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Research Paper

Gefitinib for Epidermal Growth Factor Receptor Activated Osteoarthritis Subpopulation Treatment

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ABSTRACT

Osteoarthritis (OA) is a leading cause of physical disability among aging populations, with no available drugs able to efficiently restore the balance between cartilage matrix synthesis and degradation. Also, OA has not been accurately classified into subpopulations, hindering the development toward personalized precision medicine.
In the present study, we identified a subpopulation of OA patients displaying high activation level of epidermal growth factor receptor (EGFR). With *Col2a1-creER*^{T2}*; Egfr*$^{f/f}$ mice, it was found that the activation of EGFR, indicated by EGFR phosphorylation (pEGFR), led to the destruction of joints. Excitingly, EGFR inhibition prohibited cartilage matrix degeneration and promoted cartilage regeneration. The Food and Drug Administration (FDA)-approved drug gefitinib could efficiently inhibit EGFR functions in OA joints and restore cartilage structure and function in the mouse model as well as the clinical case report.
Overall, our findings suggested the concept of the EGFR activated OA subpopulation and illustrated the mechanism of EGFR signaling in regulating cartilage homeostasis. Gefitinib could be a promising disease-modifying drug for this OA subpopulation treatment.
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1. Introduction

Osteoarthritis (OA) is one of the most prevalent musculoskeletal disorder affecting millions of people worldwide. In 2008, it was reported that 27 million Americans were afflicted by OA [1]; the number is projected to double by 2030 [2]. In China, 10% of males and 18% of females over the age of 60 were reported to have OA-related symptoms [3].

Precision medicine, or personalized medicine, is an innovative approach to tailoring disease prevention and treatment to the individual patient on the basis of a person's genes, lifestyle, and environment. However, the underlying concept of precision medicine has not yet been applied in OA as most treatments are designed as a “one-size-fits-all-approach” even with millions of patients suffering from the disease. This may be due to our limited understanding of the disease pathogenesis and mechanism; thus, more in-depth mechanistic studies need to be conducted.

Previous studies showed that the epidermal growth factor receptor (EGFR) signaling network played a role in cartilage development and OA [4–8]. Transforming growth factor-α (TGF-α), an activator of EGFR signaling, was reported to be involved in animal OA models, as well as in human [9–12]. These studies indicated the involvement of EGFR in cartilage anabolic and catabolic activities.

Cartilage damage represents the most prominent pathological feature of OA that inevitably leads to joint dysfunction [13]. Disturbances of the balance between catabolic and anabolic activities is one of the

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main characteristics of OA cartilage; therefore, the restoration of the balance is the key factor for treating OA [14, 15]. However, currently there are no effective therapeutic drugs available for OA in clinics [16]. The non-steroidal anti-inflammatory drugs (NSAIDs) only have a palliative effect, mainly aimed at alleviating the pain instead of addressing the underlying cause of OA disease progression [17]. Recently, the disease-modifying OA drugs (DMOADs) in development were introduced [14, 15] but few of them affected both anabolic and catabolic aspects and showed convincing disease-modifying efficacy [14]. Therefore, it would be beneficial to find a drug that could simultaneously and synergistically regulate both cartilage matrix synthesis and degradation.

In the present study, we examined the activation level of EGFR in human patients, and utilized *Col2a1-creER^{T2}; Egfr$^{f/f}$* mice to investigate the effect of EGFR on OA development. Intra-articular delivery of EGFR inhibitor gefitinib, encapsulated in chitosan microsphere, was applied to treat OA in the mouse model. Moreover, the regulating mechanism of EGFR was investigated.

2. Methods and Materials

2.1. Collection and Preparation of Human Tissues

Cartilage specimens were from OA patients undergoing total knee replacement surgery or non-osteoarthritis trauma patients who were undergoing arthroscopic knee surgery. The harvested cartilage tissue samples were fixed, embedded in paraffin, sliced (7 μm) and mounted on positively charged slides for immunohistochemistry and safranin O staining.

2.2. OA Animal Model and Gefitinib Treatment

For *Col2a1-creER^{T2}; Egfr$^{f/f}$* and *Egfr$^{f/f}$* male mice, tamoxifen (20 mg/ml) was administrated at the age of 2 months through intraperitoneal injection (5 μl/g) for 5 days. DMM surgery was performed 5 days after tamoxifen withdrawal, following the instructions as described previously [18]. The mice were sacrificed at 8 and 12 weeks after surgery and the joints were harvested. DMM surgery was also performed on normal 2-month old C57BL/6 male mice. For the sham surgery group, the knee joints were opened and then sutured without any treatment. For the systemic drug delivery experiment, DMM mice received either gefitinib (5, 25, 50 mg/kg) or vehicle control (normal saline, 0.9% NaCl) *via* oral gavage at 1-week post-surgery, once per day, for 8 weeks. For intra-articular treatment, chitosan microspheres with gefitinib (CM-Gefitinib) or CM in normal saline were injected into OA joints of mice, once every three days. The injections initiated at day 3 post-surgery and continued for 8 weeks until tissue samples were harvested for analysis. Each experimental group included a total of 6 mice.

2.3. Murine Tissue Fixation and Histology Processing

At the time of harvest, mice were euthanized and the surgically manipulated knee joints were dissected with the femur and tibia intact to maintain the structural integrity of the joint. Tissues were fixed and then decalcified in 10% (*w*/*v*) ethylene diamine tetraacetic acid (EDTA) solution. Subsequently, samples were embedded, sliced (7 μm) and numbered from 1 to 20. Sections numbered 5, 10 and 15 were stained with safranin O for OARSI scoring, while No. 6, 11, 16 sections were stained with Hematoxylin-Eosin (HE). Unstained sections were utilized for immunostaining.

2.4. OARSI and Synovial Inflammation Scoring of Murine Cartilage

Semi-quantitative histopathological scoring system established by the OA Research Society International (OARSI) was performed for grading mouse cartilage degeneration [19]. Summed OARSI scores were applied. Synovium was examined using the synovial inflammation grading system [20, 21]. Grading was performed by three blinded observers. The three grades for each section were averaged, and the data from each group of mice were collated.

2.5. Immunostaining

Paraffin sections for immunohistochemistry were treated with 0.4% pepsin (Sangon Biotech, Shanghai, China), 3% (*v*/v) hydrogen peroxide in methanol, 1% (*w*/*v*) BSA, primary antibodies and secondary antibodies subsequently. The DAB substrate system (ZSGB-bio, Beijing, China) was used for color development. Hematoxylin staining was utilized to reveal the cell nuclei. For quantitative analysis, 3 sections from different samples were selected for each group, and the positive/total cell ratio were calculated for each section.

Sections for immunofluorescence were incubated with 0.3% (*v*/v) Triton X-100, 1% (*w*/*v*) BSA, primary antibodies, corresponding secondary antibodies conjugated to Alexa Fluor 488 fluorescent dyes (Invitrogen) and DAPI subsequently. Images were viewed and captured under a confocal microscopy system (Olympus, BX61W1-FV1000, Japan and YOKOGAWA CV1000, Japan).

2.6. Primary Cultures of Mouse Chondrocytes

Mouse articular cartilage was obtained from the femoral condyles and tibial plateaus of postnatal day 5–6 C57BL/6 mice, and digested with 0.2% (w/v) collagenase overnight, as described previously [22]. Chondrocytes were maintained as a monolayer in DMEM/F12 supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C, 5% CO_2 environment. Cells between 0 and 3rd passage were utilized for experiments.

2.7. TGF-α and Gefitinib Treatment and In Vitro Gene Knockout

Chondrocytes were treated with 10 μM gefitinib or/and 10 ng/ml TGF-α in the culture medium. After 48 h, RNA and protein extraction were performed.

For *in vitro* gene knockout, isolated chondrocytes from *Col2a1-creER^{T2}; Egfr$^{f/f}$* mice were treated with 4-hydroxytamoxifen (1 μM) for 48 h before RNA and protein extraction. All *in vitro* experiments and assays were repeated 3 times.

2.8. qPCR Analysis

mRNA was extracted and reverse-transcripted followed by qPCR process. The relative level of expression of each target gene was calculated using the $2^{-\Delta\Delta}$Ct method. Each qPCR was performed on at least 3 different experimental samples with 3 technical replicates per sample. The representative results are displayed as target gene expression normalized to the reference gene *Gapdh*. Error bars represent one SD from the mean of technical replicates.

2.9. Western Blot Analysis

The proteins of human and mouse chondrocytes were directly extracted and the concentrations were determined using the BCA Protein Assay Kit (Pierce #23227). The extracted proteins were then separated on SDS-PAGE gels and transferred onto a polyvinylidene difluoride membrane. After blocked in 1% (*w*/*v*) BSA for 1 h at room temperature, the membrane was incubated with appropriate primary antibodies and horseradish peroxidase (HRP) conjugated secondary antibodies. The chemiluminescent signal was generated using western blot detection reagents (ECL, Beyotime Biotechnology, China and FDbio, Hangzhou, China) according to the manufacturer's protocol.

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