Glucose-derived AGEs promote migration and invasion of colorectal cancer by up-regulating Sp1 expression

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ABSTRACT

It is well established that the risk of colorectal cancer (CRC) is significantly increased in diabetic patients. As one of main forms of the advanced glycation end products (AGEs) that accumulate in vivo, glucose-derived AGEs play an important role in the pathogenesis of diabetic complications and may contribute to CRC progression. However, to date, both the contribution of glucose-derived AGEs to the course of CRC and the underlying mechanism are unclear. In the present study, the concentration of glucose-derived AGEs in the serum and tumor tissue of patients with CRC increased. A clinical data analysis demonstrated that the expression of the receptor for AGEs (RAGE), Specificity Protein 1 (Sp1), and matrix metalloproteinase – 2 (MMP2) was significantly higher in cancerous tissues compared with non-tumor tissue in Chinese Han patients with CRC and that RAGE expression was closely associated with lymph node metastasis and TNM stage. Furthermore, in vivo and in vitro experiments showed that AGEs promoted invasion and migration of colorectal cancer, and the AGEs treatment increased the expression of RAGE, Sp1, and MMP2 in a dose-dependent manner. A RAGE blocking antibody and an Sp1-specific siRNA attenuated the AGE-induced effects. Moreover, the AGEs treatment increased the phosphorylation of ERK, and reducing the phosphorylation level of ERK by MEK1/2 inhibitor decreased the expression of Sp1. In conclusion, glucose-derived AGEs promote the invasion and metastasis of CRC partially through the RAGE/ERK/SP1/MMP2 cascade. These findings may provide an explanation for the poor prognoses of colorectal cancer in diabetic patients.

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1. Introduction

With the change of life style in modern society, diabetes mellitus, with a young-onset trend, has caused a heavy social and economic burden [1] and is closely associated with the increased incidence and mortality of colorectal cancer [2], which is the fourth leading cause of cancer-related death worldwide [3]. Accumulating evidence suggests that hyperglycemia and hyperinsulinemia may contribute to the occurrence and progression of some types of cancers [4–6]. Additionally, some anti-diabetic drugs, such as metformin and thiazolidinediones, reduce the incidence of cancer and cancer-related mortality [7]. It is well

known that the progression of cancer is a complicated chronic process that includes proliferation, invasion, metastasis, and angiogenesis [8]. Here, we aimed to explore new mechanisms by which metabolites abnormally accumulate in diabetic patients to contribute to the invasion and metastasis of cancer.

As one of the most common metabolites that abnormally accumulate in diabetic patients, advanced glycation end products (AGEs) are implicated in some chronic complications of diabetes [9–13]. The accumulation of AGEs in human serum is not only related to the intake of high-temperature baking food and smoking but is also associated with age-related diseases and chronic inflammation related-diseases, such as Alzheimer's disease, cardiovascular disease, stroke, nephropathy, and cancer [14,15]. Epidemiological evidence demonstrates that the serum concentration of glyceraldehydes-derived AGEs in patients with colorectal cancer is increased and is closely related to an increased risk of rectal cancer [16]. However, whether glucose-derived AGEs accumulate in the serum and tumor tissues of patients with colorectal cancer has not been reported to date. AGEs not only directly damage the organization, structure and function through the chemical modification of

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biological macromolecules, including proteins, lipids, nucleic acids and the cross-linking of collagen [17], but they can also bind to the receptor RAGE on the surface of target cells to affect their functions. Many receptors interact with AGEs, among which RAGE plays the most important role in mediating the AGE-induced pathological effects [18]. After binding with its ligands, including AGEs, S100A4, and HMGB1, RAGE regulates multiple signaling pathways, such as the NF- κ B, Ras-MAPK, and Jak/Stat cascades, which are involved in inflammation, cell proliferation and motility [19,20]. Our previous study showed that glucose-derived AGEs promote the proliferation of colorectal cancer cells by binding to RAGE and up-regulating the expression of carbohydrate-responsive element-binding protein (ChREBP) under glucose-free conditions [21]. As the receptor for glucose-derived AGEs, RAGE was recently reported to be highly expressed in colorectal cancer tissues and was closely associated with the invasion, metastasis, and angiogenesis of colorectal cancer [22,23]. Nevertheless, the serum concentration of glucose-AGEs in colorectal patients and whether glucose-AGEs are implicated in the invasion and metastasis of colorectal cancer remain to be investigated.

As the founding member of the Sp/KLF family, Sp1 is considered a general transcription factor that regulates numerous 'housekeeping genes', which are widely involved in metabolism, cell proliferation and death [24,25]. As reported, Sp1 contributes to the growth of colon cancer, which is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) [26]. Many studies have indicated that Sp1 is over-expressed in various types of tumors, such as breast, gastric, thyroid, lung, brain (glioma), pancreatic and colorectal cancers, which regulate the expressions of genes closely related to the biological behavior of cancers, including MMP2, MMP9, VEGF, and E-cadherin [25]. Therefore, the overexpression of Sp1 in tumor tissues is closely associated with the invasion, metastasis and prognosis of many cancers. Interestingly, with the knockdown of RAGE, Sp1 expression in colorectal cancer cells is also reduced, which suggests that the activation of the RAGE receptor might regulate the expression of Sp1 and thereby contribute to colorectal cancer progression.

The present study was designed to explore the role of glucose-derived AGEs in the invasion and metastasis of colorectal cancer and the underlying mechanism. Our results confirmed that RAGE expression in the tumor tissues of patients with colorectal cancer was increased, with an increased risk of cancer invasion and metastasis, and that the serum concentration and cancer tissue content of glucose-derived AGEs were increased as well. Furthermore, a series of long-term experiments in vivo and in vitro demonstrated that the AGEs-RAGE signal promoted cancer cell invasion and metastasis by increasing the protein expression of Sp1 and its target gene MMP2. These results may help shed light on the relationship between diabetes and progression of colorectal cancer and its underlying mechanism.

2. Materials and methods

2.1. Reagents

The primary antibodies for RAGE, MMP2 were purchased from Abcam and antibodies against Sp1, AKT, p-AKT, ERK, p-ERK, JNK, p-JNK, p38, p-p38, Alexa Fluor®488 and Alexa Fluor®594 were purchased from Cell signaling technology. DAPI was purchased from Thermal Fisher Scientific (USA). U0126 (MEK1/2 inhibitor) was purchased from Selleckchem (Houston, USA).

2.2. Clinic specimens and measure of serum glucose-derived AGEs

160 sets of colorectal tumors and adjacent non-tumorous tissues (more than 5 cm away from primary tumor margin) were obtained from patients who underwent curative surgery in Ruijin Hospital affiliated to Shanghai Jiaotong University School of Medicine from 2012 to 2014. No preoperative radiotherapy or chemotherapy before surgery was performed. The pathological stage of tumor was evaluated strictly

according to the criteria of UICC TNM classification. Histological type of tumor was determined independently by at least two pathologists in a double-blind way. The blood samples of patients were collected with tubes containing EDTA before surgery. All of the related studies were approved by Medical Ethics Committee of Shanghai Ruijin Hospital. Serum was obtained and preserved at -80°C after blood was centrifuged at 3000 rpm, for 5 min. The same volume of every patient's serum was mixed with non-denatured loading for western blot analysis. The content of glucose-derived AGEs in serum was detected with glucose-derived AGEs-specific antibody, and the content of serum total protein was detected by Coomassie brilliant blue staining.

2.3. RNA extraction and real-time PCR

The total RNA of tissue samples and cell lines was extracted with TRIzol reagent (Invitrogen, USA), according to manufacturer's instruction. The isolated RNA was reverse transcribed to cDNA using reverse transcription kit (Promega, Madison, WI, USA). qRT-PCR analysis was carried out using SYBR Green reagent (Takara Bio Inc., Japan) and an Applied Biosystems 7500. The forward and reverse primers designed for RAGE were 5'-ACTACCGAGTCCGAGTCTACC-3' and 5'-CCCACCTTATTACGGACACTGG-3', respectively. The forward and reverse primers designed for GAPDH were 5'-GAGTCAACGGATTGGTCGT-3' and 5'-GACAAGCTTCCCGTTCTCAG-3', respectively.

2.4. Western blotting analysis

The protein lysates of cells or tissue specimens were prepared with RIPA buffer supplemented with protease inhibitors (Thermo Fisher Scientific, USA) and supernatant was obtained by centrifugation with 13,000 rpm for 15 min at 4°C . The protein concentration was measured using BCA Protein assay kit (Pierce, Rockford, USA). An equal amount of protein (20–50 μg) was separated by 10% SDS-PAGE gel and transferred to PVDF Membrane (Millipore, USA). After blocked with 10% non-fat milk in TBST at room temperature for 2 h, the membrane was incubated with primary antibodies overnight at 4°C , followed by incubation of secondary peroxidase-labeled antibody (Cell Signaling Technology, USA). Signals were detected by an enhanced chemiluminescence detection system.

2.5. Preparation of AGEs

AGEs were prepared and purified as described by Takeuchi et al. [27]. In brief, 50 mg/ml BSA (Sigma, USA) was incubated with 0.5 mmol/l D-glucose (Sigma, USA) under sterile conditions for 8 weeks. The unincorporated sugar was removed by dialysis against 0.2 mmol/l PBS (pH 7.4). 50 mg/ml nonglycated BSA was incubated under the same conditions except for the absence of D-glucose as a negative control. Preparations were tested for endotoxin using a Limulus Amebocyte Lysate (ACC, USA), and endotoxin was $<15\text{ EU/L}$.

2.6. Cell culture

All the colorectal cancer cell lines were purchased from ATCC (American Type Culture Collection) and kindly provided by Shanghai Digestive Surgery Institute. Leibovitz's L-15 medium containing 10% FBS (Gibco, USA), and 2 mM L-glutamine was used as culture medium for SW1116, SW480, and SW620. HCT116, Caco2, HT29, and LoVo were cultured in RPMI-1640 (Gibco, USA) supplemented with the same components. All the cell lines were cultured with or without 200 $\mu\text{g/ml}$ glucose-derived AGEs under 37°C , 5% CO_2 and saturation humidity. In order to block RAGE function, 5 $\mu\text{g/ml}$ RAGE-blocking antibody (Santa Cruz, USA) or mouse serum IgG (sigma, USA) were added for 1 h before the treatment of AGEs.

2.7. Cell viability assay

Cells were seeded at 2000 cells/well in 96-well plate for 24 h and were designed with six copies each group. After incubated in respective serum-poor medium (SPM) supplemented with 2.5% FBS in the presence of various concentrations of AGEs or non-modified BSA for three days, cell viability was assessed with CCK8 (Beyotime, China). After incubation with CCK8 at 37 °C for 2 h, OD was measured at 490 nm using spectrophotometer (Thermo Fisher Scientific, USA).

2.8. Stable gene transfection

Lentiviral expression vectors containing RAGE-specific shRNAs and green fluorescent protein (GFP) were constructed by GeneChem (Shanghai, China) and used to stably knock-down RAGE expression of LoVo cells. Briefly, LoVo cells were seeded on 6-well plates. 24 h later, cells were transfected with lentiviral expression vectors according to manufacturer's instructions (Shanghai GeneChem Co., Ltd.). For selection of stable clones, 5 µg/ml puromycin was administered for 7 weeks, followed by Western blot analysis. The RAGE shRNA duplexes were as follows: S1 forward: 5'-CCGGGTGCCAGGCAATGAACAGGAACTCGAGTTCCTGTTTCATTGCTGGCACTTTTGG-3', S1 reverse: 5'-AATTC AAAAAGTGCCAGGCAATGAACAGGAAGCTCGAGTTCCTGTTTCATTGCTGGCAC-3'; S2 forward: 5'-CCGGCACCTGACACATCTTGCAAACTCGAGTTTTCGAAGATGTGTCAGGTGTTTGG-3', S2 reverse: 5'-AATTC AAAA ACACCTGACACATCTTGCAAACTCGAGTTTTCGAAGATGTGTCAGGTG-3'.

2.9. Transwell migration and invasion assay

In order to assess the ability of invasion and migration, 3×10^5 cells/well were plated in transwell chamber (8 mm, 24-well format; Corning, Lowell, MA, USA) and cultured in complete medium for 24 h. Then the culture medium in upper chamber was changed to 200 µl respective SPM in the presence of 200 µg/ml glucose-AGEs or non-modified BSA. The upper chambers were precoated with Matrigel (BD Bioscience, CA, USA) or not. Each condition was carried out in triplicate. After 72 h of incubation, the cells on upper side of inserts were removed with cotton swab. The cells under the inserts were fixed with methanol and stained with 1% crystal violet for cell counting by an inverted microscopy.

2.10. Immunofluorescence

Cells were plated on Millicell® EZ slides (Millipore, USA) at 2000 cells/well and cultured in complete medium for 24 h. After incubated with or without 200 µg/ml AGEs for 3 days, cells were rinsed with $1 \times$ PBS for twice, fixed using 4% paraformaldehyde for 10 min at room temperature (30 min for human CRC tissues) and then increased permeability of cells using 0.1% Triton X-100 for 5 min at room temperature. Incubation of cells with primary antibody overnight at 4 °C and subsequently with fluorescent secondary antibody for 1 h at room temperature were performed in turns. Finally, the slides were washed with $1 \times$ PBS for 3 times, subsequently mounted with Prolong Gold with DAPI (Invitrogen, USA) and imaged on fluorescence microscope (Leica, Germany). The level of RAGE protein expression in tumor and normal tissues was independently examined by two pathologists who were blinded from the patients' clinical data. According to the semi-quantitative immunoreactivity score (IRS), staining intensity was evaluated as negative (0), weak (1), moderate (2), or strong (3), and percentage of positive cells was classified as follows: <10%(0), 10–25%(1), 25–50%(2), 50–75%(3), and >75%(4). The total score was the multiplication of positive cell score and staining intensity score. And cases were grouped as negative (≤ 2), weakly positive (2–5), and strongly positive (≥ 5).

2.11. Transient knockdown using siRNAs

Control and Sp1 siRNA were purchased from Obio Technology Co., Ltd. (Shanghai, China). The siRNA transfection was carried out using lipofectamine 2000 according to manufacturer's protocol. The control siRNA sequence was 5'-CCAACAGAUUAUCACAAUDDT-3'. The Sp1 siRNA sequence was 5'-AUUUGUGAUAAUCUGUUGGTTD-3'.

2.12. Gelatin zymography

To assess the matrix metalloproteinase (MMP) activities, the medium used for cell invasion was collected and centrifuged at 1000 rpm, 4 °C for 5 min. After determination of protein concentration, the samples were mixed with non-denatured gel sample loading buffer and subjected to electrophoresis on 10% SDS-PAGE gels containing 1 mg/ml gelatin (Sigma, USA). The gels were washed with the Novex zymogram renaturing buffer (Invitrogen, USA) for 30 min at room temperature and then incubated in Novex zymogram developing buffer (Invitrogen, USA) at 37 °C overnight. The Coomassie brilliant blue staining, and photographed were performed in turns.

2.13. Nude mouse xenograft model

Colorectal cancer xenografts were established in four-week-old male BALB/C nude mice (Institute of Zoology, Chinese Academy of Sciences of Shanghai). Briefly, 5×10^6 wild type or RAGE stably knock-down LoVo cells suspended in 100 µl PBS (pH 7.4) were slowly injected into the portal vein in 5 min under sterile condition. Three days later, 30 mg/kg sterile AGEs or nonglycated BSA were administered by intraperitoneal injection every other day. Mice were sacrificed two months later. CML was observed and photographed. All animal related operations were approved by the Institutional Animal Care and Use Committee (IACUC) at Shanghai Jiaotong University School of Medicine.

2.14. Statistical analysis

All of experiments were done in triplicate. Data were expressed as mean $\pm$ SD and analyzed by SAS 8.0 statistical software. Cochran-Mantel-Haenszel test was used to examine the differential expression between tumor tissue and adjacent normal tissue. Correlations between the level of RAGE expression in CRC tissues and clinicopathological parameters were analyzed by chi-square or Fisher's exact tests. Student's *t*-test was used to analyze the statistical differences between two groups. $P < 0.05$ and $P < 0.01$ were respectively considered as statistically significant.

3. Result

3.1. Co-expression of RAGE, Sp1, and MMP2 in cancer tissue and glucose-derived AGEs levels in the serum and tumor tissue of patients with colorectal cancer

To investigate the RAGE expression profile in colorectal cancer tissues, RT-PCR and immunofluorescence staining were performed on matched tumor and non-tumor tissues excised from 160 CRC patients without diabetes. RAGE mRNA expression was markedly higher in the tumor tissues than in the non-tumor tissues (Fig. 1A). Moreover, RAGE protein in the cell membrane demonstrated a higher expression in the invasive CRC tissues compared with the non-invasive CRC tissues (Fig. S1). Ninety-eight of the 160 (61.25%) colorectal cancer tissues showed RAGE positive staining. Further, a correlation analysis between RAGE expression and the clinicopathological features revealed that the increased expression of RAGE in the CRC tissues was significantly related to lymph node metastasis ($P = 0.025$) and TNM stage ($P = 0.01$) but not to other factors (Table 1).

Fig. 1. Co-expression of RAGE, Sp1, and MMP2 in clinic colorectal cancer tissues, as well as the level of glucose-AGEs in sera and cancer tissues. (A) RAGE (Receptor for advanced glycation end products) mRNA expression in colorectal cancer tissues and matched adjacent non-tumor tissues detected by qRT-PCR and normalized by GAPDH. (B) The level of glucose-AGEs in serum. Higher levels of glucose-AGEs in sera were detected in the colorectal cancer patients than in the healthy controls by western blot. (C) The confocal results showed that RAGE and Sp1 were both expressed in human normal tissues and colorectal cancer tissues. The scale bar is 25 μ m. (D) The tissue contents of the glucose-AGEs and of RAGE, Sp1 and MMP2 were simultaneously found to be highly expressed in human colorectal cancer tissues by western blot.

It is well established that AGEs accumulate under the states of hyperglycemia, age, inflammation, and in some cancers and that they contribute to many pathological sequelae [10,28]. Although Coomassie brilliant blue staining showed that the content of serum total protein in CRC patients was lower than that in normal control (Fig. S2), with age standardization, our study still revealed that the serum level of

AGEs in CRC patients without hyperglycemia was higher than that observed in healthy subjects (Fig. 1B), which suggests that AGEs might be involved in the progression of colorectal cancer.

In addition, our study revealed that RAGE and Sp1 were simultaneously expressed in human colorectal cancer tissues (Fig. 1C). Further, the expression levels of RAGE, Sp1, and MMP2, as well as the tissue content of AGEs, were higher in the tumor tissues than in the adjacent non-tumor tissues (Fig. 1D). Considering the higher concentration of AGEs in the serum of patients with colorectal cancer, all of the results indicate that AGEs, RAGE, Sp1, and MMP2 are involved in the progression of colorectal cancer.

Table 1
Association between RAGE expression and clinicopathological factors of colorectal cancer patients.

Variables	Number of cases	RAGE immunofluorescence staining		P
		Positive(high/low)	Negative	
Gender				
Male	107	64 (38/26)	43	0.596
Female	53	34 (18/16)	19	
Age(years)				
≥60	95	55 (31/24)	40	0.292
<60	65	43 (25/18)	22	
Differentiation				
Well to moderate	69	41 (23/18)	28	0.679
Poor	91	57 (33/24)	34	
Tumor location				
Colon	81	51 (29/22)	30	0.540
Rectum	79	47 (27/20)	32	
Tumor size				
≥5 cm	75	43 (27/16)	32	0.339
<5 cm	85	55 (29/26)	30	
Lymph metastasis				
Negative	70	36 (16/20)	34	0.025
Positive	90	62 (40/22)	28	
Distant metastasis				
Negative	151	91 (50/41)	60	0.295
Positive	9	7 (6/1)	2	
TNM stage				
I + II	70	35 (14/21)	35	0.010
III + IV	90	63 (42/21)	27	

3.2. AGEs promote colorectal liver metastasis in vivo

To choose suitable CRC cell lines for the in vivo and in vitro experiments, we detected the expression levels of RAGE among 8 CRC cell lines (LoVo, SW620, SW480, HCT116, RKO, SW1116, HT29, and Caco2). As shown in Fig. 2A and B, the expression abundances of RAGE were higher in the Lovo and SW620 cells, which were selected for further study.

Because RAGE mediates the AGE-induced physiological and pathological effects, we first transfected the LoVo cells with RAGE-specific shRNAs to stably knock-down RAGE expression (Fig. 2C) and explore its role in colorectal liver metastasis. According to a previous study [29], the xenograft model was established by a portal vein injection of wild type or RAGE-silenced LoVo cells and was then subjected to an AGEs intraperitoneal injection every other day for two months. The treatment with AGEs significantly promoted colorectal liver metastasis (Fig. 2D). Regardless of whether the animals received a BSA or the AGE intraperitoneal injection, the incidence of colorectal liver metastasis was reduced in the RAGE-silenced model compared with the wild type model (Fig. 2D, E). All of the results suggest that AGEs accelerate colorectal cancer progression by RAGE.

Fig. 2. Effect of glucose-AGEs on colorectal liver metastasis. (A) The RAGE (Receptor for advanced glycation end products) mRNA expression profile in eight colorectal cancer cell lines was examined by qRT-PCR and was normalized with GAPDH. (B) RAGE protein expression in the eight colorectal cancer cell lines. All experiments were repeated three times. (C) Stable suppression of RAGE expression in CRC cells. (D) The metastatic nodules in the livers of the different colorectal liver metastasis models with an intraperitoneal injection of BSA (30 mg/kg) or glucose-AGEs (30 mg/kg) every other day for 2 months. Isolated (upper row) and orthotopic liver (lower row) showing different faces of the same liver. (E) The quantification of the metastatic nodules in the liver of the different colorectal liver metastasis model treated with BSA or glucose-AGEs via i.p. (** $P < 0.01$).

Fig. 3. Effect of glucose-AGEs on the migration and invasion ability of colorectal cancer cells (200 $\times$). (A, B) Transwell assay. Representative photographs show the cells that traveled through the micropore membrane with or without matrigel. 200 μ g/ml glucose-AGEs increased the migratory and invasive cell numbers on the membranes, which were abated by a pretreatment with 5 μ g/ml of the RAGE-specific antibody for 1 h. (C, D) Histograms show the numbers of migratory cells (C) and invasive cells (D) after treatment with 200 μ g/ml glucose-AGEs or 200 μ g/ml non-modified BSA, compared with the control. All experiments were done in triplicate (* $P < 0.05$, # $P < 0.05$), and the results are presented as the mean $\pm$ SD.

3.3. AGEs promote the migration and invasion of colorectal cancer cells

It is well known that migration and invasion are crucial parts of advanced cancer progression. Consistent with the results of the *in vivo* experiments, 200 $\mu\text{g}/\text{ml}$ of the AGEs treatment for three days markedly increased the LoVo and SW620 cell migration and invasion rates (Fig. 3A–D). Pretreatment with a 5 $\mu\text{g}/\text{ml}$ RAGE-specific blocking antibody for 1 h counteracted the AGE-induced colorectal cancer cell migration and invasion (Fig. 3A–D) without any impairment on cell viability (Fig. S3A, B).

3.4. AGEs stimulate RAGE, Sp1, and MMP2 expressions in colorectal cancer cells

AGEs promote RAGE expression [30]. In the present study, a cell immunofluorescence assay demonstrated that RAGE expression on the cell membrane of LoVo and SW620 cells was up-regulated by AGEs (Fig. 4A, B). Furthermore, AGE treatment increased the protein expression of RAGE, Sp1, and MMP2 in a dose-dependent manner (Fig. 4C, D). Meanwhile, MMP2 activity was enhanced after AGE treatment (Fig. 4G). However, these AGE-induced effects were attenuated by a RAGE-

Fig. 4. Effect of glucose-AGEs on the expression of RAGE, Sp1, and MMP2, as well as MMP2 activity. (A, B) The immunofluorescence staining shows that glucose-AGEs induce RAGE expression on the cell membrane of the LoVo and SW620 cells, which was partially counteracted by pretreatment with a RAGE neutralizing antibody. The scale bar is 50 μm . (C, D) Different concentrations of glucose-AGEs promote the expression of RAGE, Sp1 and MMP2 in a dose-dependent manner in the LoVo and SW620 cells. (E, F) A RAGE neutralizing antibody reduced glucose-AGEs induced RAGE, Sp1 and MMP2 expression in the LoVo and SW620 cells. (G) A RAGE neutralizing antibody effectively reduced the glucose-AGE-induced MMP2 enzyme activity enhancement in the LoVo and SW620 cells.

specific blocking antibody pretreatment to some extent (Fig. 4E–G). These results indicate that AGEs up-regulate the expression of these three gene by binding to RAGE to influence the migration and invasion of colorectal cancer cells.

3.5. Sp1 mediates AGE-induced migration and invasion of colorectal cancer cells

As reported, Sp1 plays an important role in the progression of many cancers [25]. In our study, Sp1 was highly expressed in the cancer tissues of patients with colorectal cancer (Fig. 5A). To confirm the role of Sp1 in the AGE-induced migration and invasion of colorectal cancer, we knocked-down Sp1 expression in the LoVo and SW620 cells by siRNA (Fig. 5B). As expected, both the migration and invasion rates of the Sp1-silenced cells decreased, particularly under the AGEs treatment (Fig. 5C–F), which suggests that Sp1 mediates the AGE-/RAGE-induced migration and invasion of colorectal cancer cells.

3.6. Sp1 mediates AGEs/RAGE/ERK/MMP2 cascade in colorectal cancer

To further explore the underlying role of Sp1, the LoVo and SW620 cells were transfected with an Sp1 siRNA and were treated with 200 µg/ml AGEs for three days. AGEs increased the protein expression

of RAGE, Sp1, and MMP2 (Fig. 6A–D). As one of the Sp1 target genes, MMP2 degrades the extracellular matrix to facilitate the migration and invasion of cancer cells [31]. Sp1 knockdown partially decreased MMP2 expression and its enzymatic activity in the presence of AGEs, without a significant effect on RAGE expression (Fig. 6A, C, E). Further, we found that AGEs activated ERK, but not AKT, in the LoVo and SW620 cells (Fig. 6F) and that preincubation with U0126, a MEK1/2 inhibitor, reduced both the phosphorylation level of ERK and Sp1 expression (Fig. 6G), which indicated that the activation of ERK mediated the AGE-induced Sp1 expression.

4. Discussion

Diabetes is closely associated with the initiation and development of site-specific tumors, including cancers of the endometrium, breast, and colorectum [2,32]. As a diabetes-related cancer, colorectal cancer is the fourth most common cause of cancer-related death, and almost half of all patients with advanced colorectal cancer already suffer from or will develop colorectal liver metastasis [33]. Our present findings showed that glucose-derived AGEs increased the invasion and hepatic metastasis of colorectal cancer partially through the RAGE/ERK/Sp1/MMP2 cascade, which may give us a deeper insight into the relationship between diabetes and colorectal cancer progression.

Fig. 5. Effect of Sp1 on glucose-AGEs induced cell migration and invasion in colorectal cancer cells (200×). (A) Representative western blot images of RAGE expression in 28 paired CRC samples. (B) Sp1 expression was significantly suppressed by the transfection with Sp1 siRNA1 and siRNA2. The interference effect of Sp1 siRNA2 was better in both cell lines. (C, D) Transwell assay. Representative photographs show that the cells that traveled through the micropore membrane with or without matrigel; 200 µg/ml glucose-AGEs increased the migratory and invasive cell numbers on the membranes, which was counteracted by the Sp1 shRNA1 transfection. (E, F) Histograms show the number of migratory cells (E) and invasive cells (F). All of the experiments were done in triplicate (* $P < 0.05$, ** $P < 0.05$).

Fig. 6. Effect of Sp1 on glucose-AGEs induced RAGE and MMP2 expression, as well as the enhancement of MMP2 enzyme activity. (A, C) Interference of Sp1 expression in the LoVo and SW620 cells partially reverses the glucose-AGE-induced MMP2 expression. (B, D) The quantification of the RAGE, Sp1 and MMP2 expression in the LoVo and SW620 cells under different treatment conditions, as shown in the figures (* $P < 0.05$, # $P < 0.05$). (E) The reduced expression of Sp1 inhibits the glucose-AGEs-induced enhancement of MMP2 activity. (F, G) p-AKT, p-JNK, p-p38, p-ERK and Sp1 expressions were determined by a western blot analysis. The LoVo and SW620 cells were treated with BSA (200 µg/ml), glucose-AGEs (200 µg/ml), U0126 (10 µM), or both for 3 days.

As the products of the non-enzymatic glycation of amino-containing molecules under hyperglycemia *in vivo*, AGEs always accumulate in diabetes. AGEs used to be divided into six subtypes, including glucose-derived AGEs (AGE-1), glyceraldehyde-derived AGEs (AGE-2), glycolaldehyde-derived AGEs (AGE-3), methylglyoxal-derived AGEs (AGE-4), glyoxal-derived AGEs (AGE-5), and 3-deoxyglucosone-derived AGEs (AGE-6) [27]. Glucose-AGEs have been widely studied. Because diabetes is an important determinant of survival for colorectal cancer cases, some early researchers hypothesized that increased levels of AGEs in colorectal cancer patients with diabetes possibly contribute to the poor prognosis of the cancer [34,35]. In fact, previous studies corroborated that AGEs contribute to the advancement of many cancers,

such as hepatocellular carcinoma, melanoma, oral cancer, and breast cancer [15,36–38]. To date, the AGEs-RAGE axis is indicated to be associated with the clinical prognosis of colorectal cancer [39]. Recent epidemiological data show that glyceraldehyde-derived AGEs are enriched in the blood of patients with colorectal cancer, which is closely related to the increased risk of rectal cancer [16]. However, whether glucose-derived AGEs are involved in the course of colorectal cancer remains to be elucidated. Here, our research first uncovered that the glucose-derived AGE concentrations in both the circulation and in tumor tissues were increased in colorectal cancer and that an intraperitoneal injection of glucose-derived AGEs every other day for two months increased the incidence of colorectal liver metastasis in a nude mouse xenograft

model. Similar to the *in vivo* result, an *in vitro* AGE treatment for three days promoted the invasion and metastasis of colorectal cancer cells. Therefore, it is important to investigate the diabetes–cancer link.

RAGE, different from other AGEs receptors, including CD36, scavenger receptors class A type II, class B type I and AGE receptors 1, 2, and 3, which are involved in the detoxification of AGEs, mediate AGE-induced signaling process [40]. It is known that chronic inflammation is not only a key feature of diabetes [41,42] but also paves the way to cancer metastasis [43,44] and that RAGE bridges inflammation and cancer [45]. Thus, RAGE could be a candidate cancer-related gene. Recently, RAGE was detected in many human cancers, including brain, lung, breast, colorectal, and ovarian cancer, and it enhances cancer formation [46,47]. The overexpression of RAGE in colorectal cancer is positively correlated with colorectal cancer invasion and metastasis [23]. The result of our study on the relationship between RAGE expression and colorectal cancer progression in Chinese Han patients was consistent with those of previous studies in other ethnic patients [23,48]. The glucose-derived AGE treatment dose-dependently increased RAGE expression in the colorectal cancer cells. A RAGE-neutralizing antibody pretreatment partially reversed the AGE-induced colorectal cancer cell migration and invasion. Moreover, RAGE knock-down reduced the incidence of the AGE-induced colorectal liver metastasis in a nude mouse model. All of the findings indicate that RAGE, rather than other receptors of AGEs, mediates the effects of AGEs in the invasion and metastasis of colorectal cancer.

As a basal transcription factor implicated in the so-called housekeeping genes that contain GC boxes in their promoters, Sp1 is generally considered a negative tumor prognostic factor and is overexpressed in a number of human cancers, including colorectal, gastric, liver, pancreatic, breast, thyroid, brain (glioma), lung, and squamous cell carcinomas [25]. Many anti-inflammatory drugs, such as cyclooxygenase-2 inhibitors, aspirin, and tolfenamic acid, inhibit colon cancer cell and tumor growth by down-regulating Sp1 [26,49,50]. Recently, Qin et al. reported that Sp1 was up-regulated in colorectal cancer tissues, which silenced the tumor suppressor miR-149 by increasing its methylation level and predicted a poor clinical outcome [51]. Here, we also observed the overexpression of Sp1 in human colorectal cancer. A high expression of RAGE protein in CRC tissues was accompanied by the overexpression of Sp1 and MMP2. In a previous study [22], Sp1 expression was reduced when RAGE was knocked-down in the colorectal cancer cell line SW480. All of these data hint that Sp1 might mediate RAGE-dependent signal transduction. In our study, AGEs treatment increased the protein expression of RAGE, Sp1, and MMP2 in colorectal cancer cells and enhanced cell migration and invasion. Sp1 knockdown reversed the above effects to some extent, without an effect on AGE-induced RAGE expression. These results suggest that the binding of AGEs to RAGE regulates Sp1 expression, and Sp1 mediates the AGE-/RAGE-dependent cascade. Some studies demonstrate that many factors, including HIF1 α , ZEB2, and Sp1 itself, up-regulate Sp1 protein expression [52–54]. Specifically, the ZEB2-mediated up-regulation of Sp1, by enhancing its protein stability, induces the invasion of CRC cells. Here, we detected that the activation of ERK contributes to AGE-induced Sp1 expression. The MEK1/2/ERK signaling pathway was reported to be a complicated cascade that is involved in cancer metastasis [38]. However, the detailed mechanism underlying the increased Sp1 expression induced by AGEs remains unclear and needs to be further studied. As the effector molecule of Sp1, MMP2 degrades type IV collagen, the most abundant component of the basement membrane, to facilitate the metastasis of colorectal cancer [31]. As we know, Sp1 regulates the expression of many essential oncogenes, such as IGF, EGF, and PDGF [25], and possibly other Sp1 effectors exist.

Together, our results provide a novel insight into the poor prognosis of colorectal cancer complicated by diabetes. When diabetes coexists with colorectal cancer, glucose-AGEs/RAGE/ERK/Sp1/MMP2 signaling may contribute to the advancement of colorectal cancer progression, which may provide a rationale for targeting the glucose-AGE-RAGE axis as a novel strategy for clinical intervention.

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Conflicts of interest

The authors disclose no potential conflicts of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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