A8-sh1 | AATTCAAAAAAGGGATGACCTGAAGAAATTGCTCTCTTGAAGCAATTTCTTCAGGTCATCCCG |
| S100A8-sh2 | GATCCGTCAACACTGATGGTGCAGTTACTCGAGTAACTGCACCATCAGTGTTGATTTTTT |
| S100A8-sh2 | AATTCAAAAAATCAACACTGATGGTGCAGTTACTCGAGTAACTGCACCATCAGTGTTGACG |
| S100A8-sh3 | GATCCGAACTCTATCATCGACGTCTACTCGAGTAGACGTCGATGATAGAGTTCTTTTTT |
| S100A8-sh3 | AATTCAAAAAAGAACTCTATCATCGACGTCTACTCGAGTAGACGTCGATGATAGAGTTCG |
| S100A8-sh4 | GATCCGTGTCCTCAGTATATCAGGAACTCGAGTTCCTGATATACTGAGGACACTTTTTT |
| S100A8-sh4 | AATTCAAAAAAGTGTCCTCAGTATATCAGGAACTCGAGTTCCTGATATACTGAGGACACG |

F – forward; R – reverse.

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

| Gene | Primer sequence (5'-3') |
|---|---|
| AKT | |
| Forward | CACTGTCATCGAACGCACCT |
| Reverse | TCCATCTCCTCCTCCTCCTG |
| Cytochrome C | |
| Forward | ATGGCGGAAGACATGGAGAC |
| Reverse | TTCTGACAGCGGTGGAAGTC |
| Bad | |
| Forward | TGAGCCGAGTGAGCAGGAAGAC |
| Reverse | ATGATGGCTGCTGCTGGTTGG |
| Bax | |
| Forward | GATGCGTCCACCAAGAAGCTGAG |
| Reverse | CACGGCGGCAATCATCCTCTG |
| FOXO1 | |
| Forward | GGCAGCCAGGCATCTCATA |
| Reverse | GGCATGGTGCTTACCGTGT |

| Gene | Primer sequence (5'-3') |
|---|---|
| FOXO3 | |
| Forward | GCCAGCCTGTCACCTTCAGT |
| Reverse | AGCAGGTCGTCCATGAGGTT |
| Caspase-9 | |
| Forward | GCGTGGTGGTCATTCTCTCT |
| Reverse | CAATCTTCTCGACCGACACA |
| Caspase-3 | |
| Forward | GGAATGACATCTCGGTCTGG |
| Reverse | TCACGCATCAATTCCACAAT |
| GAPDH | |
| Forward | TGCACCACCAACTGCTTAGC |
| Reverse | GGCATGGACTGTGGTCATGAG |

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