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研究課題名（和文）　口腔がんの微小リンパ節転移の検出
ー 画像診断と分子生物学的マーカーのコラボレーション ー
研究課題名（英文）　Detection of cervical lymphnode micrometastasis in oral cancer

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研究成果の概要：
腫瘍の転移は悪性腫瘍の治療においてその予後に影響する最も重要な因子であり、口腔悪性腫瘍の大部分を占める扁平上皮がんの治療にあたっても、転移の有無が予後と深く関わっている。口腔扁平上皮がんの転移の大部分は所属リンパ節である頸部リンパ節へのリンパ行性転移で、その治療にあたっては原発巣とともに転移したリンパ節の制御が必須であり、とくに現在画像診断的に検出が困難である径5mm以下の微小リンパ節転移の有無を術前に知ることが可能であればその意義は大きい。今回の検索は、口腔がんにおける転移形質に関与する遺伝子/遺伝子産物の同定を行うことを第一の目標としている。
ヒト口腔正常粘膜上皮、白板症、口腔がん組織より試料を採取し、直ちに液体窒素で凍結保存を行い、以後の実験に用いた。試料の一部よりRNAを抽出しDNAマイクロアレイにより網羅的解析を行ったところ、数十の発現亢進する遺伝子および発現が低下した遺伝子が認められた。
これらの中で口腔がんで発現が亢進しているPim-1に注目して検索を行った。口腔扁平上皮がん細胞株６種全てにPim-1のタンパク発現が認められた。北海道大学病院で口腔扁平上皮がんと診断された早期がん症例39例についてPim-1の発現を検索したところ17例（44%）にPim-1発現が認められた。Pim-1発現症例は有意に所属リンパ節転移、遠隔臓器転移が多く、Pim-1発現と転移の関連性が示唆された。転移形質とPim-1の関連を探るため、siRNAを用いて検討を行った。その結果、Pim-1に対するsiRNA導入細胞では細胞運動能の低下がみられ、この際にRac1の活性が低下することが明らかになった。
以上の結果は、Pim-1はRac1の活性化により口腔がん細胞の運動能を高め、転移形質に深く関与しており、Pim-1をターゲットにした画像診断の有用性を示唆するものだった。

交付額

（金額単位：円）

	直接経費	間接経費	合　計
2007 年度	1,700,000	510,000	2,210,000
2008 年度	1,400,000	420,000	1,820,000
年度			
年度			
年度			
総　計	3,100,000	930,000	4,030,000

研究分野：歯学
科研費の分科・細目：外科系歯学
キーワード：口腔扁平上皮がん、転移形質、遺伝子発現、Pim-1

１．研究開始当初の背景
腫瘍の転移は悪性腫瘍の治療においてその予後に影響する最も重要な因子であり、口腔悪性腫瘍の大部分を占める扁平上皮がんの治療にあたっても、転移の有無が予後と深く関わっている。口腔扁平上皮がんの転移の大部分は所属リンパ節である頸部リンパ節へのリンパ行性転移で、その治療にあたっては原発巣とともに転移したリンパ節の制御が必須であり、とくに現在画像診断的に検出が困難である径 5mm 以下の微小リンパ節転移の有無を術前に知ることが可能であればその意義は大きい。

２．研究の目的
腫瘍の転移は悪性腫瘍の治療においてその予後に影響する最も重要な因子であり、口腔悪性腫瘍の大部分を占める扁平上皮がんの治療にあたっても、転移の有無が予後と深く関わっている。今回の検索は、口腔がんにおける転移形質に関与する遺伝子/遺伝子産物の同定を行うことを目標としている。

３．研究の方法
Cell lines and culture conditions. The human oral SCC cell lines HSC2, HSC3, HSC4, Ca9.22, OSC20 and SAS (JCRB, Osaka, Japan) were used in the study. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.
Patients and tumors. A total of 39 patients who consulted the Oral Surgery Department of Hokkaido University Hospital between January 2001 and December 2004 and were diagnosed with T1 and T2 tongue SCC were examined. The clinical features of the sample are shown in Table I. There were 27 male and 12 female patients with a mean age of 62years (range 35-83 years). TNM classification was con- ducted according to UICC criteria.
Immunohistochemical staining. A Pim-1 antibody was used to detect Pim-1 expression in oral carcinomas. Prior to its use in the clinical samples, we validated the ideal condition of the antibody using cell block samples. Pim-1-negative pancreas carcinoma KPN4 and colon carcinoma HCT117 cell lines, which were confirmed to express Pim-1, were harvested, fixed with 10% buffered formalin and embedded in paraffin according to routine histological examination procedures. Formalin-fixed paraffin-embedded tissue blocks were sec- tioned, dewaxed, rehydrated, and boiled in 10 mM sodium citrate buffer (pH 6.0) for 3 min. Slides were incubated for 5min in 0.3% hydrogen peroxide followed by 1% BSA for 10min. They were then exposed to primary rabbit polyclonal antibody for Pim-1 (Calbiochem, Tokyo, Japan) for 1 h at room temperature, followed by exposure to the Histofine Simple Stain PO (R) horseradish peroxidase-conjugated anti- rabbit secondary antibody (Nichirei, Tokyo, Japan) for 40min, then washed in PBS. Color was developed by incubation with the EnVision kit/HRP (DAB) (Dako, Tokyo, Japan). The slides were then counterstained with hematoxylin and mounted.
Statistical analysis. The independence of the clinical para- meters and histological features (age, gender, tumor size and metastasis) of Pim-1 expression was examined using Fisher's exact test.
siRNA transfection targeting pim-1. HSC3 cells were tran- siently transfected with a targeted sequence of pim-1 siRNA (siPim-1) (Qiagen HP siRNA 1027400) using HiPerfect Transfection Reagent (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Control sequence siRNA (siControl, Qiagen) was also transfected into HSC3 cells as a negative control. Cells were subjected to immunoblotting and the migration assay after 48 h.
Cell migration assay. HSC3 cells with control siRNA (nc) and siPim-1 were harvested and wounded by scraping. Phase- contrast images were captured at the 0, 1, 4 and 8 h time points, and the width of the cell-free areas was measured. A Boyden chamber (BD-Discovery Labware, Bedford, MA, USA) was also used for the cell migration assay. HSC3 cells with control nc and cells with siPim-1 were seeded into the upper chamber, and EGF was added to the lower chamber as a chemoattractant. After 24 h, the upper surfaces of the filters were wiped with a cotton swab, cells were stained with Giemsa, and the infil- trated cells were counted. All experiments were conducted in triplicate.
Immunoblotting. Cells were lysed in lysis buffer [10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl, 10%glycerol, 1% Triton X-100, 0.1% SDS and protease inhibitor cocktail(Roche, Indianapolis, IN, USA)] for 20 min on ice and clarified by microcentrifugation. The supernatant was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). A Pim-1 rabbit polyclonal antibody (Calbiochem, Tokyo, Japan) was used for Pim-1 detection in cultured oral SCC cell lines. Rac1 and Cdc42 mouse monoclonal antibodies (Upstate, Tokyo, Japan) were employed for the GTP pull-down assay.
GTP pull-down assay. Rac1/Cdc42 activity was analyzed with the Rac/Cdc42 Activation Kit (StressXpress™; Stressgen Bioreagents, Ann Arbor, MI, USA) according to the manufacturer's instructions. In brief, after washing cells were lysed in assay buffer and centrifuged to remove cell debris. To determine total Rac1/ Cdc42 levels, 40 µl of each sample was stored at -80˚C for separate analysis. The remaining 700 µl of the supernatant (containing 500 µg of total protein) was incubated with 10µg of the agarose-conjugated p21-binding domain of p21-activated protein kinase-1, which binds both activated Rac1 and Cdc42, for 1 h at 4˚C with gentle rocking. Agarose beads that bound active Rac1/Cdc42 were washed 4times in assay buffer, resuspended in 50 µl of 2X SDS sample buffer, and boiled at 95˚C for 5 min. Active (GTP-bound) and total Rac1/Cdc42 protein levels in each sample were analyzed by Western blotting. All blots were visualized using the ECL Detection System (Amersham Biosciences Corp., NJ, USA).

４．研究成果

Pim-1 protein is expressed in oral squamous cell carcinoma cell lines. To address the role of Pim-1 in oral carcinogenesis, we first examined whether it was expressed in the oral car- cinoma cell lines HSC2, HSC3, HSC4, OSC20, Ca9.22 and SAS, analyzed using Western blotting. All cell lines expressed Pim-1 protein, with HSC3 and SAS exhibiting especially high levels. No obvious expression was observed in the normal human gingival fibroblasts used as a negative control (Fig. 1).

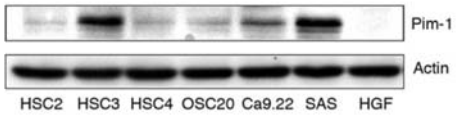


Figure 1. Western blot analysis of Pim-1 expression in oral squamous cell carcinoma cell lines. Pim-1 is observed in 6 oral carcinoma cell lines with slight (HSC2, HSC4, OSC20), moderate (Ca9.22) and high (HSC3, SAS) levels of expression. HGF serves as a negative control.

Pim-1 expression in tongue squamous cell carcinomas is related to cancer metastasis. A total of 39 tongue carcinoma cases were examined. They comprised 11 cases of T1 and 28 cases of T2, 37 cases of N0 and 2 of N2, and 37 cases of M0 and 2 of M1 according to the initial clinical TNM classification. During the follow-up period, subsequent lymph node metastasis occurred in 10 patients, and distant metastasis in 6 of the N0 and M0 cases, respectively. Patient prognoses were as follows: 13 succumbed to their tumors, 2 succumbed to other factors, and 23 are alive without tumors. The prognosis of 1 case is unknown as the patient ceased consulting the hospital during the 5-year follow-up period (Table I). Prior to addressing the clinical cases, we determined the immuno- histochemical detection conditions with cell block materials constructed using a procedure similar to that used for routine

Table I. Clinicopathological features of the examined cases.

Case	Age/gender	pTNM	Subsequent metastasis	Prognosis	Pim-1
1	47/M	T3N0M0	+; Regional lymph nodes	Deceased due to tumor	+
2	50/M	T2N2bM0	+; Distant organ	Deceased due to tumor	+
3	48/M	T1N0M0		Alive	+
4	76/F	T2N0M0		Alive	+
5	74/M	T2N0M0		Alive	+
6	71/F	T2N0M0		Alive	+
7	58/M	T2N0M0		Alive	+
8	62/M	T2N0M0		Alive	+
9	46/M	T2N0M1	+; Regional lymph nodes	Deceased due to tumor	+
10	66/M	T3N3M1	-	Deceased due to tumor	+
11	53/M	T3N0M0	+, Regional lymph nodes, distant organ	Deceased due to tumor	+
12	69/M	T3N0M0	+, Regional lymph nodes, distant organ	Deceased due to tumor	+
13	35/M	T3N0M0	+; Distant organ	Deceased due to tumor	+
14	51/M	T3N0M0	+; Distant organ	Deceased due to tumor	+
15	73/F	T2N0M0	+; Regional lymph nodes	Deceased due to tumor	+
16	74/F	T1N0M0	+; Regional lymph nodes	Deceased due to tumor	+
17	67/F	T2N0M0		Alive	+
18	67/M	T1N0M0	+; Regional lymph nodes	Alive	
19	63/M	T2N0M0		Alive	
20	72/F	T2N0M0		Alive	
21	67/F	T1N0M0		Alive	
22	68/M	T3N0M0		Alive	
23	50/M	T3N0M0	-	Alive	-
24	66/M	T3N0M0	+, Regional lymph nodes, distant organ	Deceased (other factor)	-
25	45/M	T1N0M0	-	Alive	-
26	60/M	T1N0M0	-	Deceased (other factor)	-
27	68/M	T2N0M0	-	Alive	-
28	83/F	T2N0M0	+; Regional lymph nodes	Deceased due to tumor	
29	66/M	T1N0M0		Alive	
30	28/F	T2N0M0		Alive	
31	69/M	T2N0M0		Alive	
32	65/F	T1N0M0		Alive	
33	63/M	T2N0M0		Alive	
34	69/M	T1N0M0		Alive	
35	48/M	T1N0M0	-	Alive	-
36	63/M	T3N0M0	-	Deceased due to tumor	-
37	48/F	T1N0M0	+, Regional lymph nodes	Unknown	-
38	65/F	T3N0M0	-	Deceased due to tumor	-
39	78/M	T2N0M0	-	Alive	-

pathological specimens. KPN4, a Pim-1-negative pancreas carcinoma cell line, and HCT117, a Pim-1-positive colon carcinoma cell line, were used. No obvious Pim-1-positive signal was observed in KPN4 cells (Fig. 2A), but Pim-1 was detected in the nuclei of HCT117 cells (Fig. 2B) under the conditions described in Materials and methods. Immuno-histochemical detection of Pim-1 was performed for 39 tongue SCCs. Nuclear Pim-1-positive signals (Fig. 2C) were observed in 17 of the 39 cases. The remaining 22 were Pim-1 negative. Primary and subsequent lymph node metastases were present in 12 cases, and distant metastasis was observed in 8 cases. Metastasis in the lymph nodes and/or distant organs was observed in 14/39 cases. Among the Pim-1-positive cases, 10/17 exhibited significantly high metastatic activity, whereas among the Pim-1-negative cases only 4/22 did ($p<0.005$) (Fig. 3). All 10 of the Pim-1-positive and metastasis-burdened patients succumbed to their tumors, whereas 3/22 Pim-1- negative patients experienced a poor clinical course with tumors. No significant association was noted between age or gender and Pim-1 expression.

Pim-1 is associated with cell motility through its activation of Rac-1. The role of pim-1 was further explored using HSC3 cells to examine cell motility. siPim-1-transfected HSC3 cells and HSC3 cells transfected with the negative control sequence of siPim-1 (Cont) were used for the experiments. HSC3 cells transiently transfected with siPim-1 exhibited reduced expression of Pim-1 protein (Fig. 4A). Cell motility was examined by wound-healing and Boyden chamber assays. Repair of the cell monolayer after wounding slowed upon siPim-1 treatment compared to the control (Fig. 4B). The width of migrating cells in the scratch wound was significantly narrower in siPim-1-treated HSC3 cells (Fig. 4C). Furthermore, the migration assay using the Boyden chamber revealed that siPim-1-treated HSC3 cells had an ~50% reduction in motility in comparison to control cells (Fig. 4D). This difference was statistically significant ($p<0.001$). Thus, knock-down of pim-1 extensively suppressed the malignant phenotypes of cell motility in HSC-3 cells.

Next, motility-associated protein expression was examined. The Rho family of GTPases are members of the small G protein family, and change from a GDP-bound inactive form to a GTP-bound active form. In this study, we tested GAP activity using a standard GTPase assay of Rac1 and cdc42 using immunoprecipitation from HSC3 cells transfected with control siRNA or siRNA for Pim-1. EGF stimulation (100 ng/ml) was conducted to induce cell motility, as HSC3 cells have been shown to possess an EGF receptor that induces cell migration. The GTP-binding assay for Rho family proteins revealed that HSC-3 cells with siRNA for pim-1 reduced Pim-1 protein expression (siPim-1). EGF stimulation increased the expression of GTP-bound active Rac-1 and Cdc42. Active-Rac-1 expression decreased when cells were treated with siPim-1, although the expression level of Cdc42 was not affected by siPim-1 treatment (Fig. 5).

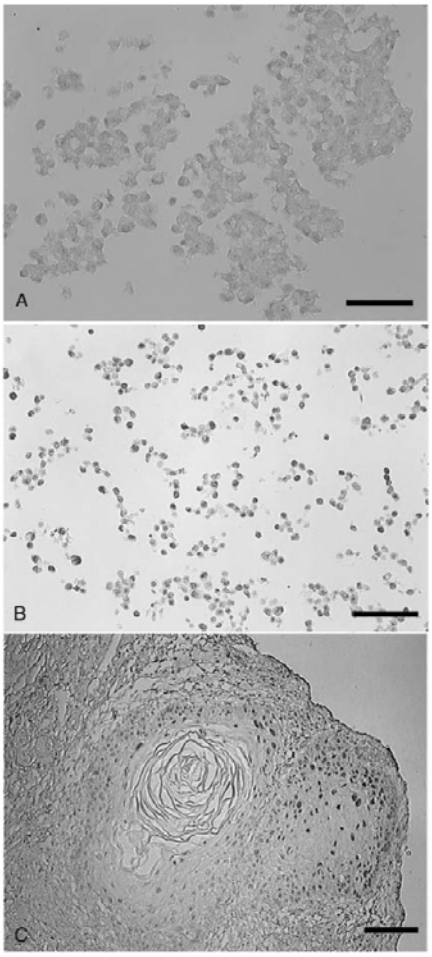


Figure 2. Immunohistochemical detection of Pim-1. (A) No obvious Pim-1-positive signal was observed in KPN4, a Pim-1-negative pancreas carcinoma cell line. (B) Pim-1 was detected in the nuclei of HCT117 cells, a Pim-1-positive colon carcinoma cell line. (C) A Pim-1-positive case of tongue squamous cell carcinoma (case 9, Table I) exhibiting nuclear Pim-1-positive signals. Bar, 200 μm.

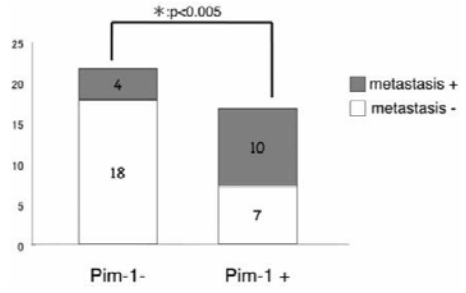
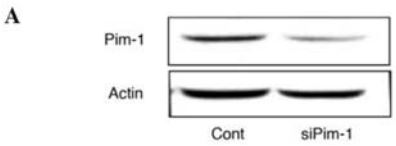
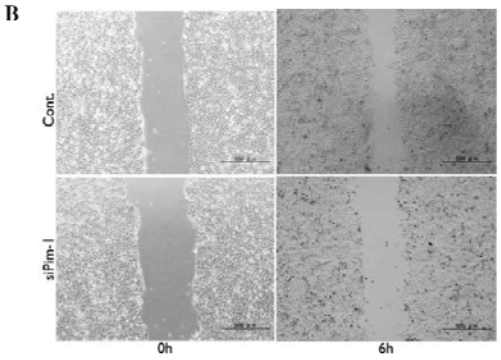
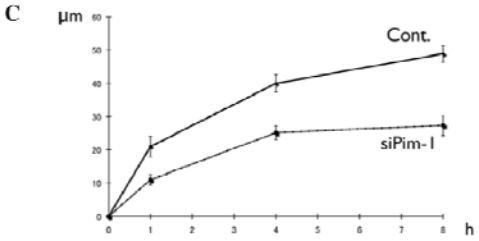


Figure 3. Statistical analysis of Pim-1 expression and metastasis. A significant correlation between Pim-1 expression and metastasis of tongue squamous cell carcinoma was noted.







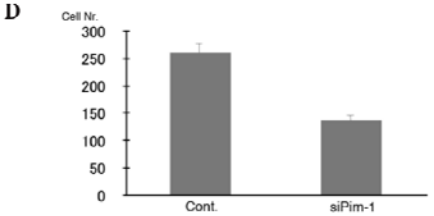


Figure 4. siRNA for Pim-1 induced Pim-1 knockdown which suppressed cancer cell motility. (A) Reduced expression of Pim-1 is observed in HSC3 cells by transfection of siRNA for pim-1. (B) The wound-healing assay shows that repair of the cell monolayer after wounding slowed with siPim-1 treatment compared to the control. Bar, 500 μm. (C) The migrating length is significantly narrower in HSC3 cells. (D) The migration assay using the Boyden chamber revealed that siPim-1-treated HSC3 cells had an ~50% reduction in motility in comparison to control cells. This difference was statistically significant ($p<0.001$).

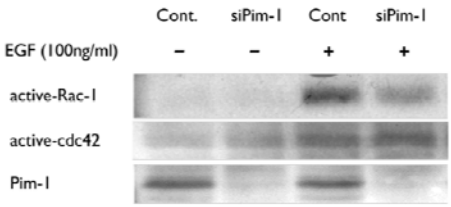


Figure 5. The active form of Rac-1 is reduced by Pim-1 knockdown. The GTP-binding assay for Rho family proteins revealed that HSC-3 cells with siRNA for pim-1 reduced Pim-1 protein expression (siPim-1). EGF stimulation (100 ng/ml) increased expression of both GTP-bound active Rac-1 and Cdc42. Active-Rac-1 expression decreased when cells were treated with siPim-1, although the expression level of Cdc42 was not affected by siPim-1 treatment.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　1 件）

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