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BMP4 promotes a phenotype change of an esophageal squamous epithelium via up-regulation of KLF4

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ABSTRACT

Introduction: Barrett's esophagus is a metaplastic lesion. However, the cellular and molecular mechanisms involved are poorly understood. The aim of this study was to investigate the roles of KLF4 and BMP4 in the pathogenesis of Barrett's epithelium.

Materials and methods: Immunohistochemistry was used to analyse the expression of KLF4, BMP4, CDX2, MUC2 and MUC5AC in human esophageal specimens. Human esophageal squamous epithelial cells were subjected to bile acid treatment and used in transfection experiments. Quantitative real-time PCR and Western blot analysis were used to detect the expression of KLF4, BMP4, CDX2, MUC2 and MUC5ac.

Results: In human tissues, Barrett's epithelium strongly expressed BMP4, p-Smad1/5/8 and KLF4. Furthermore, bile acids increased the expression of BMP4, KLF4, p-Smad1/5/8, CDX2, MUC2 and MUC5ac in esophageal epithelial cells in a time-dependent manner. Moreover, we found that BMP4 up-regulated the expression of KLF4, CDX2, MUC2 and MUC5ac, but Noggin, a specific BMP4 antagonist, can block the expression of KLF4, CDX2, MUC2 and MUC5ac induced by BMP4. However, BMP4 cannot induce the expression of CDX2, MUC2 and MUC5ac in cells with KLF4 siRNA, and Noggin cannot block the expression of KLF4, CDX2, MUC2 and MUC5ac in cells transfected with the KLF4 expression vector.

Conclusion: Our results demonstrate that BMP4 promotes a phenotype change of an esophageal squamous epithelium via up-regulation of KLF4.

1. Introduction

Barrett's esophagus (BE) is a metaplastic lesion in which the normal squamous epithelium of the distal esophagus is replaced by columnar epithelium (Spechler and Goyal, 1996; Wang and Sampliner, 2008). Gastresophageal reflux disease (GERD), in which the esophageal mucosa is chronically exposed to bile salts and acids, is the major risk factor for BE (Jankowski et al., 2000; Taddei et al., 2014; Winters et al., 1987). The most abundant bile acids in the patient's refluxate are deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) (Cronin et al., 2011; Peng et al., 2014). These bile acids cause chronic inflammation and injury of the epithelium. It is believed to be involved in the transformation of the normal squamous epithelium to BE, and eventually in the progression to esophageal adenocarcinoma (EAC) after many years (Banerjee et al., 2016; Laczko et al., 2016; Ojima et al., 2015; Peng et al., 2014; Souza, 2016). However, the molecular events responsible for the transdifferentiation of epithelial cells of the esophagus to a columnar cell type are not well understood.

A few studies used serial analysis of gene expression (SAGE), a technique to compare the expression profile of BE with a normal squamous esophagus, to identify genes specifically involved in the switching process of normal squamous esophageal mucosa to metaplastic BE (Barrett et al., 2002; van Baal et al., 2005). Bone morphogenetic protein 4 (BMP4) was detected to be highly and uniquely expressed in the SAGE library of BE but not in a normal squamous epithelium (Barrett et al., 2002; van Baal et al., 2005). Palles et al. performed a genome-wide association study (GWAS) to identify variants associated with BE and also found BMP4 may be involved in the formation of BE (Palles et al., 2015). BMP4, a member of the transforming growth factor (TGF)-β family, and its downstream targets, including the BMP4 receptor, p-Smad1/5/8, and Smad 4, comprise the BMP4 signalling pathway (Feng et al., 2015; Hogan et al., 1994). The p-Smad1/5/8 protein, in combination with Smad 4, forms a complex that translocates into the nucleus, where certain target genes can be transcribed (Heldin et al., 1997; Kretzschmar and Massague, 1998; Zhang et al., 2015). The BMP4 signalling pathway plays an important role in cell proliferation, tissue differentiation, and embryonic development (Amita et al., 2013; Cabrera-Sharp et al., 2014; Lim et al., 2000; Que et al., 2006).

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Krüppel-like factor 4 (KLF4) belongs to the Krüppel-like factors (KLFs) family of conserved zinc finger-containing transcription factors, which are widely expressed in different organs and regulate numerous biological processes, including proliferation, terminal differentiation, and apoptosis (Cuttano et al., 2015; McConnell et al., 2007; Rowland and Peeper, 2006). The function of KLF4 has been investigated in detail in cancer formation and the induction of pluripotent stem cells (Huang et al., 2015; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wei et al., 2016). However, the role of KLF4 in BE had been neglected in the development of BE. Based on the above studies, we hypothesized that KLF4 and BMP4 may cooperatively promote a phenotype change of an esophageal squamous epithelium. To further examine this hypothesis, we investigated the possible alteration of KLF4 expression in response to the change of BMP4 signalling. Furthermore, we detected the combined influence of BMP4 signalling and KLF4 on the downstream molecular markers in the development of BE. As we predicted, we found that BMP4 promotes a phenotype change of an esophageal squamous epithelium via up-regulation of KLF4.

2. Material and methods

2.1. Clinical specimens

The study enrolled cases from healthy volunteers ($n = 22$, males% = 52%, mean age: 55.7 years, range: 37–68 years) and from individuals with BE ($n = 25$, males = 57%, mean age: 52.4 years, range: 30–66 years). 2 biopsy specimens were obtained from each case. And eventually we got 44 pieces of normal esophageal tissues and 50 pieces of BE tissues. All of the BE specimens lack either dysplasia or cancerization. Fresh endoscopic biopsy specimens were fixed in 10% formalin. Paraffin sections (4 μm thick) were routinely stained with haematoxylin. Histological slides were estimated blindly and independently by two experienced gastrointestinal pathologists. None of the patients had taken antibiotics, bismuth compounds, any H_2-blockers, or proton-pump inhibitors (PPI) in the last 2 weeks before entering the study. The experiments using human materials were approved by the Bioethical Committee of the Southwest Hospital, and informed consent was obtained from all patients. Human specimens were examined histologically and by immunohistochemistry only.

2.2. Cell culture and reagents

Three cell lines, including HET-1A (an immortalized human esophageal squamous epithelial cell line; American Type Culture Collection (ATCC), Manassas, VA, USA), TE-1 (a human squamous cell carcinoma line; China Center for Type Culture Collection (CCTCC), China), and OE33 (a human esophageal adenocarcinoma cell line; American Type Culture Collection (ATCC), Manassas, VA, USA) were used in this study. All cells were cultured at 37 °C in a humidified incubator containing 5% carbon dioxide (CO_2). HET-1A cells were cultured in a BEBM (Lonza, Walkersville, MD, USA) medium supplemented with matched cytokines (Lonza, Basel, Switzerland). TE-1 cells and OE33 cells were cultured in an RPMI-1640 (Invitrogen, Carlsbad, CA, USA) medium supplemented with 2 mM glutamine and 10% foetal bovine serum (FBS). Deoxycholic acid (DCA) and dimethyl and sulph-oxide (DMSO) were obtained from Sigma-Aldrich (St Louis, MO, USA). DCA was dissolved in DMSO. Three kinds of cells were grown in a medium containing final concentrations of 200 μM DCA at a neutral pH (pH 7.1) for 4, 8 and 12 h. The purpose of using DCA was to simulate the reflux of duodenal contents with bile acid profiles in the gastresophageal refluxate of human BE patients (Kauer et al., 1997). Total RNA and protein were extracted and subjected to different analyses.

After reaching ~70% confluence, HET-1A cells were incubated with 100 ng/mL recombinant Noggin (an antagonist of BMP4) (R&D Systems, Minneapolis, MN, USA) or 100 ng/mL recombinant human BMP4 (R&D Systems, Minneapolis, MN, USA) for up to 48 h. Inhibition of BMP4 signalling was achieved by the addition of Noggin, while its activation was achieved by the addition of BMP4. Then, cells were either harvested to extract protein or dropped on glass slides for immunofluorescent staining.

2.3. RNA interference and lentivirus-mediated over-expression

A knockdown of KLF4 expression was accomplished by transfecting HET-1A cells with siRNA (GenePharma, Shanghai, China). We screened 3 effective siRNAs against KLF4 to obtain the KLF4-siRNAs mixture. The optimal concentration of siRNA (50 nM) for transfection and time for KLF4 inactivation (48 h) were assessed. HET-1A (3×10^5) cells were equally plated in six-well tissue culture plates. After 24 h, the cells were transfected with 50 nM of KLF4 siRNA for 48 h using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection, the medium was removed and replaced with a normal growth medium overnight. Then, proteins were extracted and subjected to analysis.

The cDNA coding for KLF4 (GenBank accession number NM_004235) was cloned into the lentiviral vector (LV-KLF4) (GeneChem, Shanghai, China). Lentiviral vectors were packaged by transfecting into 293T cells using Lipofectamine 2000 according to the manufacturer's instructions. HET-1A (3×10^5) cells were transduced with the lentivirus containing KLF4 by polybrene. 48 h after infection, 2 μg/mL of puromycin was added to the media for 2 weeks to select the lentivirus infected cells. The Western blot was used to detect the expression of KLF4 in the stable cell lines.

2.4. Immunohistochemistry

Human specimens were examined histologically and by immunohistochemistry only. Immunohistochemistry was performed using paraffin-embedded blocks. Freshly cut sections were deparaffinized in xylene and rehydrated through sequential graded ethanol steps. After three rinses in PBS (5 min each), the sections were dipped in 3% H_2O_2 for 30 min to suppress endogenous peroxidase activity. After rinsing in PBS, the sections were incubated with normal goat serum(cwbiotech, Beijing, China)at 37 °C for 15 min to block nonspecific antibody binding. Then, sections were incubated with primary antibodies for 2 h at room temperature (RT) and rinsed with PBS three times (5 min each). This was followed by incubation with secondary biotinylated antibodies (Beyotime Biotechnology, Shanghai, China), and bound antibodies were reacted using an avidin-biotin peroxidase complex method using Elite ABC Kit (Vectorlabs, CA, USA). Then, the slides were counterstained with haematoxylin. Finally, slides were examined under light microscopy by two experienced pathologists who were blinded to our study. Immunohistochemical staining for each marker was evaluated on a three-point scale (complete loss of expression, −; normal expression, +; over-expression, ++).

2.5. Immunofluorescence cytochemistry

Samples from HET-1A cells cultures were prepared for immunofluorescence examination. After fixating in 4% paraformaldehyde for 20 min at RT, the coverslips were rinsed in PBS and permeabilized with 0.1% Triton X-100 for 10 min at RT. Nonspecific antibody binding was blocked by placing the coverslips in 10% normal goat serum for 30 min at RT. Coverslips were then incubated for 16 h at 4 °C with buffer (control) or primary antibody. After washing, binding of the primary antibodies was detected by a 1:250 dilution of fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) conjugated secondary antibodies (Dako, Glostrup, Denmark). Coverslips were washed three times for 3 min in PBS before mounting in a 1:1000 dilution of medium with 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI; Beyotime Biotechnology, Shanghai, China). Cell images were recorded using a Nikon C1si confocal microscope equipped with three diode lasers that