様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）口腔扁平上皮癌上皮間葉移行におけるp130 Casの役割と治療標的としての可能性

研究課題名（英文）The function of p130 Cas on the epithelial-mesenchymal transition in oral squamous cell carcinoma

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研究成果の概要（和文）：TGF-β 1骨基質に豊富に存在し、腫瘍細胞の形態変化や悪性度に関与する。口腔扁平上皮がんではp130Casとリン酸Smad3が発現していた。TGF-βはSmad3とp130Casのリン酸化および上皮間葉移行（EMT）を誘導した。Smad2/3シグナルの特異的な阻害剤であるSB431542はTGF-βが誘導するp130Casのリン酸化や形態変化の誘導を解除した。p130Casがノックダウンした細胞ではTGF-βが誘導する細胞の移動や浸潤、そしてEMTが解除された。In vivo実験においてもp130Casのノックダウン細胞ではコントロールに比べて骨破壊が有意に少なかった。

研究成果の学術的意義や社会的意義
本研究ではTGF-βが誘導するがん細胞の上皮間葉移行（EMT）や骨浸潤をTGF-βの下流でp130Casが制御していることを証明した。EMTは上皮性のがん細胞がより運動性の高い間葉系細胞の表現系を獲得し、転移しやすくなった状態である。また、EMTを起こした細胞はapoptosisに抵抗性になることも報告されており、TGF-β-p130Casは口腔扁平上皮がんの骨浸潤の新しい治療法のターゲットになり得るかもしれない。

研究成果の概要（英文）：TGF-β is abundantly expressed in the bone matrix and is involved in the acquisition of aggressiveness by tumors. TGF-β is also important to cytoskeletal changes during tumor progression. TGF-β induced the phosphorilaion of Smad3 and p130Cas as well as epithelial-mesenchymal transition (EMT) accompanied by the downregulation of the expression of E-cadherinand the upregulation of the expression of N-cadherin, or Snail. SB431542, a specific inhibitor of Smad2/3 signaling, abrogated the TGF-β-induced phosphorylation of p130Cas and morphological changes. Silencing p130Cas using an shRNA or siRNA in SCCVII cells suppressed TGF-β-induced cell migration, invasion, EMT, and matrix metalloproteinase-9 (MMP-9) production. Compared with control SCCVII cells, SCCVII cells with silenced p130Cas strongly suppressed zygomatic and mandibular destruction in vivo.

研究分野：口腔外科学、口腔内科学

キーワード：口腔扁平上皮がん　上皮間葉移行　p130Cas　骨浸潤

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

口腔扁平上皮癌（OSCC）は局所浸潤しやすく、リンパ節転移を起こす。OSCC において転移の有無は予後を左右する重要な因子であり、転移の機序を明らかにして、その制御が可能になれば、治療成績は飛躍的に向上すると考えられる。OSCC が転移するには、周囲組織へ浸潤することが必要であるが、近年上皮間葉移行（EMT）が OSCC の浸潤や転移に関与することが報告されている。EMT は、上皮性の癌細胞が、より運動性の高い間葉系細胞の表現系を獲得し、転移しやすくなった状態である。また、EMT を起こした細胞は apoptosis に抵抗性になることも報告されており、EMT を制御することは新しい OSCC の治療戦略になる。

２．研究の目的

EMT は TGF-β, Notch, Wnt, Sonichedgehog などの様々な経路を介して起こることが知られているが、詳細な分子機構は不明な点が多い。p130Cas は細胞接着を制御するタンパク質の１つで、様々ながん細胞でその発現が上昇している。本研究では、TGF-β に着目し、p130Cas が口腔扁平上皮癌の EMT に果たす役割を解明し、浸潤・転移の分子機構を明らかにする。さらに p130Cas をがん化の診断や予後の予測因子としての可能性を検討する。

３．研究の方法

①口腔扁平上皮がん患者サンプル

All experimental procedures conducted in this study were reviewed and approved by the Kyushu Dental University Research Ethics Committee (approval number 16-017).

②使用した試薬

Purified recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN, USA). Anti-phosphorylated Smad3 (Ser423/425) (#9520), anti-Smad3 (#9513), anti-Smad2/3 (#8685), anti-p130Cas (#13846), anti-phosphorylated p130Cas (Tyr410) (#4011), anti-Snail (#3895), anti-phosphorylated p38 (Thr180/182) (#9216), anti-p38 (#8690) antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-phosphorylated p130Cas (Tyr165)(#PA536721) antibody was purchased from GeneTex (Irvine, CA, USA). Anti-mouse E-Cadherin (#610181) and anti-mouse N-Cadherin (#610920) antibodies were purchased from BD Bioscience (Franklin Lakes, NJ, USA). SB431542 and an anti-β-actin (AC-15) antibody were purchased from Fuji Film Wako Pure Chemical Corp. (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

③使用した細胞と処理

HSC-2 cells, a human OSCC cell line, were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Tokyo, Japan) in January 2016 and authenticated by JCRB using short tandem repeat polymerase chain reaction (STR-PCR) PowerPlex® 16 STR System (Promega, Madison, WI, USA). SCCVII, mouse squamous cell carcinoma cells were kindly provided by Dr. Shinichiro Masunaga (Kyoto University, Japan) in October 2016. The cells were tested and authenticated in the past 6 months via spectral karyotyping. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich) in a humidified atmosphere containing 5% CO_2 at 37℃. The elongation of each cell was measured using NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA).

④p130Cas をノックダウンした細胞株の作製

Generation of a stable SCCVII cell line expressing an shRNA targeting p130Cas

SCCVII cells were seeded in 35-mm plates, cultured for 24 h, and then transfected with 2 μg of the control shRNA or p130Cas shRNA expression vector using 6 μl of Lipofectamine 3000 (Thermo Fisher Scientific, Foster City, CA, USA). Transfections were performed according to the manufacturer's instructions. Forty-eight hours posttransfection, the medium was changed to complete culture medium containing 1000 μg/ml G418. After 10 days of incubation in this medium, G418-resistant colonies were selected. The cells were maintained in G418 for 6-10 days and expanded.

⑤siRNA 導入による p130Cas のノックダウン

The siRNA against mouse p130Cas and non-targeting control were used Dharmacon ON-TARGETplus siRNA reagents (#12927) (GE Healthcare, Pittsburgh, PA, USA). p130Cas and

control siRNA were transfected into SCCVII cells using Lipofectamine RNAiMAX according to the manufacturer's instruction (Thermo Fisher).

⑥ウエスタンブロッティング

For immunoblotting, whole-cell lysates were resolved on SDS polyacrylamide gels and transferred to PVDF membranes. The membranes were then incubated overnight at 4℃ with antibodies diluted 1:1,000 in a 5% dry milk or bovine serum albumin (BSA) solution containing 0.01% azide in TBST (10 mM Tris-HCl, 50 mM NaCl and 0.25% Tween-20). Finally, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody, and the immunoreactive proteins were visualized using Enhanced Chemiluminescence (GE Healthcare).

⑦細胞移動実験

SCCVII cells transfected with the sh control or sh p130Cas cells were seeded into 6-well plates and grown to 100% confluence. A linear wound was created using a pipette tip. After washing 3 times with PBS, the cells were cultured in medium containing 1% FBS in the presence or absence of TGF-β1 for 18 h. Three random images were taken at the time of wounding. The migration distance was analyzed as a reduction in the wound gap using NIH ImageJ software (National Institutes of Health).

⑧細胞浸潤実験

Cell migration was assessed in a modified Boyden chamber containing a Matrigel-coated porous membrane. SCCVII cells expressing sh control or sh p130Cas were seeded in the upper chamber. Two hours later, TGF-β1 was added to the lower chamber at various concentrations, and the chamber was incubated for an additional 24 h. Next, the cells attached to the upper surface of the membrane were scraped off, and the cells that had migrated to the lower surface were fixed and stained with DAPI.

⑨ザイモグラフィー

Serum-free conditioned medium was collected from confluent SCCVII cells expressing sh control or sh p130Cas, which were incubated in the presence of reagents for 24 h. The conditioned medium was resolved via 10% SDS-PAGE in the presence of 1 mg/ml gelatin. The resulting gel was washed in 10 mM Tris (pH 8.0) containing 2.5% Triton X-100 and then incubated for 16 h in a reaction buffer (50 mM Tris, pH 8.0, 0.5 mM $CaCl_2$, and 10^{-6} M $ZnCl_2$) at 37℃. After staining with Coomassie brilliant blue, gelatinases appeared as clear bands of lysed gelatin against the blue background.

⑩動物骨浸潤モデル

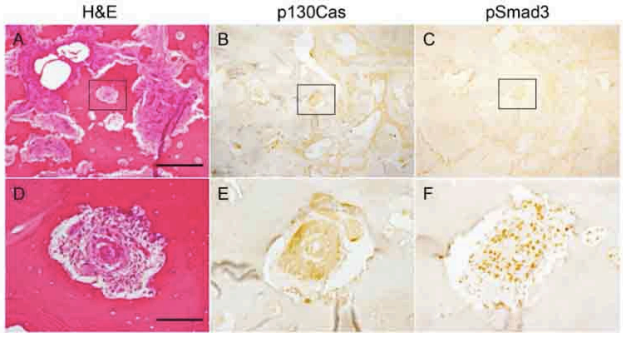
All animal experiments were reviewed and approved by the Kyushu Dental University Animal Care and Use Committee (Approved Number; 16-015).

⑪細胞増殖試験

Five-micrometer sections were stained with an anti-Ki-67 polyclonal antibody (#15580, 1:100, Abcam). Five tumor fields from different 5 mice were randomly selected from each specimen, and the number of Ki-67-positive cells in each field was quantified. The data are expressed as the number of Ki-67-positive cells/mm^2 tumor area.

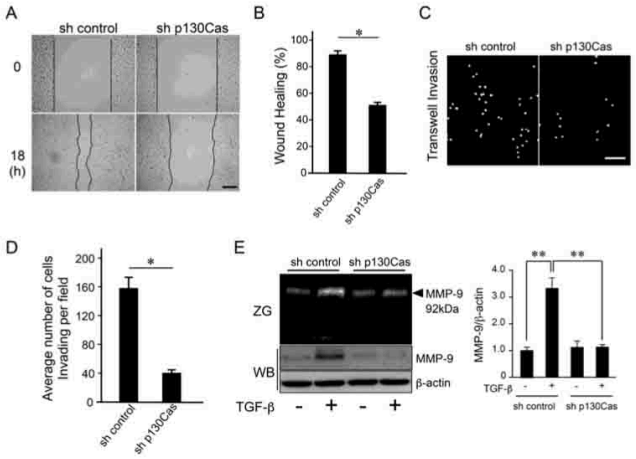
４．研究成果

図１：口腔扁平上皮がんサンプルにおける p130Cas とリン酸化 Smad3 の発現



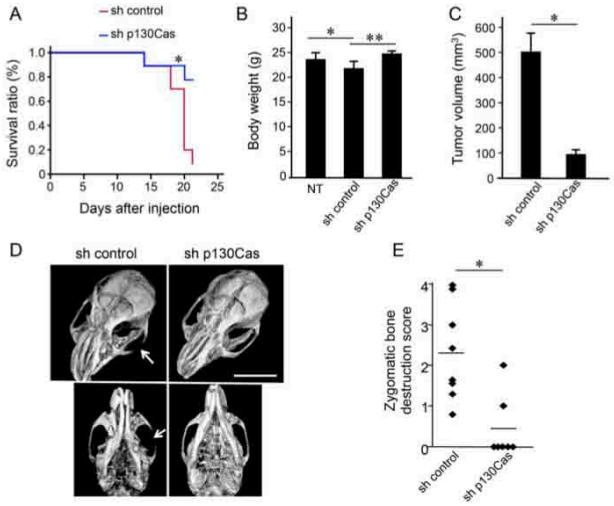
Five OSCC samples were stained with H&E (A, D), an anti-p130Cas antibody (B, E), or an anti-phosphorylated Smad3 antibody (C, F). (D-F) Magnified views of the boxed area in (A-C). One OSCC section representative of 5 samples is shown. (A-C) Original magnification 40 x. Bar = 500 μm. (D-F) Original magnification 200 x. Bar = 100 μm.

図２：p130Cas は TGF-β１が誘導する SCCVII 細胞の移動、浸潤、MMP の産生に関与する



A. SCCVII cells expressing sh control or sh p130Cas were cultured to confluence and then scratched. The cell migration of SCCVII cells expressing sh control or sh p130Cas was evaluated by a wound healing assay. Bar = 200 μm. B. The effect on cell migration as expressed as the degree of wound closure. The data are expressed as the mean ± SD (n=3). *, p<0.01. Similar results were obtained from three independent experiments. C. SCCVII cells expressing sh control or sh p130Cas were suspended in serum-free DMEM and seeded in the upper chamber above a Matrigel-coated porous membrane. TGF-β１ (5 ng/ml) was added to the lower chamber, and the cells were incubated for 18 h. Then, the cells attached to the upper surface of the membrane were scraped off, and the cells that had migrated to the lower surface were fixed, stained with DAPI and quantified. A representative image of the SCCVII cells expressing sh control or sh p130Cas is shown. Bar = 100 μm. D. The data are expressed as the mean ± SD (n=3). *, p<0.01. Similar results were obtained from three independent experiments. E. SCCVII cells expressing sh control or sh p130Cas were incubated in serum-free DMEM for 24 h in the presence or absence of TGF-β１ (5 ng/ml). The conditioned media was analyzed by gelatin zymography (ZG). Western blotting (WB) was used to measure the expression levels of MMP-9 in the SCCVII cells expressing sh control or sh p130Cas treated with TGF-β１ (5 ng/ml) for 24 h. β-actin was used as a loading control. Histogram data depicts cumulative densitometry of MMP-9 relative to β-actin. *, p<0.01. Similar results were obtained from three independent experiments.

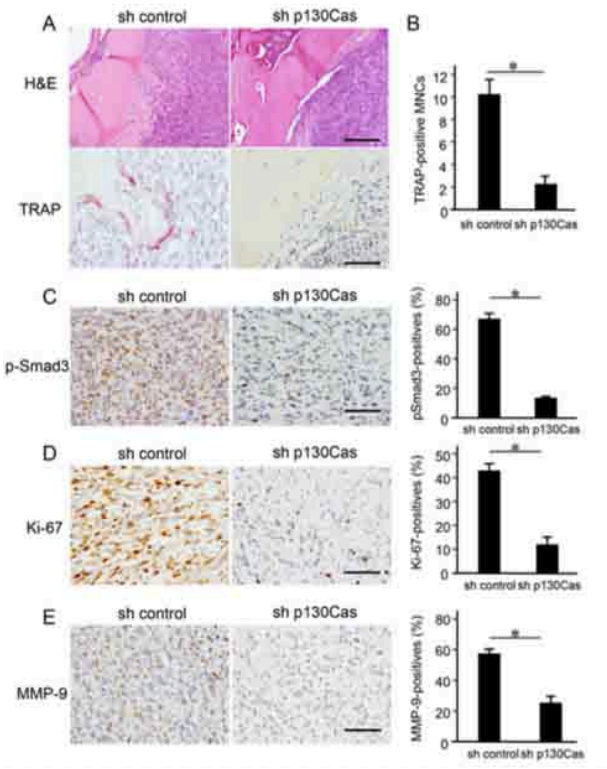
図３： sh p130Cas を発現した SCCVII では骨浸潤が減弱する



A. Overall survival with hazard ratio values of the mice injected with SCCVII cells expressing sh control of sh p130Cas. *, p<0.01. B. Body weight was measured 21 days after tumor cell inoculation. *, p<0.05, **, p<0.01. C. Tumor volume was assessed using calipers and was calculated using the following formula: width² x length x 0.52.

The data are expressed as the mean ± SD (n=5). D. Reconstructed μ-CT images of the zygoma destruction in the SCCVII cells expressing sh control or SCCVII cells expressing sh p130Cas groups. The arrowheads indicate a fracture line in the zygoma. Bar = 8 mm. E. The severity of the zygoma destruction was assessed by clinical scoring for the sh control and sh p130Cas groups. *, $p<0.01$

図４：p130Cas のノックダウンにより破骨細胞の形成、細胞増殖、MMP-9 の産生が低下する



A. Twenty-one days after SCCVII cell inoculation, tissue was fixed in 3.7% formaldehyde, decalcified in 5% formic acid, sectioned along the coronal axis and stained with H&E and TRAP. Bar = 200 μm; magnification 40 x (upper panels), Bar = 50 μm; magnification 200 x (middle and lower panels). B. For each specimen, 5 tumor fields were randomly selected, and the number of $TRAP^+$ MNCs was counted. The data are expressed as the mean ± SD of the number of $TRAP^+$ MNCs/bone surface (mm^2)/section (n=5). *, $p<0.01$. C. Histological sections were stained with anti-p-Smad3 antibodies. Bar = 50 μm; magnification 400 x. For each specimen, 5 tumor fields were randomly selected, and the number of p-$Smad3^+$ cells was counted. The data are expressed as the mean ± SD of the number of p-$Smad3^+$ cells/total number of tumor cells/field (n=5). *, $p<0.01$. D. Histological sections were stained with an anti-Ki-67 antibody. Bar = 50 μm; magnification 400 x. For each specimen, 5 tumor fields were randomly selected, and the number of $Ki\text{-}67^+$ cells was counted. The data are expressed as the mean ± SD of the number of $Ki\text{-}67^+$ cells/total number of tumor cells/field (n=5). *, $p<0.01$. E. Histological sections were stained with anti-MMP-9 antibodies. Bar = 50 μm; magnification 400 x. For each specimen, 5 tumor fields were randomly selected, and the number of $MMP\text{-}9^+$ cells was counted. The data are expressed as the mean ± SD of the number of $MMP\text{-}9^+$ cells/total number of tumor cells/field (n=5). *, $p<0.01$.

５．主な発表論文等

〔雑誌論文〕　計4件（うち査読付論文　4件／うち国際共著　2件／うちオープンアクセス　0件）

１．著者名 Yaginuma T, Gao J, Nagata K, Muroya R, Fei H, Nagano H, Chishaki S, Matsubara T, Kokabu S, Matsuo K, Kiyoshima T, Yoshioka I, Jimi E	４．巻 -
２．論文標題 p130Cas induces bone invasion by oral squamous cell carcinoma by regulating tumor epithelial-mesenchymal transition and cell proliferation	５．発行年 2020年
３．雑誌名 Carcinogenesis	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） doi: 10.1093/carcin/bgaa007	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Matsubara T, Yaginuma T, Addison WN, Fujita Y, Watanabe K, Yoshioka I, Hikiji H, Maki K, Baron R, Kokabu S	４．巻 132
２．論文標題 Plectin stabilizes microtubules during osteoclastic bone resorption by acting as a scaffold for Src and Pyk2.	５．発行年 2019年
３．雑誌名 Bone	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） doi: 10.1016/j.bone.2019	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Shiotsugu S, Okinaga T, Habu M, Yoshiga D, Yoshioka I, Nishihara T, Ariyoshi W	４．巻 39
２．論文標題 The Biological Effects of Interleukin-17A on Adhesion Molecules Expression and Foam Cell Formation in Atherosclerotic Lesions	５．発行年 2019年
３．雑誌名 J Interferon Cytokine Res	６．最初と最後の頁 694-702
掲載論文のDOI（デジタルオブジェクト識別子） 10.1089/jir.2019.0034	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Nakao Y, Hikiji H, Okinaga T, Takeuchi J, Habu M, Yoshiga D, Yoshioka I, Nishihara T, Ariyoshi W	４．巻 512
２．論文標題 Accumulation of hyaluronic acid in stromal cells modulates osteoclast formation by regulation of receptor activator of nuclear factor kappa-B ligand expression	５．発行年 2019年
３．雑誌名 Biochem Biophys Res Commun	６．最初と最後の頁 537-543
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.bbrc.2019.03.137	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

〔学会発表〕　計3件（うち招待講演　0件／うち国際学会　0件）

1．発表者名 柳沼樹
2．発表標題 p130CasはTGFβ刺激による口腔扁平上皮癌の形態変化、細胞移動および浸潤能の亢進に関与する
3．学会等名 第78回九州歯科学会総会・学術大会
4．発表年 2018年

1．発表者名 柳沼樹
2．発表標題 口腔扁平上皮癌細胞の形態変化、移動・浸潤能におけるp130Casの役割
3．学会等名 第72回NPO法人日本口腔科学会学術集会
4．発表年 2018年

1．発表者名 柳沼樹
2．発表標題 扁平上皮癌細胞の形態変化・移動・浸潤および骨破壊におけるp130Casの役割
3．学会等名 第60回歯科基礎医学会学術大会
4．発表年 2018年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

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