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Advanced glycation end products correlate with breast cancer metastasis by activating RAGE/TLR4 signaling

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ABSTRACT

Introduction This study was aimed to investigate the mechanisms of advanced glycation end products (AGEs) in promoting invasion and metastasis of breast cancer.

Research design and methods Patients with 131 breast cancer were enrolled in a cohort and followed up to investigate the association between AGEs and metastasis. Serum AGE concentrations were detected by ELISA. Breast cancer MDA-MB-231 cells were exposed to generated AGE-bovine serum albumin (BSA). CCK-8 assay was used to select the non-cytotoxic concentrations of AGE-BSA. Small interfering RNA was used to knock down Toll-like receptor 4 (TLR4). Migration and invasion were evaluated by wound healing and transwell assays. Real-time PCR and western blotting were used to detect the gene expressions.

Results In the cohort study, metastasis incidence was significantly correlated with serum AGE concentrations in patients with breast cancer (adjusted OR=1.75, 95% CI=1.20 to 2.57, p=0.004). During follow-up, metastasis interval was significantly shorter in diabetic than non-diabetic subjects. In the in vitro study, AGE-BSA incubation significantly promoted migration and invasion of cancer cells in a concentration-dependent manner. AGE-BSA dramatically increased expressions of receptor for AGEs (RAGE), TLR4, myeloid differentiation factor (MyD88), matrix metalloproteinase 9 (MMP9), promoted nuclear translocation of nuclear factor κB (NFκB) p65, but decreased the expression of inhibitor of NFκB (IκBα). *TLR4* silencing significantly suppressed migration and invasion of cancer cells exposed to AGE-BSA. *TLR4* silencing reduced the expression of MyD88 and MMP9, as well as nuclear translocation of NFκB p65 but increased IκBα expression in AGE-BSA-incubated breast cancer cells.

Conclusions AGEs are correlated with metastasis of breast cancer. AGEs' promoting effects on migration and invasion of breast cancer cells via activating RAGE/TLR4/MyD88 signaling were suggested as the involved mechanism.

Significance of this study

What is already known about this subject?

- The risks in developing malignant cancers are higher in patients with type 2 diabetes (T2DM).
- Higher prevalence of cancer metastasis which lowered survival rate was higher in patients with T2DM.
- Receptor for advanced glycation end products (RAGEs) take responsibilities in metastasis of multiple human cancers.

What are the new findings?

- Metastasis incidence of breast cancer was correlated with serum advanced glycation end product (AGE) concentrations.
- Patients with T2DM have a shorter metastasis interval than non-diabetic breast cancer patients.
- AGEs strengthened invasion and migration capabilities of breast cancer cells via activating RAGE/Toll-like receptor 4 (TLR4) signaling pathway.

How might these results change the focus of research or clinical practice?

- RAGE/TLR4 could be a novel potential therapeutic target for breast cancer patients with diabetes.

INTRODUCTION

As the prevalence of western diets and static lifestyle, the occurrence of type 2 diabetes mellitus (T2DM) has been growing fast since recent decades and becomes one of the major public health concerns worldwide. Moreover, T2DM subjects exhibited higher risks in developing malignant cancers such as bladder cancer, colorectal cancer and breast cancer.[1] A population-based study suggested that the breast-cancer-specific mortality was significantly higher in women with diabetes than non-diabetic subjects though they received similar treatments.[2] Notably, cancer patients with diabetes exhibited higher prevalence of metastasis which was accepted as the major factor lowering survival rate.[3 4] However, the underlying mechanisms are still unclear.

Advanced glycation end products (AGEs) are characterized diabetic pathological metabolites produced after Maillard reactions among biomolecules including nucleic acids, lipids and amino groups.[5] It is now believed that as ligands, AGEs induce activation of multiple intracellular signaling cascades to result many pathophysiological outcomes after binding with receptors for AGEs (RAGEs).[6] Several downstream effectors of

RAGE pathway were associated with cancer immune, cell proliferation, angiogenesis and metastasis.[7] Targeted silencing of RAGE was reported to inhibit proliferation and invasion of breast cancer.[8] Thus, it is reasonable for us to speculate that AGEs produced in diabetic individuals may promote invasion and metastasis of breast cancer via RAGE-mediated signaling pathways.

After binding with AGEs, RAGE activates its downstream signaling such as Toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88) in cytoplasm. MyD88 further triggers the activation of nuclear factor κB (NFκB) which participates in cancer invasion and metastasis by regulating the expression of targeted genes such as matrix metalloproteinase 9 (MMP9) after nuclear translocation.[9] In this study, a human breast cancer cell line MDA-MB-231 cells were exposed to synthesized AGE-BSA at non-cytotoxic concentrations. Migration and invasion of cancer cells were evaluated. The involvement of RAGE/TLR4/MyD88/NFκB signaling pathway was investigated by specific *TLR4* silencing. Moreover, a follow-up cohort study was implemented to investigate the association between AGEs and metastasis in patients with breast cancer. Results from this study would add more knowledge to our current understanding of the mechanisms concerning promoted cancer malignance in diabetes.

MATERIALS AND METHODS

Cohort study

In the period from January 2013 to January 2016, 131 female patients diagnosed as breast cancer at stage II and III received surgical treatments discharged from Department of Medical Oncology, Shaanxi Provincial People's Hospital were enrolled. These patients were followed up till metastasis was diagnosed or till January 2021 for non-metastatic subjects. Diabetes was diagnosed according to current guideline.[10] The exclusion criteria were: age below 18 years or above 80 years, pregnant women, previous cancer history, cardiac/respiratory/renal/hepatic dysfunctions, autoimmune diseases and mental disorders. Medical records were collected from all subjects. Fasting venous blood samples were collected. Serum was harvested and subjected to AGE-BSA ELISA detections with a Human AGEs ELISA Kit (CUSABIO). All patients gave informed consent to participate in this study and agreed the research purposes of blood samples for our study.

AGEs-BSA preparation and detection

The protocol of AGE-BSA synthesis was adopted from ours and others' investigations.[11 12] Briefly, BSA (Gibco) at 100 mg/mL was incubated with D-glucose (Sigma) at 90 mg/mL and sodium azide at 1.0 mmol/L in phosphate buffer (pH=7.6) at 0.4 mmol/L at 37°C in sterilized condition for 14 days. Unreacted D-glucose was eliminated by dialysis of glucose-BSA against phosphate buffer. BSA prepared without D-glucose was used as control. Concentrations of AGE-BSA were detected with an AGE Assay Kit (BioVision).

Small interfering RNA (siRNA) transfection

TLR4 was knocked down by using siRNA transfection in this study. Specific siRNA against TLR4 (*TLR4*-siRNA) was adopted from one of our previous investigations.[13] The targeting sequence was 5′-GUCUCAGAUAUCUAGAUCU-3′. *TLR4*-siRNA and scrambled control siRNA (TaKaRa) were transfected into seeded cells by using Lipofectamine RNAiMAX Reagent (Thermo) per the instruction provided by the manufacturer. Cells were then cultured for 48 hours at 37°C before subsequent experiments.

Cell line and treatment

Human breast cancer MDA-MB-231 cell line was purchased from China Cellular Institute in Shanghai. Cells were maintained in Dulbecco's modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), L-glutamine at 2 mmol/L and antibiotics mix (Santa Cruz) at 37°C in an atmosphere consisted of 5% CO_2 and 95% fresh air. Cells were exposed to

Table 1 Baseline clinical characteristics of enrolled subjects

	Total (N=131)	Metastasis (–) (n=69)	Metastasis (+) (n=62)	P value
Age (year)	55.37±13.55	56.26±11.52	54.59±15.52	0.531
Cancer history (%)	7 (5.34)	6 (8.70)	1 (1.61)	0.118
Body surface area (m^2)	1.59±0.11	1.61±0.11	1.58±0.12	0.183
TNBC (%)	19 (14.50)	9 (13.04)	10 (16.13)	0.644
Ki-67 (%)	31.92±19.91	33.46±20.52	29.75±19.21	0.386
Diabetes (%)	42 (32.06)	22 (31.88)	20 (32.26)	0.964
Neoadjuvant chemotherapy (%)	16 (12.21)	10 (14.49)	6 (9.68)	0.400
Adjuvant chemotherapy (%)	117 (89.31)	63 (91.30)	54 (87.10)	0.436
Serum AGEs (μg/mL)	5.77±1.73	5.40±1.63	6.14±1.75	0.021
HbA1c (%)	7.47±1.77	7.52±1.80	7.43±1.82	0.903

AGEs, advanced glycation end products; HbA1c, glycosylated hemoglobin A1c; TNBC, triple-negative breast cancer.

AGE-BSA at 0, 10 and 20 µg/mL for 8 hours. Cells treated with AGEs at 20 µg/mL were also transfected with above *TLR4*-siRNA 24 hours prior to the exposure.

AGE-BSA toxicity assessment

To eliminate the bias brought by the impacts on cell viability, the AGE-BSA exposure applied to MDA-MB-231 cells were at non-cytotoxic concentrations. CCK-8 assay was carried out to select these non-cytotoxic concentrations. Cells were seeded to plates at 3000 per well. Cells were then treated with AGEs at 0, 10, 20, 40, 80 and 100 µg/mL for 48 hours, respectively. CCK-8 reagent (Solarbio) of 10 µL was added to each well 4 hours before culturing termination. A plate reader (Bio-Rad) was used to detect the optical density (OD) values at 450 nm. The cell viability was calculated as Treatment OD_{450}/Control OD_{450}. Then the non-cytotoxic concentrations were selected.

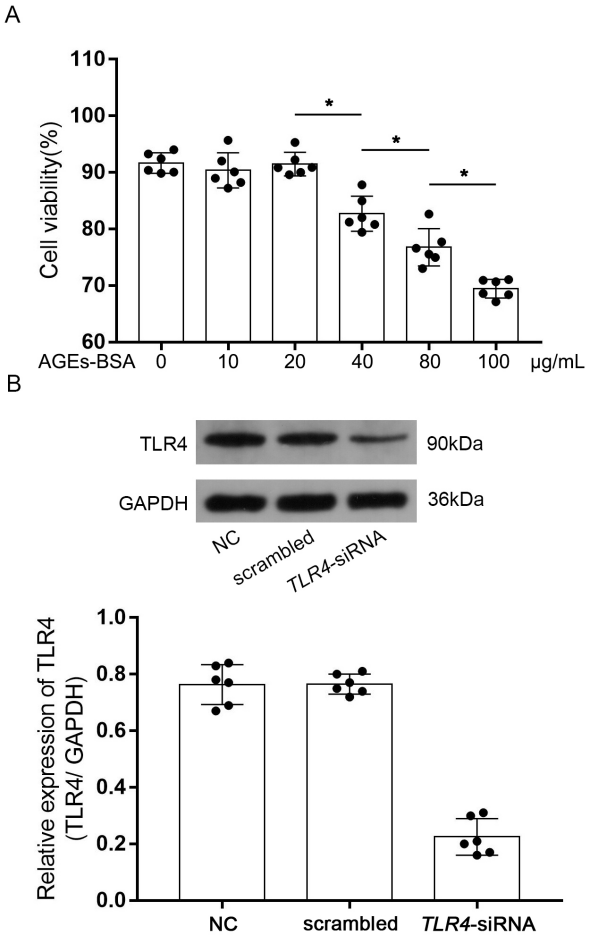

Figure 1 (A) Columns indicated the cell viabilities calculated based on CCK-8 assays in MDA-MB-231 cells incubated with advanced glycation end product-bovine serum albumin (AGE-BSA) at 0, 10, 20, 40, 80 and 100 µg/mL for 8 hours, respectively. AGE-BSA began to show significant inhibitory effects on cell viabilities starting with the concentration at 40 µg/mL. (B) Immunoblots of Toll-like receptor 4 (TLR4) and GAPDH were demonstrated. Columns indicated relative expression of TLR4 in negative control (NC) cells, scrambled small interfering RNA (siRNA) transfected cells and *TLR4*-siRNA transfected cells, respectively (n=6, *p<0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Cell migration evaluation

Wound-healing assay was used to evaluate cell migration. When at a confluence over 80%, cultured MDA-MB-231 cells were seeded in a 24-well plate and cultured for 24 hours. A 200 µL micropipette tip was used to scratch the confluent monolayer cells. After phosphate-buffered saline washing, cells were exposed to AGE-BSA at 0, 5, 10 and 20 µg/mL for 48 hours. Then a microscope was used to capture the images and the distance of migration was calculated.

Cell invasion assessment

Transwell assay was used to assess cell invasion. Cells at density of 2×10^5/mL were plated into a transwell chamber (Corning). Medium supplemented with 10% FBS was used to fill the lower chamber to induce chemotaxis. After 24-hour culturing, a cotton swab was used to scrap the non-migrated cells in the upper chamber gently. Methanol was used to fix the cells adhering to the lower surface of the insert, which were then stained by staining buffer containing 1% toluidine and 1% borax. A microscope was used to observe the membranes. The invaded cells were counted by analyzing six random fields.

Quantitative real-time PCR (qPCR)

Total RNA of cultured cells were extracted by using RNeasy plus mini kit (Qiagen) and reversely transcribed into cDNA by using ExScript RT Reagent Kit (Invitrogen) per the protocols provided by the manufacturers. SYBR Premix Ex Taq (Invitrogen) was used for qPCR. The conditions were the following: denaturing at 95°C for 15 s and annealing at 60°C for 60 s for 40 cycles. Primer for *RAGE* was: forward 5′- GGAC CCTTAGCTGGCACTTAGA-3′ and reverse 5′- GAGT CCCGTCTCAGGGTGTCT-3′; for *MMP9* was: forward 5′-TTGACAGCGACAAGAAGTGG-3′ and reverse 5′-TCACGTCGTCCTTATGCAAG-3′; for *TLR4* was: forward 5′- AATGGATCAAGGACCAGAGG-3′ and reverse 5′-CAGCCAGCAAGAAGCATCAG-3′; and for *MYD88* was: forward 5′-CGCCGGATGGTGGTGGTTGT-3′ and reverse 5′- TGTAGTCGCAGACAGTGATGAACC-3′. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was introduced as the internal reference. The primer for *GAPDH* was: forward 5′-GTCAGTGGTGGACCTGACCT-3′ and reverse 5′-AGGGGAGATTCAGTGTGGTG-3′.

Western blotting

Cell lysate were acquired after harvested cells were treated with RIPA buffer system containing protease inhibitor cocktail and PMSF (phenylmethanesulfonyl fluoride) at 1 mmol/L (Santa Cruz). Cytoplasmic and nuclear protein samples were extracted by using a Cytoplasmic Protein Extraction kit (Beyotime) and a Nuclear Protein Extraction Kit (Beyotime). Protein

concentration was determined by using a BCA Protein Assay Kit (Beyotime). After separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were then transferred to polyvinylidene fluoride membranes. After incubated with blocking buffer (Beyotime) for 1 hour, the membranes were probed by primary antibodies specific to RAGE (Abcam, 1:2500), TLR4 (CST, 1:2000), MyD88 (Abcam, 1:2500), MMP9 (Abcam, 1:2000), NFκB p65 (Abcam, 1:2000), IκBα (CST, 1:2500), GAPDH (Abcam, 1:5000) and Lamin B1 (Abcam, 1:2500) at 4°C for 10 hours. After Tris-buffered saline Tween 20 (TBST) washing, horseradish peroxidase-conjugated secondary antibodies were used to incubate the membranes at room temperature for 20 min. ECL Chemiluminescent Substrate Reagent Kit (Invitrogen) was used to develop the membranes. Image of the immunobands were captured and analyzed by a luminescent image analyzer.

Statistics

Data in this study were presented in (mean±SD) or percentage manners which were further analyzed by using SPSS 20.0 software. Participants were divided into two groups (metastasis and non-metastasis). Comparisons of baseline data between two groups were carried out by t-test (for parametric variables), Mann-Whitney U test (for non-parametric variables) and χ^2 test (for categorical variables). The association between metastasis and serum AGE concentration was evaluated by univariate and multivariate logistic regression models. Model 1 was unadjusted. Model 2 was adjusted for age, malignant cancer family history, body surface area and diabetes. Model 3 was adjusted for age, malignant cancer family history, body surface area, diabetes, Ki-67 value, triple-negative breast cancer (TNBC), neoadjuvant chemotherapy and adjuvant chemotherapy. When p<0.05,

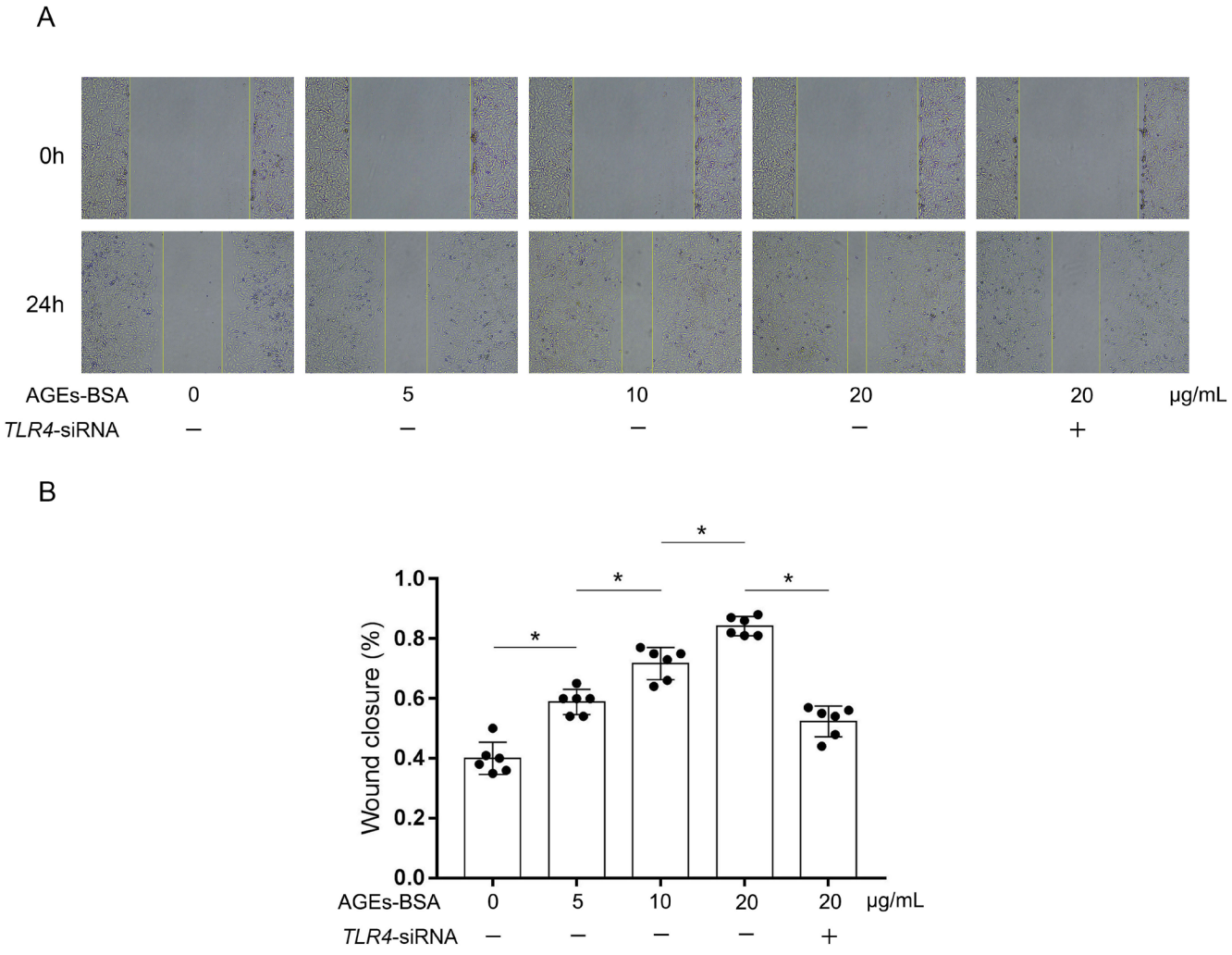

Figure 2 (A) Captured images of wound healing assay of MDA-MB-231 cells were demonstrated. (B) Columns indicated relative wound width of cells incubated with advanced glycation end product-bovine serum albumin (AGE-BSA) at 0, 5, 10 and 20 μg/mL for 8 hours, respectively. Several cells treated with AGE-BSA at 20 μg/mL were pretransfected with Toll-like receptor 4-small interfering RNA (*TLR4*-siRNA) (n=6, *p<0.05).

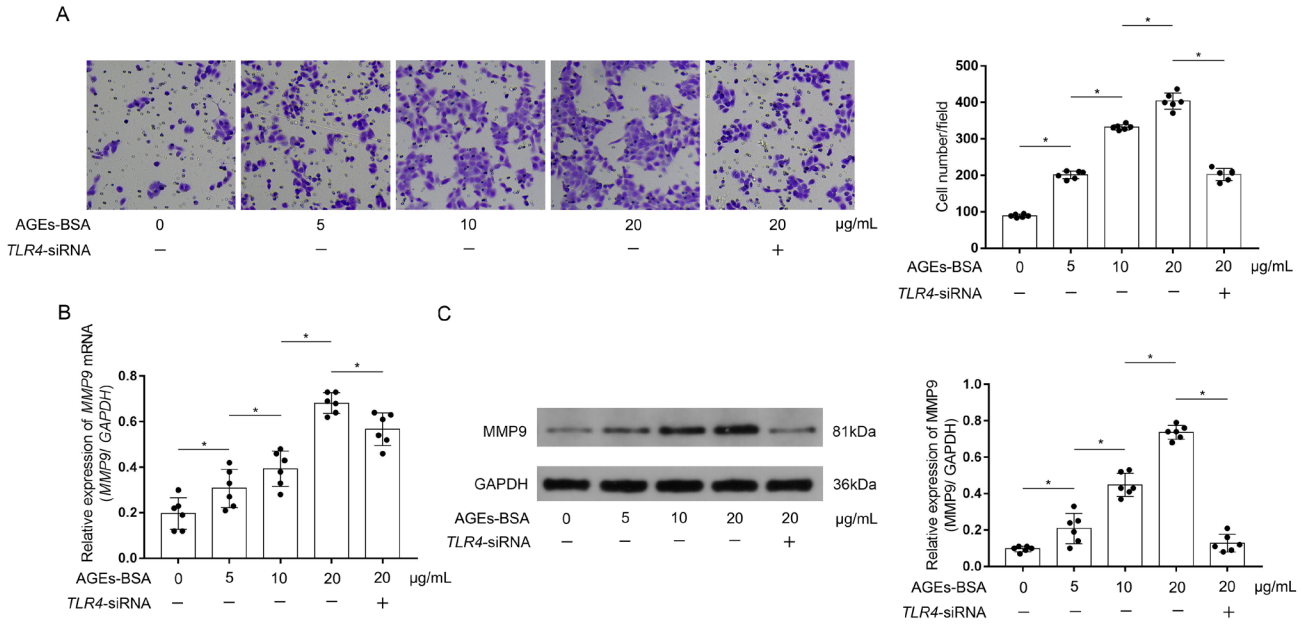

Figure 3 (A) Captured images of transwell assays were demonstrated. Columns indicated invaded cell number per field of MDA-MB-231 cells incubated with AGEs at 0, 5, 10 and 20 µg/mL for 8 hours, respectively. Several cells treated with AGEs at 20 µg/mL were pretransfected with *TLR4*-siRNA. (B) Columns indicated the relative expression levels of *MMP9 mRNA* expression levels in MDA-MB-231 cells. (C) Immunoblots of MMP9 and GAPDH were demonstrated. Columns indicated the relative expression levels of MMP9 in MDA-MB-231 cells (n=6, *p<0.05). AGE-BSA, advanced glycation end product-bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP9, matrix metalloproteinase 9; TLR4-siRNA, Toll-like receptor 4-small interfering RNA.

the compared differences would be considered statistically significant.

RESULTS

Cohort study

Baseline data of these patients were listed in table 1. Patients were divided into metastasis group and non-metastasis group. Differences of age, malignant cancer family history, body surface area, diabetes, Ki-67 value, TNBC, neoadjuvant chemotherapy and adjuvant chemotherapy were found not significant between two groups. Serum AGE concentration in metastasis group was significantly higher than non-metastasis group. As demonstrated in online supplemental table S1, the univariable Cox regression analysis (model 1, unadjusted) of AGE concentration was positively correlated with metastasis (OR=1.29, 95% CI 1.05 to 1.60, p=0.016). Multivariate regression analysis indicated serum AGE concentrations were significantly correlated with metastasis in model 2 (adjusted OR=1.78, 95% CI 1.22 to 2.60, p=0.003) and model 3 (adjusted OR=1.75, 95% CI 1.20 to 2.57, p=0.004). We further analyzed clinical characters in metastasis group which were demonstrated in online supplemental table S2. Age, body surface area, Ki-67 value, TNBC, neoadjuvant chemotherapy and adjuvant chemotherapy were found not significant different between two groups. Compared with patients without diabetes, patients with diabetes showed significant shorter metastatic interval and higher serum AGE concentrations. As demonstrated in online supplemental figure S1, serum AGE concentration was significantly and positively correlated with glycosylated hemoglobin A1c (HbA1c) level in enrolled subjects.

Selection of AGE-BSA concentrations and verification of *TLR4* silencing

As demonstrated in figure 1A, cultured MDA-MB-231 cells were exposed to AGEs at 0, 5, 10, 20, 40, 80 and 100 µg/mL for 48 hours. Cell viabilities were significantly inhibited by AGEs starting at 40 µg/mL, indicating AGE concentration above 40 µg/mL was cytotoxic against MDA-MB-231 cells. Thus, AGEs at 0, 05, 10 and 20 µg/mL were used in subsequent experiments. As demonstrated in figure 1B, *TLR4*-siRNA transfection effectively knocked down TLR4 expression in MDA-MB-231 cells.

Effects of *TLR4* silencing on migration of MDA-MB-231 breast cancer cells

MDA-MB-231 cells were exposed to AGEs at 0, 5, 10 and 20 µg/mL. As demonstrated in figure 2, evidenced by wound healing assay, AGE treatment strengthened the ability of cell migration of MDA-MB-231 cells in a concentration-dependent manner. However,

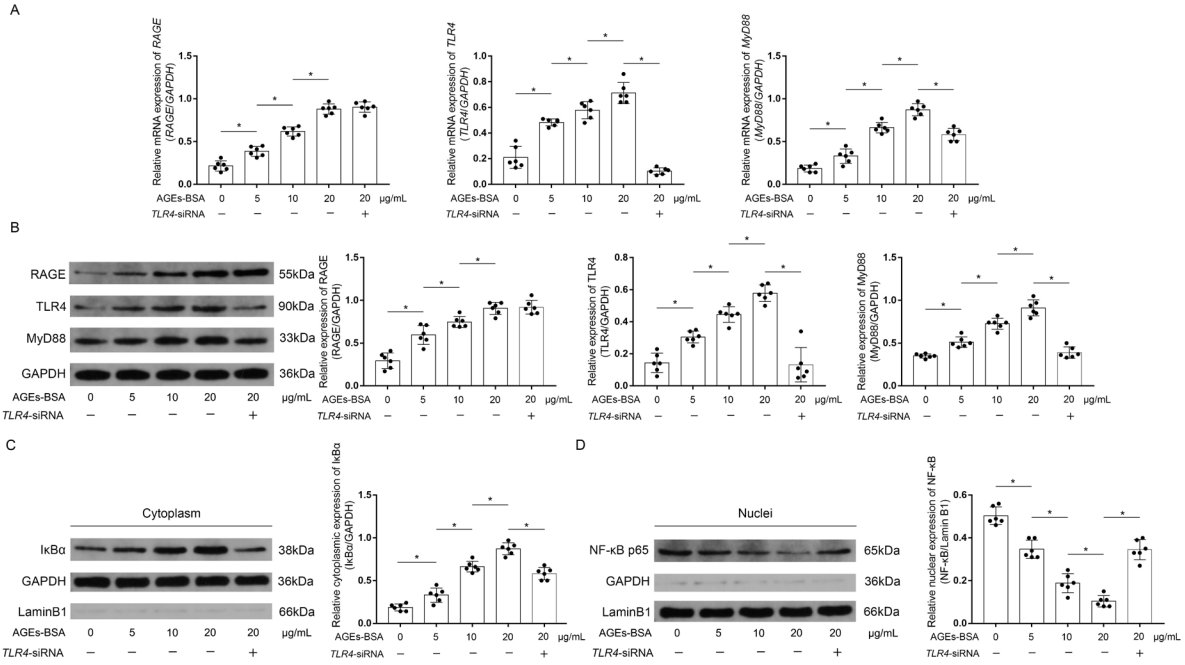

Figure 4 (A) Columns indicated the relative *mRNA* expression levels of *RAGE*, *TLR4* and *MyD88* in MDA-MB-231 cells. (B) Immunoblots of RAGE, TLR4 and MyD88 were demonstrated. Columns indicated the relative expression levels of RAGE, TLR4 and MyD88 in MDA-MB-231 cells. (C) Left panel showed detected immunoblots of IκBα, GAPDH and Lamin B1 in cytoplasmic protein samples. Columns indicated the relative expression of IκBα in cytoplasm in MDA-MB-231 cells. (D) Left panel demonstrated the detected immunoblots of NFκB p65, GAPDH and Lamin B1 in nuclear protein in MDA-MB-231 cells. Columns indicated the relative expression of IκBα in nuclei. MDA-MB-321 cells were incubated with AGE-BSA at 0, 5, 10 and 20 μg/mL for 8 hours, respectively. Several cells treated with AGE-BSA at 20 μg/mL were pretransfected with *TLR4*-siRNA (n=6, p<0.05). AGE-BSA, advanced glycation end product-bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκBα, inhibitor of nuclear factor κB; MyD88, myeloid differentiation factor; NFκB, nuclear factor κB; RAGE, receptor for advanced glycation end product; siRNA, small interfering RNA; TLR4, Toll-like receptor 4.

the AGE-enhanced cell migration was dramatically suppressed in *TLR4*-siRNA transfected MDA-MB-231 cells.

Effects of *TLR4* silencing on invasion of MDA-MB-231 breast cancer cells

Transwell assay was used to evaluate the invasion behavior of MDA-MB-231 cells, which were demonstrated in figure 3A. AGE exposure significantly enhanced the invasion in a concentration-dependent manner, which was attenuated by *TLR4* silencing in MDA-MB-231 cells. MMP9 is capable of degrading extracellular matrix (ECM) and considered taking responsibility in cancer invasion. As demonstrated in figure 3B, AGEs exposure dramatically increased MMP9 expression in MDA-MB-231 cells in an AGEs concentration- dependent manner. *TLR4* silencing significantly reduced MMP9 expression in AGE-incubated MDA-MB-231 breast cancer cells.

Effects of *TLR4* silencing on RAGE/TLR4/MyD88 signaling pathway activation in AGE-exposed MDA-MB-231 cells

As demonstrated in figure 4, AGE exposure significantly increased RAGE, TLR4 and MyD88 expressions but decreased IκBα expression in cytoplasm in a concentration- dependent manner. AGE exposure increased nuclear expression of NFκB p65 in breast cancer cells in a concentration-dependent manner. *TLR4* silencing, however, reduced MyD88 without affecting RAGE expression in AGE-incubated breast cancer cells. Moreover, *TLR4*-siRNA transfection increased IκBα in cytoplasm but decreased NFκB p65 expression in nuclei in MDA-MB-231 cells exposed to AGEs.

DISCUSSION

Breast cancer is currently one of the leading causes of cancer-associated death in women. It was reported that less than one-third patients diagnosed with metastasis failed to survive the first 5 years.[14] Evidences indicated that women with diabetes exhibited a 40% higher risk of breast cancer-specific mortality than non-diabetic patients.[15] Metastasis takes responsibility for the cancer-related mortality. However, the mechanisms are still in vague. In this study, 131 patients with breast cancer participated our follow-up investigation. Our results suggested that breast cancer patients with diabetes showed shorter metastatic interval than non-diabetic ones. Moreover, serum AGE concentrations in these patients were significantly higher. Our further investigation proposed serum AGE concentration was closely correlated with metastasis.

Thus, it is reasonable to speculate AGEs may be the pathological metabolite promoting cancer metastasis under circumstance of diabetes.

A large amount of AGEs are produced and accumulated in diabetes due to sustained glucose metabolism disorder. AGEs are the most representative metabolites with various biological activities. In this study, a human breast cancer cell line of MDA-MB-231 cells was exposed to AGE-BSA at non-cytotoxic concentrations. Our results suggested that AGE-BSA exposure significantly promoted migration and invasion abilities of MDA-MB-231 cells in a concentration-dependent manner. AGEs induce biological effects mainly via binding with their receptor RAGE, which takes responsibilities in regulating various cell functions such as inflammation, differentiation and immune response.[16] It was reported that RAGE- associated pathway activation was associated with the progression of several human cancers.[17]

TLR4/MyD88 signaling plays vital roles in various diseases including cancer. Our previous study showed TLR4 pathway was the downstream effector of RAGE signaling.[13] Activation of TLR4 would further mobilize MyD88. Previous investigations reported activation of TLR4/MyD88 pathway potentiated the metastatic behavior of human breast cancer cells.[18] MyD88 relives the inhibition of IκBα on NFκB and thus facilitates the activation of NFκB p65, which translocates into nuclei to initiate the transcription of targeted genes.[19] In this study, we found that AGE exposure significantly activated RAGE/TLR4/MyD88/NFκB signaling, resulting in elevated expression of MMP9 which expedites cancer migration and invasion by degrading ECM. Targeted *TLR4* silencing dramatically impaired migration and invasion of AGE-incubated breast cancer cells by deactivating this MyD88/NFκB signaling.

In summary, results from the present study suggested that the characterized diabetic metabolite AGEs participated in promoting breast cancer metastasis. Activation of RAGE/TLR4/MyD88 signaling pathway may involve in the underlying mechanisms. We believe that our study would be benefit in further understating the mechanisms of the clinical scenario that breast cancer was exacerbated by diabetes. Moreover, RAGE/TLR4/MyD88 signaling could be a novel potential therapeutic target for breast cancer patients with diabetes.

LIMITATION

There were several limitations of this study. First, though the human breast cancer cell line MDA-MB-231 was characterized with the abilities of invasion and metastasis, investigation on multiple cell lines would be more convincing. Second, establishment of an AGE-exposed nude mice bearing breast cancer animal model would add more evidence to support our conclusion. Third, the employment of high-performance liquid chromatography might be more accurate in evaluating AGE levels. Fourth, the detection of AGEs-BSA by using a human ELISA-based assay was not accurate enough. It would be more persuasive if fluorescence or chromatography-based methods were employed.

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Contributors ZL, YG, SP and YM implemented experiments and wrote the manuscript; QC and ZT accomplished statistical analysis; SP, YM, JL and CZ participated in implementing experiments; JJ and ZL revised the manuscript; LZ designed and participated in the statistical analysis; YZ and JJ reviewed and revised the manuscript.ZL is the guarantor.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study involves human participants and was approved by Ethics Committee of Shaanxi Provincial People's Hospital (Approval No 20121204HT). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request.

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