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Acid sphingomyelinase, a novel negative biomarker of ovarian cancer

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Abstract. – OBJECTIVE: Ovarian cancer is the sixth most common cancer and the main cause of death in women. However, the molecular mechanism for the cause of the ovarian cancer has not been fully elucidated. Acid sphingomyelinase (ASM), a lipid hydrolase, has been suggested for treating cancer and may affect the development of ovarian cancer. We want to find the function of ASM in the development of ovarian cancer.

PATIENTS AND METHODS: Human ovarian cancer cells HO 8910 (HOCC) and human primary ovarian cells (HPOC) were transfected with ASM gene and ASM RNAi. Real-time qPCR and western blot analysis was carried out to examine the level of ASM. The growth rate of transfected and non-transfected cells was measured. Ovarian biopsies were collected from 80 ovarian cancer patients and 20 healthy subjects.

RESULTS: The growth rate of HOCC and HPOC was decreased by 22% and 19% in the ASM-transfected group compared with non-transfected group. Inversely, the growth rate of HOCC and HPOC was increased by 16% and 35% in the ASM-RNAi-transfected group compared with non-transfected group. In the transfected and non-transfected cells, the change level of SAM was approved by Real-time qPCR and western blot analysis. The levels of SAM were reducing with the development of ovarian cancer.

CONCLUSIONS: SAM is higher expressed in normal cell than that in ovarian cancer, and can be a negative biomarker for the diagnosis of ovarian cancer. SAM can be developed a new drug for the ovarian cancer therapy.

Key Words:
Acid sphingomyelinase, Ovarian cancer, Real-time qPCR, Western blot, Biomarker.

Introduction

Ovarian cancer is the sixth most common cancer and the main cause of death in women[1]. It is urgent to find a novel way for the early diagnosis of the cancer and novel medicine to improve the treatment. Biomarkers may be an effective way for the early diagnosis of various cancers and has been widely reported[2-5]. MS-275 and adiphenine have been identified a group of small molecules which can be exploited as adjuvant drug to improve the therapy of ovarian cancer[6]. Propofol has been found to inhibit invasion and metastasis, enhance ovarian cancer cell apoptosis[7]. However, these biomarkers and drugs are still from the actual clinical use. Hypertriglyceridemia is a potential side effect of propofol sedation in critical illness[8]. Thus, it is still necessary to find novel biomarkers and drugs of ovarian cancer.

Acid sphingomyelinase (ASM), a lipid hydrolase, cleaves the sphingolipid into ceramide[9]. Altered sphingolipid metabolism occurs in many cancers, which can decrease the level of ceramide, lipid and sphingosine-1-phosphate. All the changes result in tumorigenicity[10]. Ceramide can induce cell death, which can be used for many cancer therapies[11]. ASM can also break down the membrane lipid sphingomyelin. ASM is positively charged and interacts with the lipid Bis Monoacylglycero Phosphate (BMP) in the membranes of vesicles in lysosome. CADs (cationic amphiphilic drugs), the positively charged molecules, can kill cancer by displacing ASM from vesicular membranes. In the cancer cells, CAD can block the activity of ASM, resulting in higher level of sphingomyelin. The result will lead to the permeabilization of lysosome membrane and, thus, cathepsins will be released into the cytoplasm. Finally, the cell-death pathways are triggered[12]. Thereafter, ASM may be used for the cancer therapy.

ASM exerting multiple anti-tumor effects has been widely reported in many cancers. For instance, ASM can used as an adjuvant treatment with sorafenib to treat hepatocellular carcinoma. The combination of ASM and sorafenib combination showed a synergistic function for decreasing the size of liver tumor and the density of blood vessel density[13]. ASM can regulate

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the homeostasis of sphingolipids, sphingosine-1-phosphate (SIP) and ceramides. The regulation can control carcinogenesis and cell proliferation, survival, apoptosis and so on. ASM from the liver reduces the growth rate of cancer via the accumulation of macrophage and tissue inhibitor of metalloproteinase 1. ASM may be a novel drug for treating liver metastasis of colon cancer[14].

However, the inhibitory effects of ASM on the ovarian cancer have not been fully reported. Thus, we want to know whether ASM ameliorates ovarian cancer by transfecting the ovarian cancer with ASM gene and ASM RNAi and what change can be found for the growth rate of ovarian cells.

Patients and Methods

Patients' Samples and Cell Lines

All protocols were approved by the Ethics Committee from the Affiliated Shengjing Hospital, China Medical University. All participants should submit the informed consent. From 2011 December to 2013 March, a total of 100 patients were diagnosed as ovarian cancers. The biopsy samples were placed in liquid nitrogen and stored at –80°C until use. In all patients, the diagnoses of ovarian cancers were confirmed histologically. None of the patients received any additional therapies. The stages of ovarian cancer were classified according to International Union Against Cancer (UICC)/American Joint Committee of Cancer (AJCC) TNM staging classification[15]. Meanwhile, 20 healthy subjects were selected as a control group.

Human ovarian cancer cells HO 8910 (HOCC) were purchased from Shanghai Bluegene Biotech CO., LTD (Shanghai, China). Human primary ovarian cells (HPOC) were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Immunohistochemical Analysis

ASM antibody was used for immunohistochemical staining for ASM. Different ovarian biopsies were ground in the buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.2% Sodium Dodecyl Sulphate SDS, 200 mM NaCl, and 200 μg/ml proteinase K). Endogenous peroxidase was blocked by 5% hydrogen peroxide for 5 min. Nonspecific binding sites were blocked by 2% normal horse serum for 20 min. The samples were incubated with the primary antibodies for 60 min. Immunoreactivity was visualized by using DAKO Envision HRP System (1:10,000 dilution) (DAKO, Carpinteria, CA, USA). Immunoreactivity was quantitatively evaluated using analyzed with NIH image program. A score of 0-300 was calculated for each case as the product of the intensity score and the percent of immunoreactivity.

Reconstruction of pcDNA3.1-ASM

ASM gene (GenBank No.: M59916.1) was amplified using the primers (Sense primer, 5'-GTGAGCTAGCATGCCCCGCTACGGAGCGT-CACTCC-3'; Antisense primer, 5'-CTGA-GAATTCTTAGCAAAACAGTGGCCTTGGC-CAC-3'). The PCR product was cleaved by the enzymes *Nhe* I and *EcoR*I, and inserted into the sites of pcDNA3.1. pcDNA3.1-ASM was constructed and transferred into *E. coli*. The plasmid was isolated with isolated using TaKaRa MiniBEST Plasmid Purification Kit Ver.4.0 (TaKaRa, Dalian, China) and verified by automated DNA sequencing.

ASM Gene-Silencing Constructs

The pTZU6+1 expression plasmid donated from Dalian Medical University (Dalian, China). Here, the ASM coding sequence and the reverse complementary sequence were synthesized as follows: siKDM5B, sense 5'TCGA-CATAGACCTTTGCAGATTGGGCTTGGGC-CCAATCTGCAAAGGTCTATTTTTTT-3', antisense 5'-CTAGAAAAAAATAGACCTTTGCA-GATTGGGCCCAAGCCCAATCTGCAAAG-GTCTATG-3'; SalI and XbaI restriction sites were incorporated on the either end of the oligos for the cloning into the pTZU 6+1 vectors. Thus, pTZU6+1-shRNA-SAM vectors were constructed.

HOCC and HPOC Proliferation Assay

HOCC and HPOC were harvested using 0.05% trypsin. Cells were suspended (40,000 cells/ml) in Dulbecco's Modified Eagle Medium (DMEM) with 20% BCS (bovine calf serum), plated onto gelatinized 96-well culture plates (0.1 ml/well), and incubated at 37°C, 5% CO_2 for 24 h. The media were replaced with 0.1 ml of DMEM with 5% BCS and incubated for 24, 48 and 72 h, at 37°C,

5% CO_2. Cell proliferation was determined using 3-(4,5-dimethylthiazol-2-gl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Real Time qPCR

Real time qPCR were performed using in a Stratagene Mx3005P® QPCR System instrument (La Jolla, CA, USA) according to manufacturer's protocol. RNA was isolated from HOCC and HPOC, followed by cDNA synthesis and data analysis as described previously[16]. Primers used for the real time qPCR were as following: ASM, 5'-ATCCTCTTCCTCACTGACCTG-3'and 5'-CTGGTGCCAGACATCATGTGC-3'; GAPDH, 5'-CCCTTCATTGACCTCAACTAC-3' and 5'-CCACCTTCTTGATGTCATCAT-3'. GAPDH was used as an internal control.

Western Blotting Analysis

All HOCC and HPOC cells were homogenized in RIPA buffer (150 mM Sodium chloride, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0) and protease inhibitor cOmplete Mini (Roche Diagnostics, Rochester, MN, USA) was included. After the debris was removed, supernatants were boiled and mixed with an equal volume of 20% glycerol containing 0.02% bromophenol blue. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in TBST (10 mM Tris (pH 7.5), 100 mM NaCl and 0.1% Tween 20) and incubated with ASM antibody (ab83354, ABCAM, Hangzhou, China) in TBST with 0.5% skim milk overnight at 4°C. The membrane was treated with horseradish peroxidase conjugated secondary antibody (1:3000) (Amersham Biosciences, Piscataway, NJ, USA). Immunoreactive bands were visualized by ECL (electrochemiluminescence) (GE Healthcare, Cleveland, OH, USA) and quantified by densitometry with Image J software 1.45 (NIH, Bethesda, MD, USA).

Statistical Analysis

The association between the levels of ASM and the stages of ovarian cancer was compared by one-way analysis of variance (ANOVA). Data was analyzed using Statview 5.0 software (Abacus systems, Berkeley CA, USA) with a p value < 0.05 accepted as significant.

Results

The Characters of the Patients with Ovarian Cancer

The age of all the patients was more than 40 years old and the average age was 48 ± 6 years old. All patients have no tobacco smoking and alcohol drinking habits. According to UICC/TNM staging classification[15], all the subjects for each category were: n = 20 for healthy subjects, n = 37 for the patients with stage I ovarian cancer, n = 22 for the patients with stage

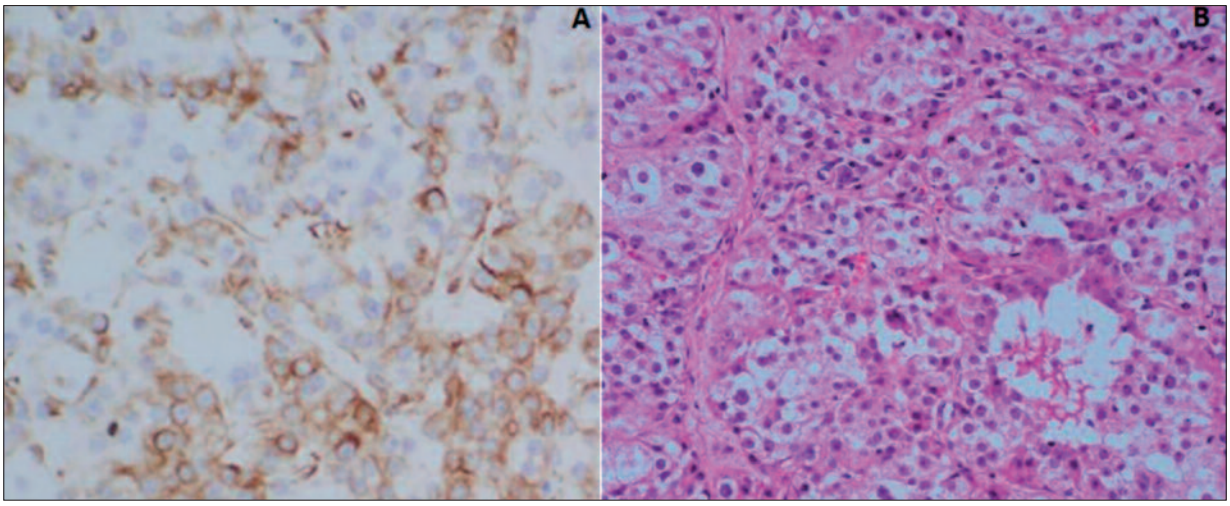

Figure 1. Immunohistochemical analysis of ASM expression in ovarian tissues. **A**, ASM is highly expressed in normal ovarian tissues. **B**, ASM is lowly expressed in ovarian cancer tissues. Original magnifications ×200.

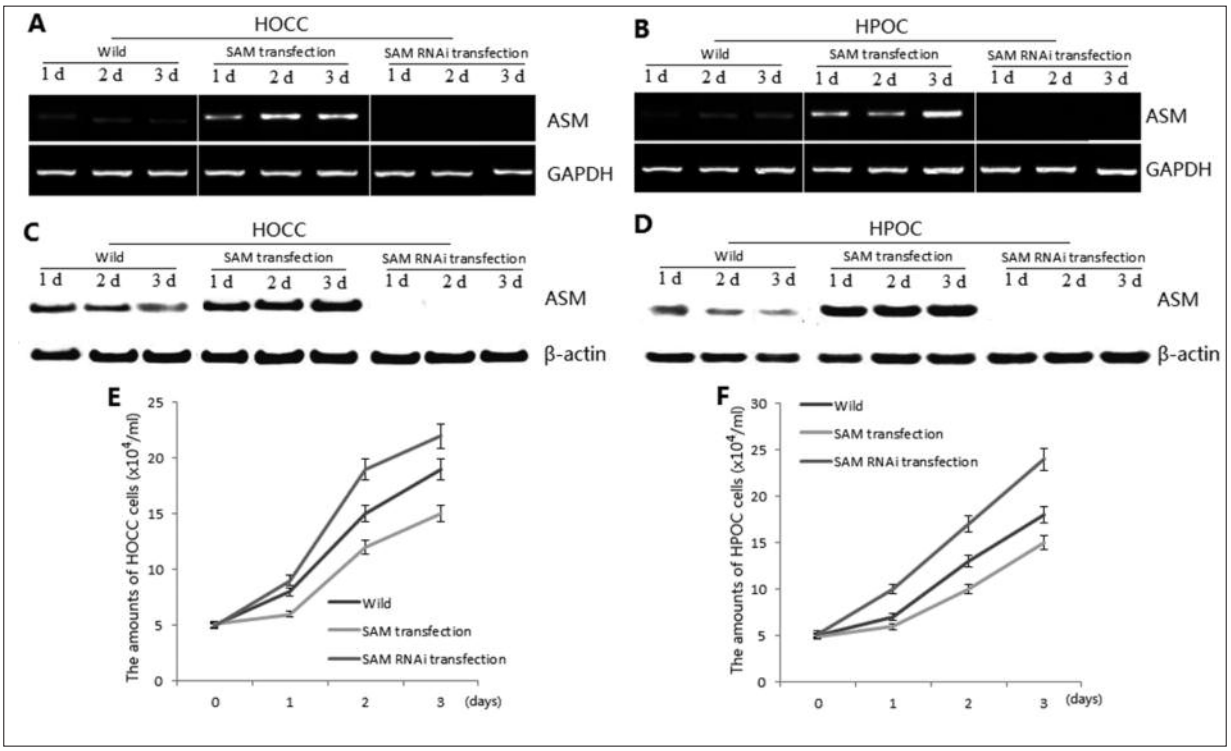

Figure 2. The levels of ASM in ovarian cancer cell lines and the growth rate of HOCC and HPOC. **A**, the mRNA levels of ASM in the HOCC transfected with ASM gene or ASM RNAi. **B**, the mRNA levels of ASM in the HPOC transfected with ASM gene or ASM RNAi. **C**, the protein levels of ASM in the HOCC transfected with ASM gene or ASM RNAi. **D**, the protein levels of ASM in the HPOC transfected with ASM gene or ASM RNAi. E, the growth rate of the transfected and non-transfected HOCC. F, the growth rate of the transfected and non-transfected HPOC. n =5, results are shown as mean TSD.

II ovarian cancer, n = 28 for the patients with stage III ovarian cancer, and n = 13 for the patients with stage IV ovarian cancer. To determine whether a lack of ASM is existed in ovarian cancer, immunohistochemical analysis was used to examine ASM expression in normal ovarian tissues and ovarian cancer tissues. The results showed that ASM was highly expressed in normal ovarian tissues. Inversely, ASM was lowly expressed in ovarian cancer tissues (Figure 1). Also, no cases of normal ovarian tissues were considered to be the ovarian cancer. All the normal tissues showed brown color after immunohistochemical analysis compared with that from cancer tissues (Figure 1).

The Levels of ASM and the Growth Rate of HOCC and HPOC

After we found that ASM was highly expressed in normal tissues than that in ovarian cancer, we wanted to know whether ASM could regulate the growth of the ovarian cancer cells. Thus, two kind of ovarian cancer cell lines, HOCC and HPOC, were selected. The HOCC and HPOC were incubated in DMEM with 5% BCS for 24, 48 and 72 h, the levels of ASM and cell proliferation were shown in Figure 2. In HOCC, the mRNA levels of ASM were highest in the cells transfected with ASM gene while the levels came to zero when the cells were transfected with ASM RNAi (RNA interference) (Figure 2A). The protein levels showed the similar changing trend with the mRNA levels (Figure 2C). In HPOC, the mRNA levels of ASM were also the highest in the cells transfected with ASM gene while the levels came to zero when the cells were transfected with ASM RNAi (Figure 2B). The protein levels also showed the similar changing trend with the mRNA levels (Figure 2D). All the results suggested that the two kinds of cells were successfully transfected with ASM gene and ASM RNAi.

For HOCC, the growth rate could be increased by 16% when the cells were transfected with ASM RNAi while the growth rate was decreased by 22% when the cells were transfected with

ASM after three-day culture (Figure 2E). Similarly, for HPOC, the growth rate could be increased by 35% when the cells were transfected with ASM RNAi while the growth rate was decreased by 19% when the cells were transfected with ASM after three-day culture (Figure 2F). The results suggested that high levels of ASM inhibited the growth of HOCC and HPOC while the silence of ASM would promote the growth of the two kinds of cells.

The mRNA Levels of ASM were Decreasing with the Development of Ovarian Cancers

The ASM mRNA levels reflects directly the transcriptional activity of the ASM, so the relative mRNA levels of ASM were firstly analyzed via real time quantitative RT-PCR. The mRNA levels of ASM were the highest in benign ovarian tissues compared with those from ovarian cancers ($p < 0.01$) (Figure 3). Furthermore, the levels of ASM were decreasing with the development of ovarian cancer. The mRNA levels of ASM reached the lowest concentration in the patients with the ovarian cancer at stage IV (Figure 3). The results suggested that the transcriptional activity of ASM was low in the ovarian cancer and reached the lowest level at stage IV.

The Protein Levels of ASM were Decreasing with the Development of Ovarian Cancers

The ASM protein levels can also reflect the overall activity of the ASM, so the protein levels of ASM were analyzed via western blot. Just as above mRNA levels, the protein levels of ASM were the highest in benign ovarian tissues compared with those from ovarian cancers (Figure 4A). On the other hand, the levels of ASM were also decreasing with the development of ovarian cancer. The protein levels of ASM reached the lowest concentration in the patients with the ovarian cancer at stage IV (Figure 4A). The statistics data also suggested that the overall activity of ASM was low in the ovarian cancer and reached the lowest level at stage IV (Figure 4B).

Discussion

Ovarian cancer is the most common women cancer. Pathological diagnosis is a key factor for correct and on-time treatment. Pathological diagnosis is made by the biopsy of the tumor. Clinical diagnosis is made by using modern diagnostic procedures (ECHO, CT, MRI, PET scan)[17-19]. Moreover, a handful of cancer biomarkers are

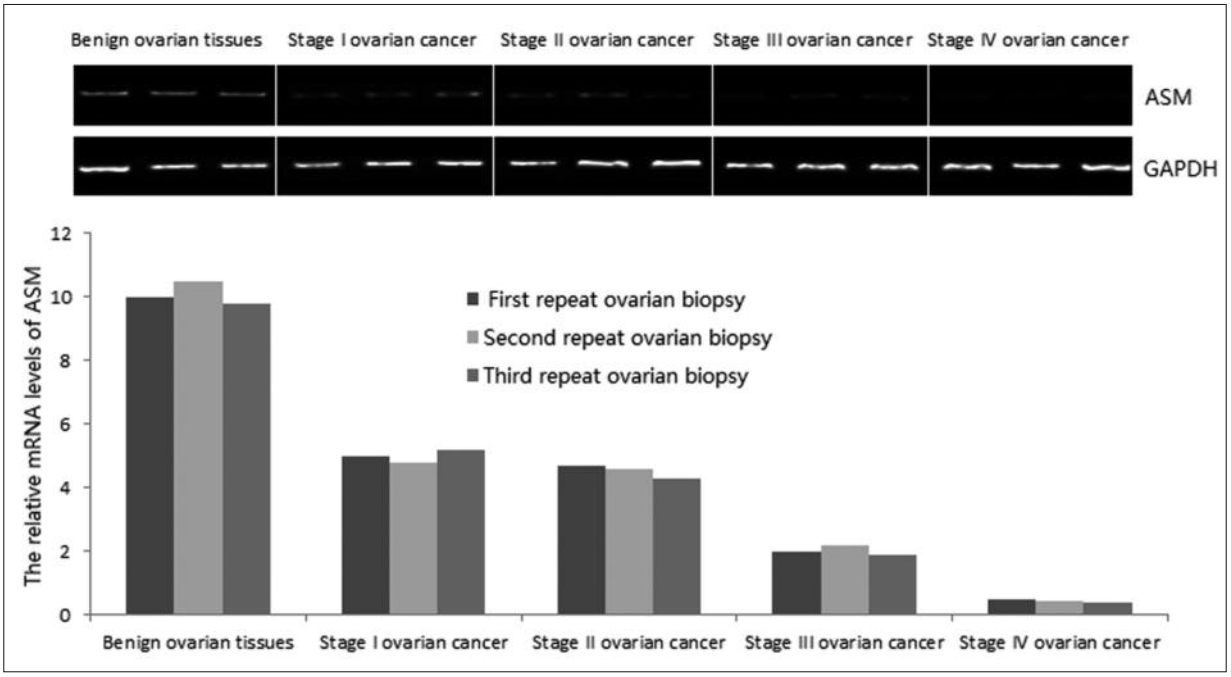

Figure 3. The real time quantitative RT-PCR analysis of the relative mRNA levels of ASM in different ovarian tissues. The analysis of each sample was repeated in three experiments with similar results.

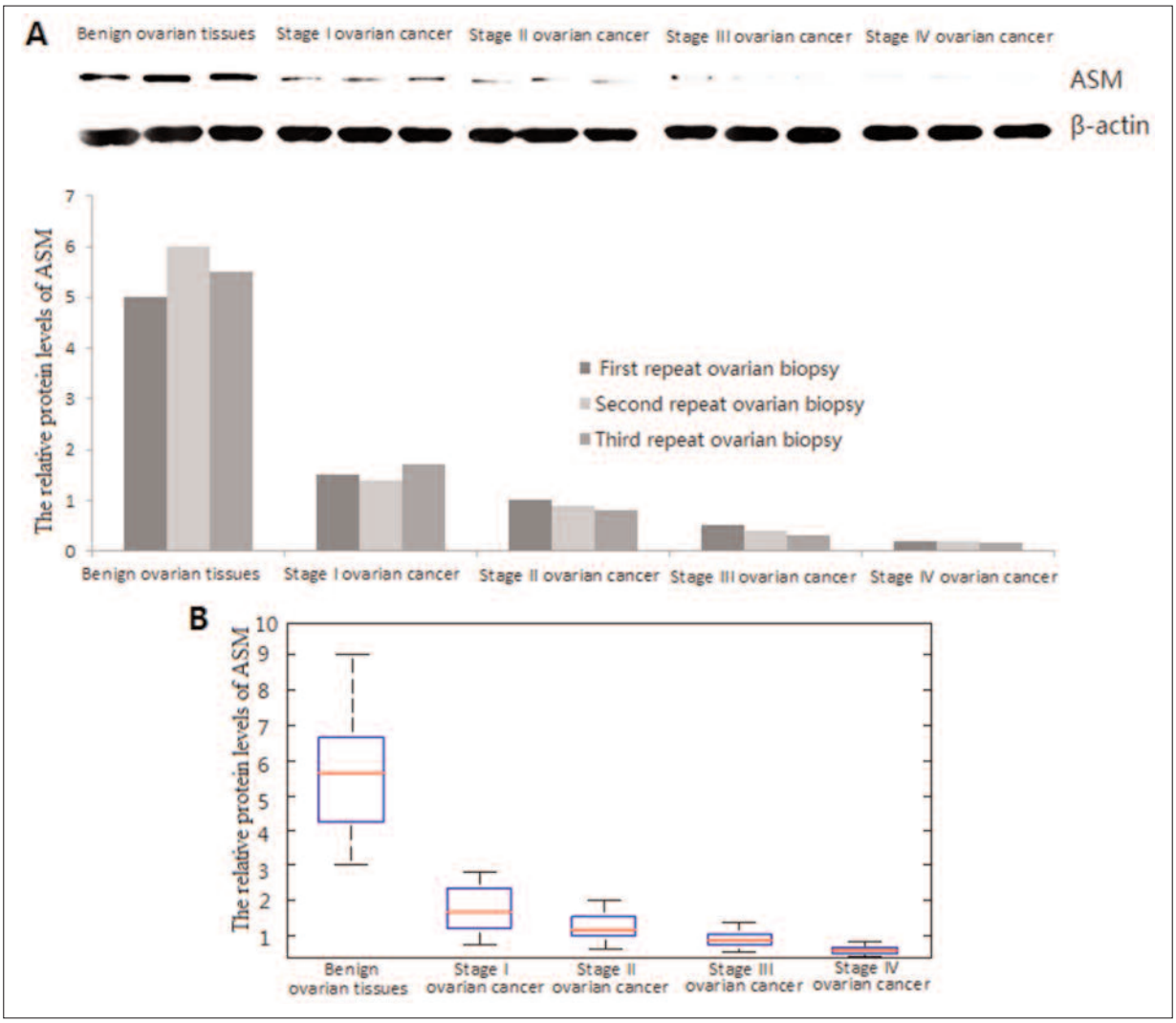

Figure 4. Western blot analysis of the protein levels of ASM in different ovarian tissues. A, western blot analysis for the relative protein levels of ASM in different ovarian tissues. The analysis of each sample was repeated in three experiments with similar results. B, the relative protein expression of ASM in normal ovarian tissues and ovarian cancers. The bars in the boxes showed the average activities and the boxes represented 90% of the samples at the same stage. The error bars were above or below the boxes. All the subjects for each category were: n = 20 for normal ovarian tissues, n = 37 for stage I ovarian cancer, n = 22 for stage II ovarian cancer, n = 28 for stage III ovarian cancer, and n = 13 for stage IV ovarian cancer.

currently used routinely for population screening, disease diagnosis, prognosis, monitoring of therapy, and prediction of therapeutic response[20]. Stress-induced phosphoprotein 1 (STIP1) has been recently identified as a released biomarker in human ovarian cancer. STIP1 expression may be related to prognosis and that the STIP1 pathway may represent a novel therapeutic target for human ovarian cancer[21]. The ovarian cancer biomarker CA125 has been extensively investigated over the last 30 years. Independent verification of CA125 identity in characterization studies will help establish a refined model of its molecular structure that will promote the development of new approaches for diagnosis, prognosis and therapy of ovarian cancer[22]. The serum CUB and zona pellucida-like domain-containing protein 1 (CUZD1) appears to be a highly promising novel serum biomarker for ovarian cancer diagnosis[23].

Although many biomarkers have been widely reported, none of these biomarkers really comes into actual clinical use by far now. Thus, it is still necessary to find the novel biomarker for the diagnosis of ovarian cancer. The sphingomyelin-ceramide pathway is a conserved signalling system and related with the cell apoptosis. Different

ASMs can hydrolyze sphingomyelin to ceramide, which can cause the apoptosis in many kinds of cells[24-26]. Thus, ASM can promote the apoptosis of many cancers and inhibit the growth of many cancers[14,27,28]. ASM may inhibit the growth of ovarian cancer and can be an adjuvant negative value for ovarian cancer diagnosis. Our results showed that the high level of ASM could inhibit the growth of the both kinds of ovarian cancers, HOCC and HPOC significantly compared with the non-transfected cell lines ($p < 0.01$) (Figure 2E and F). Inversely, the ASM RNAi would result in increasing growth rate of both kinds of ovarian cancers (Figure 2E and F). The results suggested that the lack of ASM would result in the overgrowth of the cell lines, which is the main reason of carcinogenesis.

Based on above method, the mRNA and protein levels of ASM were examined in different ovarian tissues. The clinical tests showed that the high level of ASM only could be found in the normal ovarian tissues. The levels of ASM became very low in all the ovarian cancers. The levels of ASM reached the lowest in the stage IV ovarian cancer. The results just we expected that ASM could inhibit the growth of cell and would become lesser with the development of ovarian cancer. Thus, the data may suggest a negative value to include ASM immunological activity as an ancillary study in the cases clinically suspicious for primary ovarian malignancy. Additional effects of increasing the protein levels of ASM will further help reducing the risk of ovarian cancer.

In future work, it is necessary to explore the molecular mechanism for the function of the novel potential medicine for curing ovarian cancer. It is difficult to quantitatively assess the contribution of ASM in any particular case. The apoptosis function of ASM in cancer needs to be explored[27,29-33]. Furthermore, to understand the molecular mechanism of ASM for inhibiting the development of ovarian cancer, the combination with other molecules may be necessary[29-32].

Conclusions

Our data demonstrate that ASM is higher expressed than that in ovarian cancer and can inhibit the growth of ovarian cancer cells. ASM is the useful medicine for inhibiting the progression of ovarian cancer and can be a potential medicine for curing the cancer[34].

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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