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Overexpressed GRP78 affects EMT and cell-matrix adhesion via autocrine TGF-β/Smad2/3 signaling

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ABSTRACT

Glucose-regulated protein of 78 kD (GRP78) is a multifunctional protein belonging to the heat shock protein 70 family. Overexpression of GRP78 triggered by environmental and physiological stresses is positively correlated with the occurrence and progression of various tumors, but the molecular mechanisms have not been well established. The present study indicated that overexpression of GRP78 in colon cancer cells could promote cell-matrix adhesion through the upregulation of fibronectin, integrin-β1 and phosphorylated FAK. Meanwhile, it resulted in a visible epithelial–mesenchymal transition in DLD1 cells, and the Snail-2 played the key role during the process. More importantly, the data indicated that GRP78 overexpression facilitated the expression and secretion of TGF-β1, which further activated the downstream Smad2/3 signaling module to effectuate the cell-matrix adhesion and epithelial–mesenchymal transition. Taken together, this study provides a novel molecular mechanism involving in the effects of GRP78 on colon cancer metastasis.

1. Introduction

Colorectal cancer is a common malignancy of human digestive system (Jemal et al., 2011), and liver metastasis of colorectal cancer is the main cause of therapy failure (Manfredi et al., 2006). The metastasis of epithelial cancer involves complex processes, which are related with the alterations of cell-ECM (extracellular matrix) adhesion and the conversion between epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) (Geiger and Peeper, 2009; Voulgari and Pintzas, 2009).

Enhanced cell adhesion to the ECM is an early step of cell migration. ECM composition is complicated and variable in specific tissues. As an important component of the ECM, the changes of fibronectin abundance in ECM have profound effects on cell adhesion (Clark et al., 1982; Hazlehurst et al., 2000). Integrin-β1, a transmembrane protein, is a major adhesion molecule to interact with ECM and regulates the signal transduction between inside and outside of cells (Piwko-Czuchra et al., 2009). Focal adhesion kinase (FAK), the downstream target of integrin, is a crucial signaling molecule to modulate cellular responses to integrin-mediated adhesion (Michael et al., 2009; Sieg et al., 1999).

EMT takes place during embryonic development and adult epithelial wound healing, and associates closely with the metastasis of epithelial tumors. In cancer progression, EMT is regulated by a numbers of transcription factors and signaling pathways. Snail-2, a zinc-finger transcription factor, is attributed to the activation of mesenchymal molecules like Vimentin and the repression of epithelial makers like E-cadherin during EMT. Victoria Bolós and co-workers have reported that snail-2 could bind directly to the E-box of E-cadherin promoter to inhibit E-cadherin expression (Bolós et al., 2003). TGF-β/Smad2/3 signaling is a ubiquitous regulatory signaling pathway in metazoan biology, and TGF-β is a key player of EMT in cancer progression and metastasis. It is synthesized in

Abbreviations: GRP78, glucose-regulated protein of 78 kD; EMT, epithelial–mesenchymal transition; MET, mesenchymal–epithelial transition; HSP, heat shock protein; ER, endoplasmic reticulum; CRC, Colorectal cancer; ECM, extracellular matrix; FAK, focal adhesion kinase; TGF-β, transforming growth factor β; TGF-β1, transforming growth factor β1; GFP, green fluorescent protein; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; α-SMA, α-smooth muscle actin.

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cytoplasm, and functions through autocrine and paracrine. Smad2 and Smad3, as downstream targets of TGF-β, are activated by type I transmembrane receptor of TGF-β through the direct phosphorylation. The phosphorylated Smad2 and Smad3 form trimers with Smad4. Then, the trimer translocates into the nucleus, in which it associates with transcription factors to regulate gene transcription (Massague, 2008).

The glucose-regulated protein of 78 kD (GRP78), a member of the heat shock protein (HSP) 70 family, harbors a signal peptide sequence targeting the endoplasmic reticulum (ER). It is implicated in modulating protein fold and assembly, misfolded protein degradation, and Ca^{2+} homeostasis (Lee, 2001; Zhang and Zhang, 2010). The expression of GRP78 can be induced by ER stress mainly resulted from glucose deprivation, hypoxia and acidosis, which frequently occur in solid tumors due to poor vascularization and shortage of nutrition (Lee, 2007). High expression of GRP78 in tumor cells contributes to the acquisition of several cancer hallmarks, such as cell proliferation (Cali et al., 2014), survival (Chang et al., 2012), immune resistance (Corrigall et al., 2009; Wang et al., 2007), and metastasis (Dong et al., 2011; Li et al., 2012). However, the mechanisms of these processes caused by GRP78 have not been very clear yet.

The present results showed that high expression of GRP78 in colon cancer cells elevated the expression of fibronectin, integrin-β1 and the phosphorylation of FAK and further enhanced the cell-matrix adhesion. In addition, Overexpressed GRP78 could increase the expression of mesenchymal markers of N-cadherin, Vimentin and α-SMA (α-smooth muscle actin), whereas the expression of epithelial marker E-cadherin was reduced. Moreover, the results implicated that the transcription factor Snail-2 stimulated by GRP78 played the key role in the context of EMT. We further demonstrated that the effects of GRP78 were achieved by facilitating the expression and secretion of TGF-β1, which subsequently activated the downstream of Smad signaling module. These findings provide a novel understanding of GRP78 in CRC metastatic progression and are of considerable therapeutic significance for malignant CRC.

2. Materials and methods

2.1. Materials

The medium and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, USA). Trizol, PrimeScript RT Master Mix and SYBR green PCR master mix were from Takara (Shiga, Japan). GRP78 antibody was bought from Abcam (Cambridge, UK). E-cadherin antibody was from Sino Biological, Inc (Beijing, China). FAK antibody was from MBL (Nagoya, Japan). The active conformation of integrin-β1 (12G10) was purchased from Millipore (Bedford, USA). GFP antibody was from Beyotime (Shanghai, China). N-cadherin and Vimentin were from Bioworld Technology (Minneapolis, USA). TGF-β1, Snail-2, integrin-β1, phospho-FAK (Tyr397) and phospho-smad2/3 antibodies were purchased from Cell Signaling Technology (Beverly, USA). GAPDH antibody was from Abmart (Shanghai, China). FITC-, TRITC- and HRP-conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, USA). Rhodamine Phalloidin was from Cytoskeleton, Inc (Denver, USA). The Triciribine, LY294002, Wortmannin, BAY11-7082 and SB431542 were from Sigma–Aldrich (St. Louis, USA).

2.2. Cell culture

Human colon cancer cell lines DLD1, HCT-116 and SW480 were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin. Human embryonic kidney 293 T cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (FBS) and 1% penicillin/streptomycin. All of those cell lines were obtained from the Chinese Type Culture Collection and cultured at 37 °C in a humidified tissue culture incubator containing 5% CO_2.

2.3. Cell transfection and selection

For stable cell lines selection, GRP78 overexpression or GRP78 shRNA plasmid was co-transfected with both psPAX2 and pMD2.G plasmids into 293 T cells using the calcium phosphate method at a ratio of 15:10:5 μg (for a 10-cm dish). Media containing virus were collected and concentrated using 100-kD ultrafiltration membranes (Millipore, USA). DLD1 cells were infected with the viruses in the presence of polybrene (8 μg/ml) for 24 h and subjected to puromycin (5 μg/ml) selection.

For transient transfection, GRP78 overexpression or GRP78 shRNA plasmid were transfected into HCT116 and SW480 cells using turbofect transfection reagent (Thermo) as recommended by the manufacturer. After 24 h of transfection, the cells were selected by puromycin (5 μg/ml) for 12 h.

Primer sequences of GRP78 were listed in the supplemental Table 1, and shRNA sequences of GRP78 were listed in the supplemental Table 2.

2.4. Quantitative real-time PCR

Total RNA was extracted from the cells using Trizol reagent (Takara, Shiga, Japan). 500 ng RNA was reverse transcribed into cDNA with PrimeScript RT Master Mix (Takara). All the RT-PCR samples were performed using SYBR Green PCR Master Mix (Takara) on an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, CA). Primer sequences were listed in the supplemental Table 3.

2.5. Western blot assays

After appropriate treatment, lysates of cells were prepared and centrifuged at 13,000 × *g* at 4 °C to remove cell debris. 80 μg of the obtained supernatant proteins were mixed with 5× SDS sample buffer, boiled for 5 min and separated by 10% SDS-PAGE before being transferred onto PVDF membranes (Millipore, USA). After blocking in 5% skim milk for 1 h, the membranes were rinsed and incubated overnight at 4 °C with the appropriate diluted primary antibodies. The membranes were subsequently incubated with HRP-conjugated secondary antibody for 2 h at room temperature. The bands were visualized using an enhanced chemiluminescence detection kit (Engreen, China) and radiographic film exposure.

2.6. Immunofluorescence analysis

Cells were plated on 6-well glass slides. After the required treatments, the cells were fixed in 4% paraformaldehyde in PBS for 30 min, and permeabilized in PBS containing 0.3% Triton X-100 for 10 min in the process of cytoplasmic protein staining. Next, slides were blocked in PBS containing 5% bovine serum albumin (BSA) for 30 min at 37 °C, and incubated with the primary antibodies at 4 °C overnight. Slides were then washed and incubated with corresponding FITC- or Rhodamine-conjugated secondary antibodies. DNA was identified by staining with DAPI. After three washes of PBS, slides were mounted in gelvatol for immunofluorescence analysis.

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