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・Title: Periostin secreted by cancer-associated fibroblasts promotes cancer progression and drug resistance in non-small cell lung cancer

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Abstract:

Cancer-associated fibroblasts (CAFs) are important components in the tumor microenvironment, and we sought to identify effective therapeutic targets in CAFs for non-small cell lung cancer (NSCLC). In this study, we established fibroblast cell lines from the cancerous and non-cancerous parts of surgical lung specimens from patients with NSCLC and evaluated the differences in behaviors towards NSCLC cells. RNA sequencing analysis was performed to investigate the differentially expressed genes between normal fibroblasts (NFs) and CAFs, and we identified that the expression of periostin (POSTN), which is known to be overexpressed in various solid tumors and promote cancer progression, was significantly higher in CAFs than in NFs. POSTN increased cell proliferation via NSCLC cells' ERK pathway activation and induced epithelial-mesenchymal transition (EMT), which improved migration *in vitro*. In addition, POSTN knockdown in CAFs suppressed these effects, and *in vivo* experiments demonstrated that the POSTN knockdown improved the sensitivity of EGFR-mutant NSCLC cells for osimertinib treatment. Collectively, our results showed that CAF-derived POSTN is involved in tumor growth, migration, EMT induction, and drug resistance in NSCLC. Targeting CAF-secreted POSTN could be a potential therapeutic strategy for NSCLC.

・Keywords: lung cancer, periostin, cancer-associated fibroblasts, tumor microenvironment

Abbreviations:

αSMA: alpha-smooth muscle actin

CAF: cancer-associated fibroblasts

FAP: fibroblast activation protein

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

EMT: epithelial-mesenchymal transition

MAPK: mitogen-activated protein kinase

NF: normal fibroblasts

NSCLC: non-small cell lung cancer

POSTN: periostin

TME: tumor microenvironment

Introduction

Treatment strategies for non small cell lung cancer (NSCLC) have greatly improved with the advent of therapies targeting oncogenic driver mutations or immune checkpoint molecules [1]. However, it is common for most molecular-targeted therapies to initially show great effect before cancer cells eventually develop drug resistance. Efforts are being made to overcome this acquired drug resistance.

It is essential to consider that tumor growth is determined by cancer cells and the tumor microenvironment (TME), which consists of various cellular and non-cellular components, when considering the mechanism of cancer progression and treatment strategies [2]. Among TME stromal cells, cancer-associated fibroblasts (CAFs) are key players due to their abundance in most solid tumors and active crosstalk with cancer cells [3]. CAFs provide paracrine signals and alter tumor tissues' extracellular matrix components to encourage tumorigeneses: promoting the initiation of tumor formation, tumor growth, angiogenesis, metastasis, and therapeutic resistance [4].

To understand these features of CAFs, we compared expression profiles of CAFs with those of normal fibroblasts (NFs) and identified genes that were upregulated in CAFs. Among them, we focused on periostin (POSTN), a disulfide-linked 90 kDa secretory protein [5]. POSTN, originally isolated as an osteoblast-specific factor, is usually absent in normal adult tissues but is highly expressed in injured tissues, inflammatory sites, and tumor stroma [6]. POSTN is overexpressed in various solid tumors, such as lung, breast, colorectal, ovarian, pancreatic, prostate, and liver cancer, and promotes cancer development and progression [7]. Furthermore, since high POSTN levels in serum are associated with patient prognosis for various malignancies [8-11], it is of growing concern as a valuable biomarker.

While it is known that POSTN is mainly secreted from CAFs in TME [12], the function of POSTN in cancers remains unclear. In this study, we demonstrate that CAF-derived POSTN plays an important role in tumor progression and therapeutic resistance of NSCLC cells, suggesting that it is a potential therapy target for lung cancers.

Material and Methods

Cell Lines and Reagents

Four human NSCLC cell lines, A549, HCC827,HCC4006 (American Type Culture Collection), and EBC-1 (RIKEN Cell Bank, Tsukuba, Japan) were used in this study. The study used paired cell lines of NFs and CAFs isolated from a normal lung part and a non-necrotic tumor part, respectively, of lung cancer patients' surgical specimens [13]. All human cell lines were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) at 37˚C in a humidified incubator under 5% CO_2 gas. Additional reagents used in this study include cisplatin (CDDP) (AdipoGen Life Sciences), osimertinib (AZD9291), SB431542 and SCH772984 (Selleckchem), and recombinant human POSTN, transforming growth factor (TGF)-β1 and TGF-β3 (R&D Systems). All experiments were performed with mycoplasma-free cells.

Western Blotting

Cell lysate was extracted from cell lines with lysis buffer, a mixture of RIPA buffer, phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich), and Complete Mini (Roche). Western blot analysis was performed using the following primary antibodies: rabbit anti-human fibroblast activation protein (FAP), alpha-smooth muscle actin (αSMA), E-cadherin, vimentin, N-cadherin, ZEB1, mitogen-activated protein kinase (MAPK), phospho-MAPK (Erk1/2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology), and rabbit anti-human POSTN (Abcam). Anti-rabbit IgG-conjugated horseradish peroxidase (Santa Cruz Biotechnology) was used as a secondary antibody. Specific signals were detected on membranes using the ECL Prime Western Blotting Detection System (GE Healthcare) and LAS-3000 (Fujifilm).

Transwell Migration Assay

Transwell inserts (Corning) were used to evaluate the migratory capacities of NSCLC cells *in vitro*. A total of 4×10^4 cells (A549 and HCC4006) in 300 μL serum-free media were

added to the upper chamber, and 800 μL medium containing 10% FBS with or without 5 × 10^4 fibroblasts was added to the lower chamber. After 24-h incubation at 37˚C, cells on the upper surface were removed with a cotton swab and cells that migrated were fixed with methanol and stained using 0.5% crystal violet (Sysmex). Migrated cells were enumerated in eight randomly selected fields under a microscope.

Colony Formation Assay

NSCLC cells were seeded at 2 × 10^4 cells (A549) or 1 × 10^4 (HCC827) cells per well in 6-well plates and cultured for 7 days. They were treated with or without cisplatin or osimertinib at the indicated doses, and NFs or CAFs (1 × 10^5 cells) were placed in the top chamber of transwell polyethylene terephthalate membrane cell culture inserts (Sarstedt). Cells were washed with PBS and fixed with 4% formalin for 20 min followed by staining with 0.2% crystal violet. Plates were then rinsed with water and dried prior to scanning.

RNA Sequencing

Total RNA was extracted from NFs and CAFs using the RNeasy mini Kit (Qiagen), and the RNA Integrity Number (RIN) of samples was assessed with TapeStation Instrument (Agilent Technologies). RNA sequencing libraries were prepared using TruSeq RNA Access Library Prep Kit (Illumina). Sequencing was performed on the Illumina HiSeq system (Illumina) in a 2×150 bp paired-end sequencing protocol. All sequence reads were converted to FASTQ format using bcl2fastq software. CLC Genomics Workbench 20.0.2 (Qiagen) was used to count the reads mapped onto each gene via RNA-seq analysis pipeline. Human genome (hg38) was used as a reference sequence. TPM (Transcripts per Million) was calculated for further normalization to account for sample variations. The sequencing coverage and quality statistics for each sample are summarized in Supplementary Table 1.

Immunohistochemical Analysis

Dissected lung cancer tissues were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4-μm-thick sections. The sections were deparaffinized and rehydrated and endogenous peroxidase was inhibited by a 5-min incubation with 3.0% H_2O_2 solution. Sections were blocked with normal horse serum and then incubated with anti-POSTN primary antibody (Abcam) at 4˚C overnight. After a brief wash, sections were incubated with the second antibody (Vector Laboratories) for 30 min at room temperature. Antibody binding was detected using the ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories), and sections were counterstained with Mayer's hematoxylin. For immunofluorescence, The sections were incubated at 4◦C overnight with anti-POSTN primary antibody and anti-αSMA. Next, the preparations were incubated for 1 hour with anti-rabbit secondary Alexa Fluor 488 conjugated antibody and anti-mouse Alexa Fluor 555 conjugated antibody (CST). The preparations were mounted in a Prolong DAPI Mounting Medium (Thermo Fisher Scientific).

Single-Cell RNA Sequencing Data Analysis in Clinical Tissue Samples

Accession number GSE131907 [14] identified a single-cell RNA sequencing data set of 208,506 single-cell mRNA expression profiles from 44 patients pathologically diagnosed with lung adenocarcinoma (NCBI Gene Expression Omnibus database). A total of 45,149 cells derived from cancer tissues (tLung) and 42,995 cells derived from normal tissues (nLung) were selected to investigate the population of POSTN-positive fibroblasts in lung cancer and normal lung tissues. Cell clustering and tSNE visualization were performed using the FindClusters and RunTSNE functions of Seurat v4.0.

Gene Expression and Survival Analysis in Clinical Cohort

TNMplot database (http://www.tnmplot.com) was used to verify the expression of POSTN in various cancers [15]. Transcriptome RNA sequencing data and clinical survival information of NSCLC patients was analyzed to assess gene expression impact on clinical prognosis via cBioPortal (https://www.cbioportal.org/) using The Cancer Genome Atlas (TCGA) PanCancer Atlas data set, such as 566 samples of Lung Adenocarcinoma data

set (https://www.cbioportal.org/study/summary?id=luad_tcga_pan_can_atlas_2018) and 487 samples of Lung Squamous Cell Carcinoma data set (https://www.cbioportal.org/study/summary?id=lusc_tcga_pan_can_atlas_2018). As a gene expression data, mRNA expression z-scores relative to normal samples (log RNA Seq V2 RSEM) was used for both datasets. The overall survival curves were analyzed by the Kaplan–Meier method with log-rank test.

mRNA Expression Analysis by qRT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen) and reverse-transcribed with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The quantitative reverse transcription PCR (qRT-PCR) was performed on the StepOnePlus™ Real-Time PCR System using the TaqMan™ assay (Thermo Fisher Scientific). Three replicates per sample were assayed for each gene. The delta-delta CT method was used to quantify the relative changes in gene expression, and reactions were normalized to the endogenous control gene, GAPDH, expression levels. The TaqMan gene expression assays used were as follows: GAPDH: Hs02786624_g1 and POSTN: Hs01566750_m1.

Cell Proliferation Assay

A total of 3×10^4 cells (A549), 5×10^4 (HCC4006), or 5×10^4 (EBC-1) cells were plated in quadruplicate on 6-well plates with or without recombinant POSTN at indicated concentrations. Cell proliferation was counted at 36, 72, and 108 h using TC20 Automated Cell Counter (Bio-Rad).

RNA Interference

Two pLKO.1-shRNA plasmids against POSTN and a non-target control shRNA plasmid were purchased (Sigma-Aldrich). The target sequences were as follows: shPOSTN-1: CGGTGACAGTATAACAGTAAA, shPOSTN-2: CGGATCTTGTGGCCCAATTAG, non-

target control shRNA: no known gene targets from any species. Lentivirus particles were produced by transient transfection of shRNAs targeting POSTN and pLKO.1 non-target control along with packaging vectors (pVSVG and psPAX2) in 293T cells. CAFs were transduced with shRNA and underwent cell selection in 2 μg/mL puromycin, and expression levels of POSTN were analyzed by qPCR and western blot.

Animal Models

Pathogen-free immunodeficiency female BALB/c-nu mice 6 to 8 weeks old were purchased (CLEA Japan). HCC4006 cells (1×10^6 cells) and CAFs (1×10^6 cells) were resuspended in RPMI 1640, and a 200 μL 1:1 mixture of resuspended cells and Matrigel was subcutaneously injected into the bilateral flanks of mice. In the treatment conditions, when tumor volumes reached around 130 mm^3, mice were orally administered osimertinib at a dosage of 5 mg/kg/day for 5 days in a week. Throughout the study, mice were monitored twice a week for signs of pain or distress and loss of body weight. Tumor growth was also monitored twice a week, and individual tumor volumes were measured using a digital caliper and approximated according to the formula $V=1/2ab^2$ (a = the long diameter, b = the short diameter of the tumor). All mice were sacrificed at the endpoint by CO^2 inhalation.

Statistical Analysis

All statistical analyses were performed with GraphPad Prism, version 9.2.0 (GraphPad Software). Group differences were compared using Mann–Whitney U test for repeated measurements. Values of $p< 0.05$ were considered statistically significant.

Results

CAFs induce EMT and accelerate migration and drug resistance of NSCLC cells

CAFs and paired NFs were isolated from primary NSCLC and adjacent normal lung tissues and cultured in RPMI with 10% FBS. Morphological differences between NFs and CAFs could be observed under light microscope; NFs were thin and small spindle-shaped cells, while CAFs appeared as large, plump spindle-shaped cells (Figure 1A). Interactions between CAFs and NSCLC cells using conditioned media (CM) and co-culture methods were examined to determine the properties of isolated CAFs and how CAFs affect tumor cells. A549 and HCC827 cells cultured in NFs' or CAFs' CM showed morphological changes to a spindle shape and elongated formation as compared to control cells cultured in normal media (Figure 1B). In western blot analysis, an upregulation in the mesenchymal marker vimentin, N-cadherin, ZEB1 and a downregulation in the epithelial marker E-cadherin could be observed in CM groups, especially in CAF-CM (Figure 1C), indicating that these NSCLC cells underwent an epithelial-mesenchymal transition (EMT) due to CAF stimulations. Transwell migration assay revealed that NSCLC cells' migration ability was significantly increased in the presence of fibroblasts (Figure 1D). Interestingly, the effect was clearly higher with CAFs than with NFs. The colony formation assay demonstrated that CAFs conferred significant resistance to CDDP on A549 cells, and HCC827 cells showed acquired resistance to osimertinib in the presence of CAFs but not NFs (Figure 1E). To evaluate the difference between established NFs and CAFs, we compared CAF activation markers FAP and αSMA, which are used to identify CAFs [16]. Unexpectedly, there were no clear differences between each pair of NFs and CAFs (Supplementary Figure 1A). However, when NFs or CAFs were treated with CM from NSCLC cells, expression differences were confirmed with CAFs showing upregulation in FAP compared to NFs (Figure 1F and Supplementary Figure 1B).

Periostin is a secretory protein expressed in CAFs of NSCLC

We performed RNA sequencing analysis to investigate the differentially expressed genes (DEGs) between NFs and CAFs. Three pairs of NFs and CAFs were cultured with or

without tumor CM obtained from A549 or HCC827 cells for 48 hours and submitted for RNA-seq to evaluate the differences in their innate expression and dynamic reactivity to tumor stimulation. First, we performed unsupervised hierarchical clustering of 9 CAFs and 9 NFs using 125 DEGs, including 68 upregulated genes and 57 downregulated genes in CAFs: the 68 upregulated genes had > 1 of average log2 ratio (CAF/NF) for 3 pairs and > 10 TPM in CAF1, and the 57 downregulated genes had < −1 of average log2 ratio (CAF/NF) and > 10 TPM in NF1. TPMs of 125 DEGs were log transformed and centered with a median value of 18 samples. A comprehensive summary of the 125 DEGs is provided in Supplementary Table 2. These analyses identified two distinctive subsets of CAFs and NFs (Figure 2A). Gene set enrichment analysis (GSEA) identified activated gene signatures in CAFs (Supplementary Table 3), and multiple gene sets related to upregulation of MYC, TGF-β, and HIF-1α signature were included in the most highly ranked DEG sets (Supplementary Figure 2). Next, we explored the candidate molecules that significantly contributed to the TME. Of interest, several genes encoding cytokines, chemokines, and growth factors (e.g., TGFBI, IGFBP3) were the most significantly upregulated genes in CAFs (Figure 2B).

We focused on POSTN for further study, which RNA-seq showed overexpression in CAFs and upregulation in response to the tumor-CM stimulation (Supplementary Figure 3). Western blot analysis of multiple pair samples confirmed that CAFs had higher levels of POSTN protein expression than NFs, while there was no clear difference between FAP and αSMA protein expression levels (Figure 2C). In addition, western blotting using CM was performed to evaluate extracellular secretion from each cell (Figure 2D). CAFs expressed significantly more POSTN than NSCLC cells and NFs in both the cell lysate and CM, indicating that POSTN is secreted from CAFs in the TME of NSCLC. Immunohistochemistry staining on clinical specimens of lung cancer showed that POSTN was strongly expressed in the stromal region where CAFs are abundant, while very little expression was observed in tumor cells or normal lung tissue apart from the tumor region (Figure 2E). The immunofluorescence imaging revealed the colocalization of αSMA and POSTN (Figure 2F).

Furthermore, single-cell RNA sequencing data of 44 patients diagnosed with lung adenocarcinoma was used to investigate cellular POSTN expression at the single-cell

level in lung tumor and normal lung tissues (data obtained from the NCBI Gene Expression Omnibus database: GSE131907) [14]. Among the available 208,506 cells, 45,149 cells from lung tumor tissues and 42,995 from normal lung tissues were cataloged into 8 distinct cell lineages annotated with canonical marker gene expression. Most of the fibroblasts expressed COL1A1, a canonical marker for fibroblasts in both normal and tumor tissues. By contrast, POSTN-positive fibroblasts were highly enriched in lung tumor tissues but rarely observed in normal lung tissues, suggesting CAF-specific POSTN expression in lung cancer (Figure 2G).

High POSTN expression correlates to poor prognosis in NSCLC

Expression levels of POSTN were verified with the TNMplot database and RNA sequencing data from The Cancer Genome Atlas (TCGA), Genotype-Tissue Expression (GTex), and Therapeutically Applicable Research to Generate Effective Treatments (TARGET). The POSTN gene was upregulated in multiple malignancies compared to matched normal tissues, including NSCLC (Supplementary Figure 4). Next, we analyzed the prognostic value of POSTN on patients with lung adenocarcinoma (n = 501) and lung squamous cell carcinoma (n = 478) using the TCGA database. As expected, higher expression of POSTN was correlated to poor survival in lung adenocarcinoma and squamous cell carcinoma patients (Figure 2H). Taken together, these results suggest that POSTN expression may play a crucial role in lung cancer progression.

TGF-β3 and POSTN regulate POSTN expression in CAFs

The TGF-β family is a well-known inducer of POSTN [17]. Our study showed that adding recombinant human TGF-β1 or TGF-β3 promoted POSTN expression in CAFs. TGF-β3 showed more of an effect than TGF-β1, which is consistent with previous findings [12] (Figure 3A). In addition, TGF-β3 promoted extracellular secretion of POSTN and inhibiting TGF-β3 signaling with the TGF-β type I receptor inhibitor, SB431542, suppressed POSTN expression (Figure 3B). This suggests that TGF-β3 is a key regulator for POSTN expression in CAFs. Moreover, we found that recombinant human POSTN (rhPOSTN)

was able to significantly upregulate the POSTN expression in CAFs in a dose-dependent manner, suggesting the existence of a POSTN autocrine mechanism (Figure 3C and 3D).

POSTN induces EMT and accelerates cell proliferation and migration of NSCLC cells through the MAPK/ERK pathway

We used rhPOSTN to investigate the role of POSTN protein in NSCLC cell proliferation and EMT. rhPOSTN increased A549, HCC4006, and EBC-1 cell proliferation in a dose-dependent manner (Figure 3E). Western blot analysis demonstrated that rhPOSTN slightly increased phosphorylation of ERK1/2 and SCH772984 (ERK inhibitor) suppressed the proliferation effect of adding POSTN, suggesting that the MAPK/ERK pathway is one of the aspects of the POSTN-mediated cell proliferation process (Figure 3F and 3G). Moreover, we observed increased expression of mesenchymal markers and decreased E-cadherin expression when treated with rhPOSTN. It suggests that POSTN induces EMT in NSCLC cells, while the tendency to undergo EMT differs depending on the cell line (Figure 3H). Furthermore, rhPOSTN significantly facilitated the migration ability of A549, HCC4006, and EBC-1 cells (Figure 3I).

Knockdown of POSTN alleviated the CAF-induced enhancement of an aggressive tumor phenotype

To understand the roles of CAF-derived POSTN *in vitro* and *in vivo*, we used the shRNA system to knockdown POSTN in CAFs. We established CAF-shNT, which was transduced with non-target shRNA, and two kinds of CAF-shPOSTN. Intra- and extracellular protein knockdown levels of CAF-shPOSTN were confirmed (Figure 4A). While CAF-shNT clearly facilitated the migration of A549 and HCC4006 cells, CAF-shPOSTN significantly decreased this effect (Figure 4B). We then assessed tumor growth *in vivo* using the HCC4006 xenograft model in BALB/c nude mice. As shown in Figure 4C, CAFs could not form stable nodules by themselves. CAF-shNT triggered xenograft tumor growth of HCC4006 cells, while CAF-shPOSTN exhibited significantly less stimulative effects on tumor growth. We then performed osimertinib treatment in combination with POSTN

knockdown. Osimertinib treatment was started after HCC4006 cells with or without CAFs formed primary nodules around 130 mm^3. In HCC4006 cells, CAF-shNT induced drug resistance to osimertinib (Figure 4D), whereas POSTN knockdown significantly decreased CAF-mediated drug resistance. These results suggest that inhibition of CAF-derived POSTN in the TME can be an effective treatment approach in suppressing tumor progression and overcoming drug resistance.

Discussion

Recent studies have demonstrated that targeting the TME could be an effective strategy for cancer treatment [18]. CAFs are found in high proportions in the TME and are particularly important since they secrete many regulatory factors that promote tumor initiation and progression [19]. Therefore, CAFs are potential targets for cancer therapy [20]. However, there are a lot of unknowns behind how CAFs promote tumor progression. This study aimed to identify effective therapeutic targets in CAFs for NSCLC by establishing pair samples of NFs and CAFs from clinical specimens and using RNA sequencing to comprehensively compare them.

Our study focused on POSTN, among the genes that were upregulated in CAFs, because little is known about POSTN's effect on NSCLC. POSTN has been implicated in tumor progression in various malignancies and high expression levels of POSTN in tumor tissue, and serum leads to poor prognosis for NSCLC patients [11, 21-25]. Most of previous studies on POSTN focused on its role in tumor cells; therefore, this study is the first to reveal the effects of POSTN on NSCLC cells as a secretory signal from CAFs.

First, we showed that POSTN expression was clearly elevated in CAFs than in NFs. CAF identification markers FAP and αSMA did not show a distinct pattern between the two types of fibroblasts in our study; furthermore, previous studies have found that FAP and αSMA levels in NFs were sometimes comparable to those in CAFs [26]. This suggests that POSTN is a more sensitive marker to distinguish CAFs in NSCLC.

In this study, POSTN was revealed to regulate multiple biological behaviors of NSCLC cells, including proliferation, EMT, migration, and drug resistance, as seen in other malignancies [27]. Acquisition of EMT features in tumor cells has been considered as a major factor of resistance to various drugs through several mechanisms, such as enhanced efflux pumps, enhanced DNA repair mechanisms, and activation of survival pathways [28]. We also demonstrated that POSTN stimulation activated the ERK pathway in NSCLC cells, consistent with other studies [29,30].

Identifying mutations in the gene encoding the epidermal growth factor receptor (EGFR) tyrosine kinase in NSCLC patients and the subsequent development of EGFR tyrosine

kinase inhibitor (TKI)-targeted therapy has revolutionized lung cancer treatment. Osimertinib (AZD9291), the third-generation TKI, has overcome the “gatekeeper” T790M mutation, which is the most commonly acquired resistance mechanism to TKIs [31,32]. However, patients inevitably develop secondary resistance to osimertinib, which is of clinical significance due to the lack of post-osimertinib options [33]. In this study, we showed that POSTN knockdown in CAFs improved the sensitivity for osimertinib treatment in cell mixtures consisting of HCC4006 (EGFR-mutant cell line) cells and CAFs, mimicking the TME. POSTN knockdown inhibited EMT induction in HCC4006 that contributed to overcoming drug resistance. In addition, recent studies have demonstrated that the combination of ERK inhibition and osimertinib enhanced the induction of apoptosis and improved the drug response by preventing ERK reactivation in EGFR-mutant NSCLC cell lines [34]. rhPOSTN activates the ERK pathway in NSCLC cells, suggesting that the POSTN knockdown in CAFs might reduce the ERK pathway activation, which could enhance the effect of osimertinib.

Our study has some limitations. According to the latest studies, CAFs are not single-cell populations in terms of their origin and function and have pleiotropic actions on tumor cells. In addition, CAFs exhibit plasticity with context-dependent effects on cancer [35,36]. Our study does not address these aspects of CAFs; therefore, further research on POSTN derived from each subset of CAFs could be useful.

In conclusion, we demonstrate that CAF-derived POSTN is involved in tumor growth, migration, EMT induction, and drug resistance in NSCLC. Our study strongly suggests that targeting POSTN secreted from CAFs in the TME could be a novel therapeutic strategy for NSCLC.

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Statements & Declarations:

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Competing Interests

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All authors have read and approved the final manuscript.

Data Availability

The raw RNA-seq data generated in this study are publicly available in Gene Expression Omnibus (GEO) at GSE205814. Data sources and handling of publicly available data are described in the Materials and Methods. Further information is available from the corresponding author upon request.

Ethics approval

Studies using clinical specimens were approved by the Okayama Medical School and Hospital's Research Ethics Committee (Approval Number: # 1906-033). The protocol for animal models was approved by the Animal Care and Use Committee of Okayama University (Okayama, Japan: permit number: OKU-2016398), and this study was carried out in accordance with the Guidelines of the Okayama University.

Consent to participate

Informed consent was obtained from all individual patients for the use of their materials.

Figure legends:

Figure 1. Interactions between NSCLC cells and NFs/CAFs

(A) CAFs and paired NFs were isolated from primary NSCLC and adjacent normal lung tissues. Scale bar: 100 μm. (B) Representative microscopy images of A549 and HCC827 cells treated with NF- or CAF-CM for 2 days. (C) Cells treated with CM for 2 days and then subjected to immunoblotting. (D) NSCLC cells were starved with serum-free RPMI overnight and then co-cultured with NFs or CAFs for 24 h, followed by a migration assay. (E) Colony formation assay of NSCLC cells co-culturing with or without NFs/CAFs. (F) NFs and CAFs treated with CM obtained from NSCLC cells for 2 days. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Figure 2. POSTN is a secretory protein highly expressed in CAFs in NSCLC tissues

(A) Unsupervised hierarchical clustering of 9 CAFs and 9 NFs using 125 DEGs, including 68 upregulated genes and 57 downregulated genes, which show more than a twofold change for 3 CAF/NF pairs with more than 10 TPMs. TPMs of 125 DEGs were log transformed and centered with a median value of 18 samples. (B) Volcano plot of differences in gene expression between NFs and CAFs. The level of enrichment is represented as a difference in gene expression (X axis) and its significance (P value, Y axis). A total of 1643 genes that represented changes of 10 TPMs or more, on average, in 9 pairs of NFs and CAFs are shown. (C) The protein levels of POSTN and commonly used CAF markers (αSMA and FAP) were determined by western blot analysis. (D) Protein specimens [cell lysates (left) and their corresponding CM (right)] from the indicated cells were analyzed for POSTN. GAPDH was used as a control for loaded amounts of cell lysate and for a successful preparation of secreted proteins without contamination of cellular proteins. (E) Representative immunohistochemical images with POSTN expression in NSCLC [lung adenocarcinoma (left) and squamous cell carcinoma (right)]. The red and blue frames represent magnified images of tumor and normal tissues, respectively. Scale bar: 500 μm. (F) The localization of αSMA and POSTN in the NSCLC tissues was detected by confocal laser scanning microscopy, as indicated (red: αSMA,

green: POSTN, blue: DAPI). Scale bar: 20 μm. (G) Single-cell RNA sequencing analysis for whole cells derived from primary sites of lung tumor (45,149 cells) and normal lung tissues (42,995 cells). tSNE projection within each tissue origin, color-coded by major cell lineages. (H) Kaplan–Meier curve showing overall survival of patients with lung adenocarcinoma and squamous cell carcinoma bearing high or low POSTN expression. High expression was defined as lung cancer tissue with more than twice the POSTN expression compared to normal lung tissue, and low expression was defined with less than twice the POSTN expression.

Figure 3. The modulators of POSTN expression and functional analysis of POSTN effects on NSCLC cells

(A) The mRNA levels of POSTN in NF and CAF were detected 48 h after adding PBS, TGF-β1 (10 ng/ml), and TGF-β3 (10 ng/ml) by real-time PCR. (B) Blocking of TGF-β3 (10 ng/ml) with the TGF-β receptor inhibitor (SB431542, 10 μM) suppressed the expression of extracellular POSTN in CAFs. (C, D) POSTN expression induced by rhPOSTN in CAFs in a dose-dependent manner. Protein expression by western blot analysis (C) and mRNA expression by real-time PCR (D) were measured at the indicated rhPOSTN concentrations. (E) POSTN increased the cell proliferation of A549, HCC4006, and EBC-1 cells in a dose-dependent manner. (F) Expression of MAPK-ERK pathways as detected by western blotting in NSCLC cells cultured with rhPOSTN (100 ng/ml) for 48 h. (G) Blocking of rhPOSTN (100 ng/ml) with the ERK inhibitor (SCH772984, 5 μM) suppressed the cell proliferation of A549, HCC4006, and EBC-1. (H) Expression of EMT-associated markers. (I) A549, HCC4006 and EBC-1 cells were starved with serum-free RPMI overnight and then cultured with rhPOSTN (100 ng/ml) for 24 h, followed by a migration assay. rhPOSTN significantly promoted the migration ability of NSCLC cells. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Figure 4. Knockdown of POSTN alleviated the CAF-induced enhancement of an aggressive tumor phenotype

(A) The expression levels of POSTN in CAF-shNT and CAF-shPOSTN were confirmed by western blot analysis. (B) A549 and HCC4006 cells were co-cultured with CAF-shNT or CAF-shPOSTN cells for 24 h, and then migration assay was performed. (C) Tumor sizes were monitored on the indicated days after transplantation of the prepared cells [HCC4006 cells alone (1×10^6 cells), HCC4006 (1×10^6 cells) mixed with CAFshNT (1×10^6 cells), HCC4006 (1×10^6 cells) mixed with CAFshPOSTN (1×10^6 cells), CAFshNT cells alone (1×10^6 cells), CAFshPOSTN cells alone (1×10^6 cells)]. (D) Xenograft tumors were treated with osimertinib 5 mg/kg (n = 7 mice for each) administered five times in a week by oral gavage. Tumor volumes were measured over time from the start of treatment (mean ± SE). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)