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Original Article

LFG-500, a novel synthetic flavonoid, suppresses epithelial–mesenchymal transition in human lung adenocarcinoma cells by inhibiting NLRP3 in inflammatory microenvironment

Dan Yang [a, 1], Xin Cao [a, 1], Fan Wang [a, 1], Haijing Jiang [a], Dingding Feng [a], Hao Guo [a], Lei Du [a], Yingliang Jin [b], Yansu Chen [b], Xiaoxing Yin [a, **], Chenglin Li [a, *]

[a] Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Jiangsu Center for the Collaboration and Innovation of Cancer Biotherapy, Xuzhou Medical University, Xuzhou, PR China
[b] School of Public Health, Xuzhou Medical University, Xuzhou, PR China

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ABSTRACT

Increasing evidence indicates that inflammatory microenvironment facilitates tumor metastasis. Here, we found that LFG-500, a novel synthetic flavonoid, significantly inhibited epithelial–mesenchymal transition (EMT) in human lung adenocarcinoma A549 and H1299 cells co-cultured with LPS-challenged THP-1 cells or cultured in THP-1 cell-derived conditioned medium. Moreover, we found that TNF-α is a direct and decisive factor for promoting EMT and LFG-500 suppressed TNF-α-induced EMT and cell motility. *NLRP3* knockdown inactivated NLRP3 inflammasome, which subsequently inhibited EMT and blocked cell migration, indicating that TNF-α-induced EMT requires the NLRP3 inflammasome. LFG-500 inhibited the activation of the NLRP3 inflammasome, thus inhibiting EMT. Moreover, LFG-500 treatment significantly inhibited metastasis *in vivo* by downregulating NLRP3 expression. Importantly, we found that NLRP3 was highly expressed in high-grade lung adenocarcinoma and that its expression was correlated with lymph node metastasis. NLRP3 and vimentin levels were significantly increased in matched metastatic lymph nodes. Moreover, a significant positive correlation was observed between their levels. Together, these results suggest that LFG-500 markedly suppresses EMT by inhibiting the NLRP3 inflammasome in the inflammatory microenvironment and that NLRP3 is a potential biomarker of lung adenocarcinoma metastasis.

Introduction

Lung cancer, the most common cancer worldwide, is responsible for >1.5 million deaths every year [1]. Despite remarkable advances in lung cancer therapy, the average five-year survival rate of patients with lung cancer is very low, i.e., only 16% in the USA and <10% in the UK [2]. Histologically, adenocarcinoma, the most common type of lung cancer, always metastasizes at an early stage [3], which is in turn responsible for its high mortality [4]. Therefore, it is important to improve effective therapeutic strategies for preventing metastasis in patients with lung adenocarcinoma.

Epithelial–mesenchymal transition (EMT) is an important event in the initiation of cancer metastasis [5,6], during which epithelial cells lose their apical–basal polarity and develop a mesenchymal phenotype. During EMT, epithelial carcinoma cells undergo phenotypic changes that increase their motility and invasive capacities, thus facilitating their migration from primary foci to distal sites [7]. Molecular changes underlying EMT include the expression of epithelial and mesenchymal markers and transcription factors that regulate EMT [8].

Inflammation increases the risk of cancer and promotes carcinogenesis by providing bioactive molecules from cells infiltrating into tumor microenvironment [9]. Of these bioactive molecules, inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, are important constituents of inflammatory microenvironment and are believed to induce

Abbreviations: CM, conditioned medium; H&E, hematoxylin and eosin; IRS, immunoreactivity score; IL-1β, interleukin-1β; IL-6, interleukin-6; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NLR, NOD-like receptors; TMA, tissue microarray; TNF-α, tumor necrosis factor-α.

* Corresponding author. Tongshan Road 209#, Xuzhou 221004, PR China. Fax: +86 516 83262630.
** Corresponding author. Tongshan Road 209#, Xuzhou 221004, PR China. Fax: +86 516 83262630.
E-mail addresses: yinxx@xzhmu.edu.cn (X. Yin), licl@xzhmu.edu.cn (C. Li).
[1] These authors contributed equally to this work.

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metastasis by promoting EMT [10,11]. TNF-α is involved in the pathogenesis of cancer [12] and is often overexpressed in the inflammatory microenvironment. Importantly, recent research has shown that TNF-α is the major inflammatory cytokine involved in Snail stabilization, which is essential for inflammation-induced EMT and cancer cell migration, invasion, and metastasis [13]. However, detailed mechanisms underlying the induction of EMT in cancer cells in the inflammatory microenvironment remain unclear.

NOD-like receptor (NLR) family pyrin domain-containing 3 (NLRP3) is a member of a nucleotide-binding domain and leucine-rich repeat-containing protein family of intracellular sensors. Activation of NLRP3 inflammasome, which includes NLRP3, the adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment domain, and the effector protein procaspase-1, promotes the activation of caspase-1 and secretion of inflammatory cytokines such as IL-1β and IL-18 [14]. Recent studies have shown that NLRP3 inflammasome plays an important role in the development of different cancers [15–17]. Moreover, NLRP3 inflammasome promotes the metastasis of hepatocellular carcinoma cells [18]. However, its role in the progression of lung adenocarcinoma, especially during EMT, is unclear.

In recent years, antitumor activity of flavonoids, particularly their anti-metastatic activity, has been widely recognized and investigated [19,20], indicating their potential clinical use in cancer therapy. Our previous studies have shown that LFG-500 ($C_{30}H_{32}N_2O_5$; Fig. 1A), a novel synthetic flavonoid, exerts effective anti-inflammatory and anticancer effects [21,22]. Particularly, recent data have shown the potential anti-metastatic effects of LFG-500 [23]. Findings of the present study showed that LFG-500 markedly suppressed EMT in lung adenocarcinoma cells by downregulating the NLRP3 inflammasome in the inflammatory microenvironment. Importantly, our results suggest that NLRP3 is a potential biomarker of lung adenocarcinoma metastasis and can be used as a therapeutic target.

Materials and methods

Co-culture system

Human lung adenocarcinoma A549 and H1299 cells were co-cultured with 10 μg/mL LPS-treated THP-1 cells at a ratio of 1:5 in six-well tissue culture plates containing Transwell inserts (0.4-μm-pore-size polycarbonate membrane, #3412; Corning Costar, Cambridge, MA), as reported previously [24]. Next, the co-cultured A549 and H1299 cells were exposed to different concentrations (2, 4, or 6 μM) of LFG-500 for 24 h.

Preparation of conditioned medium derived from LPS-challenged THP-1 cells

THP-1 cells were incubated with 10 μg/mL LPS for 24 h, and culture supernatant was centrifuged at 4000 rpm at 4 °C for 15 min to collect conditioned medium (CM) according to a previous study [25]. Next, A549 and H1299 cells were treated with THP-1 cell-derived CM and different concentrations (2, 4, or 6 μM) of LFG-500 for 24 h.

Transfection

A549 and H1299 cells were transfected with *NLRP3* shRNA (5′- gcCUUCUUGGUAGGAGUGGAA-3′ [sense] and 5′- UUCCACUCCUACCAAGAA GGC-3′ [antisense], #34189; Shanghai GeneChem Co., Ltd., Shanghai, China), according to the manufacturer's instructions.

In vivo tumor metastasis assay

A549 cells were diluted in PBS to achieve an appropriate concentration (1×10^6 cells/mL), and 0.2 mL of the cell suspension was injected into athymic BALB/c nude mice through the tail vein [26]. The mice were randomized into the following three groups (n = 5/group): blank group, normal saline group (control group), and 30 mg/kg LFG-500 group. After 24 h of inoculation, LFG-500 was intravenously injected into the mice every alternate day for 20 days. Next, the mice were weighed and sacrificed. Their lungs were rapidly excised, washed, and fixed in Bouin's solution. The number of metastatic nodules on the lung surface was counted under a dissecting microscope. Moreover, histological sections of the lungs were stained with hematoxylin and eosin (H&E) to confirm the presence of malignant nodules and to determine the location and extent of micrometastatic foci.

Immunohistochemical analysis

NLRP3 and vimentin staining in tissue microarray (TMA) specimens was scored independently by two pathologists (who were blinded to the clinical data) by using a semiquantitative immunoreactivity score (IRS), as reported previously [27]. Category A documented the intensity of immunostaining on a scale of 0–2, with a score of 0 indicating negative staining, 1 indicating weak staining, and 2 indicating strong staining. Category B documented the percentage of immunoreactive cells on a scale of 1–4, with a score of 1 indicating 0%–25% immunoreactive cells, 2 indicating 26%–50% immunoreactive cells, 3 indicating 51%–75% immunoreactive cells, and 4 indicating 76%–100% immunoreactive cells. IRS was obtained by multiplying scores from categories A and B and ranged from 0 to 8. NLRP3 or vimentin expression was classified as low (IRS, 0–3) or high (IRS, 4–8).

Other materials and methods

This information has been mentioned in Supplementary material.

Results

LFG-500 inhibits EMT in human lung adenocarcinoma cells in the inflammatory microenvironment

To investigate the effects of LFG-500 on EMT in human lung adenocarcinoma cells in the inflammatory microenvironment, we established a co-culture system that simulated physiological conditions. After co-cultivation for 24 h, A549 cells showed an EMT-like morphology characterized by the loss of their normal cobblestone-like appearance and formation of fibroblast-like spindle shape. Treatment with LFG-500 (at 4 or 6 μM, which did not affect cell viability; Supplementary Fig. S1A) inhibited these EMT-like changes in co-cultured A549 cells (Fig. 1B). Because EMT is associated with enhanced cellular motility, we evaluated the effects of LFG-500 on the migration of co-cultured A549 cells. We found that the rate of wound closure increased significantly in co-cultured A549 cells and that treatment with LFG-500 (4 or 6 μM) significantly delayed wound closure (Fig. 1C). Similarly, LFG-500 prevented the migration of co-cultured A549 cells across the Transwell chamber (Fig. 1D). Importantly, co-cultivation decreased the expression of epithelial markers E-cadherin and ZO-1 and increased the expression of mesenchymal markers N-cadherin and vimentin. However, treatment with LFG-500 reversed these changes (Fig. 1E). These results indicate that LFG-500 inhibited the migration of A549 cells co-cultured with LPS-challenged THP-1 cells and altered the expression of EMT markers in these cells. Similar inhibitory effects of LFG-500 on EMT and cell mobility were also observed in co-cultured H1299 cells (Supplementary Fig. S2).

Next, we examined the effect of CM derived from LPS-challenged THP-1 cells, which simulates an inflammatory microenvironment *in vitro* [24]. LFG-500 remarkably suppressed the mobility of A549 and H1299 cells cultured in THP-1 cell-derived CM (Fig. 2A and B, and Supplementary Fig. S3A). Moreover, LFG-500 significantly reversed the changes in the expression of EMT markers in the cells cultured in THP-1 cell-derived CM (Fig. 2C–G and Supplementary Fig. S3B). In addition, LFG-500 treatment significantly decreased the levels of Snail, Slug, and ZEB1 (Fig. 2H and Supplementary Fig. S3B), which are the key regulators of EMT [28]. These results suggest that LFG-500 inhibited EMT in and suppressed the motility of human lung adenocarcinoma cells in the inflammatory microenvironment.

TNF-α in THP-1 cell-derived CM promotes EMT

To explore mechanisms underlying the inhibitory effects of LFG-500 on EMT induced by the inflammatory microenvironment, we detected the major inflammatory factor in THP-1 cell-derived CM