β-elemene inhibits non-small cell lung cancer cell migration and invasion by inactivating the FAK-Src pathway

HONGBO ZHANG¹*, SHAOBING LI²*, JUN BAO³, NING GE¹, FU HONG¹ and LITING QIAN¹

¹Department of Radiology, The First Affiliated Hospital of USTC, Division of Life and Medicine, University of Science and Technology of China, Hefei, Anhui 230031; ²Department of Anatomy, College of Basic Medicine, Anhui Medical University, Hefei, Anhui 230032; ³National Synchrotron Radiation Laboratory, University of Science and Technology of China, Hefei, Anhui 230029, P.R. China

Received December 14, 2019; Accepted January 21, 2021

DOI: 10.3892/etm.2021.10529

Abstract. Despite sustained effort, the prognosis of lung cancer remains poor and the therapeutic responses are limited. Cell movement ability is a prerequisite for lung cancer metastasis, which involves focal adhesion kinase (FAK)-mediated cell migration and invasion via complex formation with Src. Hence, FAK-Src signaling might be an effective target for anti-cancer treatment. β-elemene, the major component of elemene extracted from Curcuma Rhizoma, exhibits broad-spectrum anti-tumor properties. However, the role of β-elemene in lung cancer cell motility and invasion abilities may be potential targets for preventing metastasis. Therefore, the migration and invasion abilities of NSCLC through FAK-Src signaling.

Introduction

Lung cancer is one of the most invasive malignancies worldwide. Non-small cell lung cancer (NSCLC) is the major type of lung cancer, with an estimated 5-year survival rate of only 16% in 2014 in the United States (1). While great effort has been made to improve the prognosis of NSCLC, metastasis is still a serious hurdle for its treatment. Metastasis is a complex process; its foundation depends on the activity of cellular movement machinery (2). Therefore, the migration and invasion abilities of NSCLC through FAK-Src signaling.

Correspondence to: Dr Liting Qian, Department of Radiology, The First Affiliated Hospital of USTC, Division of Life and Medicine, University of Science and Technology of China, Hefei, Anhui 230031, P.R. China

E-mail: drqianliting@163.com

*Contributed equally

Key words: β-elemene, non-small cell lung cancer, migration, invasion, FAK-Src pathway
signaling pathway. The effects of β-elemene on cell migration and invasiveness were investigated in two NSCLC cell lines (A549 and H1299) using wound-healing and Transwell assays. The impact of β-elemene on the expression levels of mRNA and protein associated with FAK-Src signaling was determined. The results suggest that β-elemene could suppress NSCLC cell motility via the inhibition of FAK-Src signaling.

Materials and methods

Reagents. β-elemene was obtained from CSCP Pharmaceutical Group Ltd. Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM/H) were purchased from Cytivia. Dimethyl sulfoxide (DMSO) was purchased from BioFROXX and 3-(4,5-dimethylthiazol-2-yl)-2, 5-depethyltetrazolium bromide (MTT) was obtained from (Shanghai Ica Biotechnology Co., Ltd.; cat. no. MO105-1G). A Matrigel-coated Transwell chamber was purchased from BD Biosciences. Antibodies against phosphorylated (p)-FAK Tyr397, total (t)-FAK, p-Src Tyr416, p-Src Tyr527, and t-Src were obtained from Abcam. Crystal violet was obtained from Beyotime Institute of Biotechnology.

Cell culture and treatment. A549 and H1299 cells (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained in a 37°C incubator supplemented with 5% CO₂. β-elemene was diluted to 25, 50, 100 and 200 µg/ml with complete medium. A vehicle control consisting of 0 µg/ml β-elemene.

MTT assay. In total, 3,000 cells were plated into each well of a 96-well plate. After incubating overnight, different concentrations of β-elemene (0, 50, 100 and 200 µg/ml) were added with fresh medium to each well. Subsequently, cells were washed with phosphate-buffered saline (PBS) and further incubated with 10 µl MTT for 4 h. Subsequently, the MTT was aspirated and the cells were washed with PBS. DMSO (100 µl) was added to dissolve the formazan crystals. The absorbance at 570 nm was measured with a microplate reader (Molecular Devices SpectraMax i3; Bio-Rad Laboratories, Inc.). Cell numbers were normalized to the 0 µg/ml group.

Migration assay. A549 and H1299 cells were plated into 12-well plates (2x10⁵ each well). Once cells reached 80-90% confluence, a 200-µl pipette tip was used to generate a scratch. Floating cells were washed with PBS. Then, different concentrations of β-elemene (0 and 50 µg/ml) with fresh serum-free medium were added to each well. The wound gap was imaged with a light microscope (magnification, x4 and x10; XDS-1A; Precision Instruments) after 0, 12 and 24 h of incubation. The migration ability was determined by measuring the width (the shortest measurement horizontally across the gap) of the scratch and was normalized to the 0-µg/ml group.

Invasion assay. The invasion capacity of cells was determined using a Matrigel-coated Transwell chamber (BD Biosciences). After treating with 0 or 50 µg/ml β-elemene with serum-free medium, 5x106 cells were plated on the top chamber of the Transwell insert and stimulated with 500 µl medium with 50% FBS added to the bottom chamber. After 24 h of incubation (5% CO₂ at 37°C), a cotton swab was used to remove the non-invasive cells on the inside of the upper chamber. The invasive cells on the underside of the upper chamber were fixed using 4% paraformaldehyde (PFA) for 10 min at room temperature and stained with 2% crystal violet (20 min at room temperature). Random fields were imaged (magnification, x100) and measured with a light microscope.

Cell adhesion assay. After treatment with 0 or 50 µg/ml β-elemene, cells were digested and resuspended in complete medium. Then, the cells (2,000 cells/well) were plated in each well of a 96-well plate precoated with 20 µl Matrigel for 1 h. The cells were subsequently gently washed with PBS. The remaining cells were fixed with 4% PFA for 10 min at room temperature and stained with 0.2% crystal violet for 20 min at room temperature. The plates were placed directly under a light microscope (magnification, x100) to measure the adhesive cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from A549 and H1299 cells treated with β-elemene (0 or 50 µg/ml) using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. Reverse transcription was performed with a RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.) using 100 ng of total RNA (60 min at 42°C, 5 min at 25°C followed by 60 min at 42°C and 5 min at 70°C). The primers used to amplify (Table I) Rhoa, Rac1, Cdc42, matrix metalloproteinase (MMP) 2 and MMP9 were designed with Primer Premier 5.0 software (BBI Life Sciences). The thermocycling parameters for RT-qPCR using SybrGreen qPCR Master mix (cat. no. F-415XL; Thermo Fisher Scientific, Inc.) were: 94°C for 10 min followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. Relative changes in mRNA expression were calculated using the 2-ΔΔCT method with β-actin as the internal control (19).

Table I. Primer sequences for PCR amplification.

| Names                       | Sequences, 5' to 3' |
|-----------------------------|---------------------|
| Rhoa-Human-RT-F             | GGAACACGTAGTGTTGCC  |
| Rhoa-Human-RT-R             | GGCTGTCGAGAAACACAT  |
| Rac1-Human-RT-F             | ATGTCGGTACAGTGTATAC |
| Rac1-Human-RT-R             | CTGGATGCTTTCTGAAACA |
| Cdc42-Human-RT-F            | CACCTAGGAATATGCAGCT |
| Cdc42-Human-RT-R            | CTCACGGGCTCTATGTCA  |
| MMP2-Human-RT-F             | GATACCCCTTTAGCGTAAGGA |
| MMP2-Human-RT-R             | CTTCTTCGAAAGTCCATAGC |
| β-actin-Human-RT-F          | GGGACGCAGAGCATCGTATC |
| β-actin-Human-RT-R          | AGCCGAGCATCCCCAAAGTT |
| β-actin-Human-RT-R          | GGGCACGAAGGCCCTCATATT |

F, forward; R, reverse; MMP, matrix metalloproteinase.
Western blotting. A549 and H1299 cells were treated with 50 µg/ml of β-elemene for 0, 1, 3, 6, 12 and 24 h. Then, radiomunoprecipitation assay lysis buffer and phenylmethylsulfonyl fluoride were added to the cells, and incubated on ice for 2 h. After centrifuging at 161 x g for 10 min at 4°C, 30 µg of total protein was loaded on 12% gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was run for 1 h at 120 V. The proteins were transferred to polyvinylidene difluoride membranes and run at 100 V for 1 h. Subsequently, the membranes were blocked with 5% nonfat milk in TBST at room temperature for 1 h. Next, the membranes were incubated with specific primary antibodies in blocking buffer against p-FAK\(^{397}\) (Abcam; cat. no. ab81298; 1:1,000), t-FAK (Abcam; cat. no. ab40794; 1:2,000), p-Src\(^{540}\) (Abcam; cat. no. ab4066; 1:1,000), p-Src\(^{527}\) (Abcam; cat. no. ab32078; 1:5,000), and t-Src (Abcam; cat. no. ab109381; 1:10,000) at 4°C overnight. The membranes were then washed with TBST three times and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Biosharp Life Sciences; cat. no. BL003A; 1:4,000) in blocking buffer for 1 h at room temperature. The membranes were washed with TBST three times and the bands were visualized using an electrochemiluminescence system (Bio-Rad Laboratories, Inc.) in a dark room according to the manufacturer's instructions. The expression levels of specific proteins in A549 and H1299 cells were normalized to those in cells of the 0 µg/ml group.

Statistical analysis. All data are shown as the mean ± standard error of the mean. More than three independent replicates were performed for each set of experiments. All statistical analyses were performed using GraphPad Prism (version 5; GraphPad Software, Inc.). The differences between treatment groups were compared using the Student's t-test and one-way analysis of variance (ANOVA) with Dunnett's post hoc test. P<0.05 was considered to indicate statistically significant difference.

Results

β-elemene decreases the viability of A549 and H1299 cells. To investigate the effects of β-elemene on cell viability and determine the suitable concentration for subsequent experiments, A549 and H1299 cells were treated with different doses of β-elemene (0-200 µg/ml) for 0-48 h. Cell viability was determined with the MTT assay. A concentration-dependent decrease in viability was observed in both cell lines after exposure to β-elemene (Fig. 1). Treatment with 50 µg/ml β-elemene for 24 h showed no inhibitory effect on either cell line and was selected for subsequent experiments.

β-elemene decreases the mRNA expression of motility-associated genes in A5469 and H1299 cells. RhoA, Rac1 and Cdc42, which are small GTP-binding proteins in the Rho family, have been reported to regulate the cellular cytoskeleton and cell migration (20). To determine whether β-elemene regulates the expression of genes associated with migration and invasion, the mRNA expression of RhoA, Rac1 and Cdc42 was compared in A549 and H1299 cells by RT-qPCR, following inoculation with 0 and 50 µg/ml of β-elemene for 24 h. β-elemene treatment
ZHANG et al.: β-ELEMENE INHIBITS MIGRATION AND INVASION OF NSCLC CELLS

β‑elemene remarkably decreased the mRNA expression levels of RhoA, Rac1 and Cdc42 (Fig. 4A and C). MMP2 and MMP9 are key members of the MMP family, which may also facilitate cell migration and invasion (21,22). As shown in Fig. 4B and D, the mRNA levels of MMP2 and MMP9 were decreased after incubation with β‑elemene. The data suggest that β‑elemene inhibits the expression of genes associated with cell motility.

β‑elemene inhibits FAK‑Src activation in A5469 and H1299 cells. To elucidate whether FAK‑Src signaling was involved in the anti‑migration effect of β‑elemene on NSCLC cells, the activity of FAK and Src was evaluated by western blotting. As shown in Fig. 5A‑D, treatment with 50 µg/ml β‑elemene decreased the phosphorylation of FAK (p‑FAK) on Y397 and the phosphorylation of Src (p‑Src) on Y416 in both A549 and H1299 cells. However, p‑Src on Y527 was increased after β‑elemene inoculation. Overall, β‑elemene may inhibit the activity of the FAK‑Src pathway in lung cancer cells.

Discussion

Metastasis is a multi-step process that includes the following: i) Tumor cells detach from the primary tumor by altering cell‑cell adhesion; ii) tumor cells infiltrate adjacent tissues; iii) tumor cells migrate into the vasculature; iv) tumor cells survive in the circulation; and v) tumor cells extravasate and proliferate in a new tissue (23). Hence, migration capacity is critical for successful metastasis. In the present study, the role of β‑elemene in the motility of NSCLC cells was investigated. The data indicate that β‑elemene treatment inhibited the viability, migration, invasion and adhesion of A549 and H1299 cells. Moreover, β‑elemene decreased the mRNA expression levels of motility‑associated genes, including RhoA, Rac1, Cdc42, MMP2 and MMP9. Finally, it was demonstrated that the anti‑migratory and anti‑invasive effects of β‑elemene might be regulated by the FAK‑Src signaling pathway.

β‑elemene, an organic compound extracted from Curcuma Rhizoma, has attracted scientific interest due to its good performance in anti‑cancer treatment. β‑elemene has been reported to inhibit cell growth, induce apoptosis, and block the EMT in different types of cancer cells (12,14,24). Using the MTT assay, it was also demonstrated that β‑elemene treatment inhibited the cell viability of NSCLC cells. In A549 and H460 cells, β‑elemene induced cell death through G2‑M regulation and apoptosis‑modulating proteins, including Cdc2, Bcl‑2 and...
cleaved caspase-9 (25). In H1299 cells, β-elemene inhibited cell growth through the AMPKα- and ERK1/2-regulated inhibition of Sp1 (26). β-elemene also inhibited the migration and the invasive ability of tumor cells in gastric cancer (27). In the present study, it was also found that migration was inhibited in A549 and H460 cells following β-elemene treatment.

FAK and Src are key regulators of integrin-mediated cell adhesion and migration. FAK upregulation and hyperphosphorylation have been demonstrated to increase invasive capability in a variety of human cancer types including gastric cancer and breast cancer (28). The phosphorylation of FAK on Tyr397, which is the major site of phosphorylation, led to the progression of tumor cells by promoting migration and invasion (29). FAK phosphorylation on Tyr397 creates a high-affinity binding site for the recognition of the SH2 domain in Src family kinases. Moreover, phosphorylation of FAK on Tyr397 is important for the activation and recruitment of Src via the formation of the FAK-Src complex (30). Numerous studies have demonstrated that FAK-Src signaling is important in the regulation of cell migration (4). Here, we found that β-elemene treatment decreased the phosphorylation of FAK on Tyr397 and of Src on Tyr416 in both A549 and H460 cells while the phosphorylation of Src on Tyr527 was elevated. The phosphorylation of Src on Tyr527 decreases its recruitment to FAK, while the phosphorylation of Src on Tyr416 activates its recruitment (31). In a previous study, breast cancer cells with FAK-Src inactivation showed decreased metastatic potential (10). The present results suggest that the suppression of FAK-Src signaling by β-elemene may decrease NSCLC metastasis by decreasing cell migration.
The formation and remodeling of FAK-Src-Paxillin contributes to cell migration in a dynamic process, under the regulation of GTPases in the Rho family. Cell movement is dependent on the dynamic organization of the protrusion of filopodia and lamellipodia, whose formation is regulated by Cdc42 and Rac1 (32). RhoA also acts to promote tension in the organization of actin. In addition, the regulation of Rho, Rac, and Cdc42 is coordinated within cells. RhoA is downstream of Rac1, which in turn is downstream of Cdc42. In the present study, treatment with β-elemene led to a remarkable
Inhibition of epithelial cell migration and 

Beta-elemene inhibits breast cancer metastasis

Not applicable.

Patient consent for publication

Ethics approval and consent to participate

all raw data.

the corresponding authors on reasonable request.

The datasets used and analyzed in this study are available from the corresponding authors on reasonable request.

Authors' contributions

The study was conceived and designed by LQ and HZ. HZ and SL conducted most of the experiments with assistance from JB, NG and FH. The manuscript was written by HZ and SL, with contributions from LQ. All authors read and approved the final manuscript. HZ and LQ confirm the authenticity of all raw data.

Acknowledgements

Overall, β-elemene exposure inhibits NSCLC cell migration and invasion by suppressing the activity of FAK-Src signaling. Moreover, β-elemene leads to dysregulated expression of motility-associated Rho GTPases and MMPs. The present results suggest that β-elemene holds promise as an anti-metastatic therapy to prevent tumor cell migration.

References

1. Siegel R, Ma J, Zou Z and Jemal A: Cancer statistics, 2014. CA Cancer J Clin 64: 9-29, 2014.

2. Paul DW, Mistrick E P and Konstantopoulos K: Cancer cell motility: Lessons from migration in confined spaces. Nat Rev Cancer 17: 131-140, 2017.

3. Graham ZA, Gallagher PM and Cardozo CP: Focal adhesion kinase and its role in skeletal muscle. J Muscle Res Cell Motil 36: 305-315, 2015.

4. Parsons JT, Martin KH, Slack JK, Taylor JM and Weed SA: Focal adhesion kinase: A regulator of focal adhesion dynamics and cell movement. Oncogene 19: 5606-5613, 2000.

5. Dy GK, Ylagan L, Pokharel S, Miller A, Brese E, Bshara W, Morrison C, Cance WG and Golubovskaya VM: The prognostic significance of focal adhesion kinase expression in stage non-small-cell lung cancer. J Thorac Oncol 9: 1278-1284, 2014.

6. Liu C, Li Y, Xing Y, Cao B, Yang F, Yang T, Ai Z, Wei Y and Jiang J: The interaction between cancer stem cell marker CD133 and Src protein promotes focal adhesion kinase (FAK) phosphorylation and cell migration. J Biol Chem 291: 15540-15550, 2016.

7. Baquero P, Jiménez-Mora E, Santos A, Lasa M and Chiloeches A: TGFB1 induces epithelial-mesenchymal transition of thyroid cancer cells by both the BRAF/MEK/ERK and Src/FAK pathways. Mol Carcinog 55: 1639-1654, 2016.

8. Lee JH, van de Ven RAH, Zaganjor E, Ng MR, Barakat A, Demmers JJ, Finley LWS, Gonzalez Herrera KN, Hung YP, Harris IS, et al: Inhibition of epithelial cell migration and Src/FAK signaling by SIRT3. Proc Natl Acad Sci USA 115: 7057-7062, 2018.

9. Ward KK, Tancioni I, Lawson C, Miller NL, Jean C, Chen XL, Uruy S, Kim J, Tarin D, Stupack DG, et al: Inhibition of focal adhesion kinase (FAK) activity prevents anchorage-independent ovarian carcinoma cell growth and tumor progression. Clin Exp Metastasis 30: 579-594, 2013.

10. Chikara S, Lindsey K, Borowicz P, Christofidou-Solomidou M and Reinidl K: Enterolectone alters FAK-Src signaling and suppresses migration and invasion of lung cancer cell lines. BMC Complement Altern Med 17: 30, 2017.

11. Logue JS, Cartagena-Rivera AX and Chadwick RS: c-Src activity is differentially required by cancer cell motility modes. Oncogene 37: 2104-2121, 2018.

12. Li P, Zhou X, Sun W, Sheng W, Tu Y, Yu Y, Dong J, Ye B, Zheng Z and Lu M: Elemene induces apoptosis of human gastric cancer cell line BGC-823 via extracellular signal-regulated kinase (ERK)/1/2 signaling pathway. Med Sci Monit 23: 809-817, 2017.

13. Pan Y, Wang W, Huang S, Ni W, Wei Z, Cao Y, Stupack DG, et al: Inhibition of focal adhesion kinase (FAK) activity prevents anchorage-independent ovarian carcinoma cell growth and tumor progression. Clin Exp Metastasis 30: 579-594, 2013.

14. Chang Z, Gao M, Zhang W, Song L, Jia Y and Qin Y: Beta-elemene treatment is associated with improved outcomes of patients with esophageal squamous cell carcinoma. Surg Oncol 26: 333-337, 2017.

15. Liu C, Wang W, Huang S, Ni W, Wei Z, Cao Y, Yu S, Jia Q, Wu Y, Chai C, et al: Beta-elemene inhibits breast cancer metastasis through blocking pyruvate kinase M2 dimerization and nuclear translocation. J Cell Mol Med 23: 6846-6858, 2019.

16. Guo Z, Liu Z, Yue H and Wang J: Beta-elemene increases chemosensitivity to 5-fluorouracil through down-regulating microRNA-191 expression in colorectal carcinoma cells. J Cell Biochem 119: 7032-7039, 2018.

17. Jiang J, Jacob JA, Loganathanatti DS, Nainangu P and Chen B: β-Elemene: Mechanistic studies on cancer cell interaction and its chemosensitization effect. Front Pharmacol 8: 105, 2017.

18. Wu L, Zhao RQ, Wang W, Cui LN, Hu LL, Jiang XX, Shuai J and Sun YP: Nuclear receptor coactivator 6 promotes HTR-8/SVneo cell invasion and migration by activating NF-kB-mediated MMP9 transcription. Cell Prolif 53: e12876, 2020.

The authors declare that they have no competing interests.
22. Lu Q, Huang Y, Wu J, Guan Y, Du M, Wang F, Liu Z, Zhu Y, Gong G, Hou H, et al: T-cadherin inhibits invasion and migration of endometrial stromal cells in endometriosis. Hum Reprod 35: 145-156, 2020.

23. van Zijl F, Krupitza G and Mikulits W: Initial steps of metastasis: Cell invasion and endothelial transmigration. Mutat Res 728: 23-34, 2011.

24. Zhang X, Li Y, Zhang Y, Song J, Wang Q, Zheng L and Liu D: Beta-elemene blocks epithelial-mesenchymal transition in human breast cancer cell line MCF-7 through Smad3-mediated down-regulation of nuclear transcription factors. PLoS One 8: e58719, 2013.

25. Wang G, Li X, Huang F, Zhao J, Ding H, Cunningham C, Coad JE, Flynn DC, Reed E and Li QQ: Antitumor effect of beta-elemene in non-small-cell lung cancer cells is mediated via induction of cell cycle arrest and apoptotic cell death. Cell Mol Life Sci 62: 881-893, 2005.

26. Zhao SY, Wu J, Zheng F, Tang Q, Yang LJ, Li L, Wu WY and Hann SS: β-elemene inhibited expression of DNA methyltransferase 1 through activation of ERK1/2 and AMPKα signalling pathways in human lung cancer cells: the role of Sp1. J Cell Mol Med 19: 630-641, 2015.

27. Deng M, Zhang Y, Liu B, Chen Y, Song H, Yu R, Che X, Qu X, Liu Y, Hu X, et al: β-Elemene inhibits peritoneal metastasis of gastric cancer cells by modulating FAK/Claudin-1 signaling. Phytother Res 33: 2448-2456, 2019.

28. McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG and Frame MC: The role of focal adhesion kinase in cancer - a new therapeutic opportunity. Nat Rev Cancer 5: 505-515, 2005.

29. Lai IR, Chu PY, Lin HS, Liu JY, Jan YJ, Lee JC and Shen TL: Phosphorylation of focal adhesion kinase at Tyr397 in gastric carcinomas and its clinical significance. Am J Pathol 177: 1629-1637, 2010.

30. Tungsukruthai S, Sritularak B and Chanvorachote P: Cycloartabiloxanthone inhibits migration and invasion of lung cancer cells. Anticancer Res 37: 6311-6319, 2017.

31. Dwyer SF and Gelman IH: Cross-phosphorylation and interaction between Src/FAK and MAPKAP5/PRAK in early focal adhesions controls cell motility. J Cancer Biol Res 2: 2, 2014.

32. Aspenström P: The intrinsic GDP/GTP exchange activities of Cdc42 and Rac1 are critical determinants for their specific effects on mobilization of the actin filament system. Cells 8: 8, 2019.

33. Webb AH, Gao BT, Goldsmith ZK, Irvine AS, Saleh N, Lee RP, Lendermon JB, Bheemreddy R, Zhang Q, Brennan RC, et al: Inhibition of MMP-2 and MMP-9 decreases cellular migration, and angiogenesis in in vitro models of retinoblastoma. BMC Cancer 17: 434, 2017.