行政院國家科學委員會專題研究計畫 成果報告

基質細胞衍生因子(SDF-1)及其受體(CXCR4)在口腔黏膜下纖維化及口腔癌的表現及其機制探討(第 3 年)

研究成果報告(完整版)

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中 華 民 國 100 年 10 月 31 日

Stromal Cell-Derived Factor-1 promotes the migration via urokinase plasminogen activator of oral cancer

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Abstract

Stromal cell-derived factor-1 (SDF-1; CXCL12), the C-X-C subfamily of chemokine, has been found to involve in cell migration. Here, we found that SDF-1 increased migration and urokinase plasminogen activator (u-PA) secretion and expression in human oral cancer cell. U-PA small interfering RNA inhibited the SDF-1-induced u-PA expression and thereby significantly inhibited the SDF-1-induced cell migration. We also found that the transcriptional regulation of u-PA by SDF-1 was mediated by phosphorylation of extracellular signal-regulated kinases (ERKs) and PI3K/Akt pathway and SDF-1 induced migration activity was inhibited by the specific inhibitor of Erk 1/2 and PI3K/Akt pathway. Taken together, these results indicate that SDF-1 enhanced migration of oral cancer cells through the increase of u-PA production.

Keyword: SDF-1, migration, u-PA, MAPK pathway

Introduction

Oral cancer is the sixth leading cause of cancer death in Taiwan [1], and more than 85-90 % of cases are oral squamous cell carcinoma (OSCC) [2]. The clinical presentation of this disease is complex and affected areas may include lip, tongue, major salivary glands, gums and adjacent oral cavity tissues, floor of the mouth, tonsils, oropharynx, nasopharynx, hypopharynx and other oral regions, nasal cavity, accessory sinuses, middle ear, and larynx [3].

The development of carcinogenesis requires not only proliferation of tumor cells, but also infiltration of organs, which promote tumor growth and metastases [4-12]. Stromal cell-derived factor-1 (SDF-1; CXCL12), the C-X-C (Cys-Xxx-Cys) subfamily of chemokine [4], has been found to involve in cell proliferation, [5,6] cell migration, [7,8] and cell invasion[5]. The interaction between SDF-1 and its seven-transmembrane G-coupled receptor, CXCR4, is associated with cell proliferation, [7,9] extramedullary organ infiltration, [8] cell migration, [7,10,11] tumor grade development, [11] transcriptional activation[6,10], and metastases [12,13]. As well, over expression of SDF-1 and CXCR4 are associated with the development and metastasis of human oral squamous cell carcinoma [14-16].

The urokinase plasminogen activator (u-PA) system is a serine proteinase system involved in extracellular matrix (ECM) degradation [17]. Many experimental and

clinical studies have demonstrated the association of u-PA system activity with prognosis of cancer patient [17-19]. The levels of u-PA system are highly expressed in invasive carcinomas, but are very low in normal tissues [19, 20]. Therefore, the levels of u-PA system in tumor tissues are implicated as prognostic biomarkers in a wide range of malignancies [18, 19, 21]. Additionally, the involvement of u-PA system members on human cancer progress has been explored in a recent study [22].

The contribution of SDF-1 to cancer migration has been intensively studied. U-PA modulates the cell migration and invasion of several types of cancer cells. However, the effect of SDF-1 and u-PA on migration activity in human oral cells is still unknown. Here, we show that SDF-1 increase migration and up-regulate u-PA expression in human oral cancer cells. In addition, Erk 1/2 nad PI3K/ Akt pathway signaling pathways are involved.

Materials and Methods

Materials.

Cell culture materials and fetal bovine serum (FBS) were obtained from Gibco-BRL (Gaithersburg, MD). An enhanced chemiluminescence kit was purchased from Amersham (Arlington Heights, IL). Antibodies specific for u-PA, Erk1/2, JNK, PI3K, Akt, p38 and β-actin were obtained from Santa Cruz Biotechnology Inc. (Santa

Cruz, CA, USA). Unless otherwise specified, other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture

OECM were were cultured in DMEM supplemented with 10 % FBS and penicillin (100 U/mL), streptomycin (100 μg/mL), and 25 mM HEPES (pH 7.4) in a humidified 37 °C incubator.

2.3. Cell migration assays

Cell migration were assayed according to the methods described by Yang et al [23]. After treatment with SDF-1 (0~100 ng/mL) for 24 h, surviving cells were harvested and seeded in a Boyden chamber (Neuro Probe, Cabin John, MD, USA) at a density of 10^4 cells/well in serum free medium, and then incubated for 24 h. For invasion assay, 10 μl Matrigel (25 mg/50 mL; BD Biosciences, MA, USA) was applied to 8 μm pore size polycarbonate membrane filters and the bottom chamber contained standard medium. The invaded cells were fixed and stained with 5 % Giemsa. Cell numbers were counted under a light microscope.

2.4. casein zymography

The activities of u-PA in conditional medium were measured using casein zymography protease assays as described previously [24]. Collected media of an appropriate volume were subjected to 0.1 % casein-8 % SDS–PAGE electrophoresis. After electrophoresis, gels were washed with 2.5 % Triton X-100 and incubated in reaction buffer (40 mM Tris–HCl, pH 8.0; 10 mM $CaCl_2$ and 0.01 % NaN_3) for 12 h at 37 °C. The gel was, then, stained with Coomassie brilliant blue R-250.

2.5. RNA preparation and TaqMan quantitative real-time PCR

Total RNA was isolated from oral cancer cells using Trizol (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Quantitative real-time PCR analysis was carried out using Taqman one-step PCR Master Mix (Applied Biosystems). 100 ng of total cDNA was added per 25 µl reaction with MMP-2 or GAPDH primers and Taqman probes. The MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-12, MMP-13, MMP-14, u-PA, u-PAR, PAI-1 and GAPDH primers and probes were designed using commercial software (ABI PRISM Sequence Detection System; Applied Biosystems). Quantitative real-time PCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was

detected.

2.6. Western blot analysis

The total cell lysates or nuclear extracts were prepared as previously described[25] . Western blot analysis was performed using primary antibodies against u-PA, u-PA, Erk1/2, JNK, PI3K, Akt, p38 and β-actin. The relative photographic densities were quantitated by scanning the photographic negatives using a gel documentation and analysis system (AlphaImager 2000, Alpha Innotech Corporation, San Leandro, CA, USA).

2.7. Small interfering RNA transfection

Silencer pre-designed small interference RNA (siRNA) for human u-PA and a non-related control siRNA-A were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). After OECM-1 cells had grown to 70% confluence, the cells were transfected with the siRNA duplexes at a final concentration of 20 nM using GenMute^{TM} siRNA Transfection Reagent (SignaGen Laboratories) according to the manufacturer's instructions.

2.8. Statistical analysis

Statistical significances of differences throughout this study were analyzed by One-way ANOVA test to compare differences between treatments and followed up using Dunnett's multiple comparison post-hoc test. A p value < 0.05 was considered to be statistically significant. Values represent the means ± standard deviation and the experiments were repeated three times.

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Acknowledgment

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Figure 1

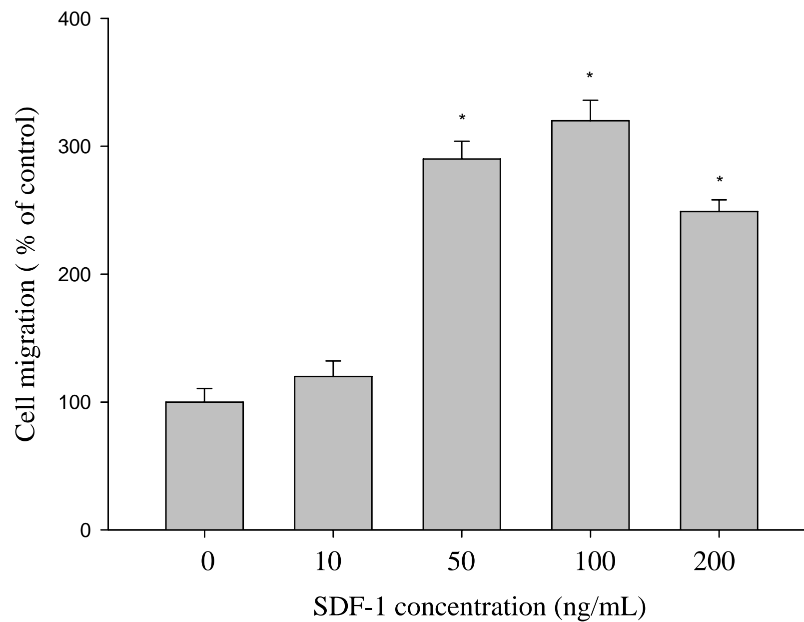


Figure 1. Effects of SDF-1 on migration of OECM-1 cells. Cell migration was measured using a Boyden chamber for 24 h with polycarbonate filters. The migration abilities of OECM-1 cells were quantified by counting the number of cells that invaded to the underside of the porous polycarbonate as described in the *Materials and Methods* section. The values represented the means ± SD of at least three independent experiments. *$p < 0.05$ as compared with the vehicle group.

Figure 2

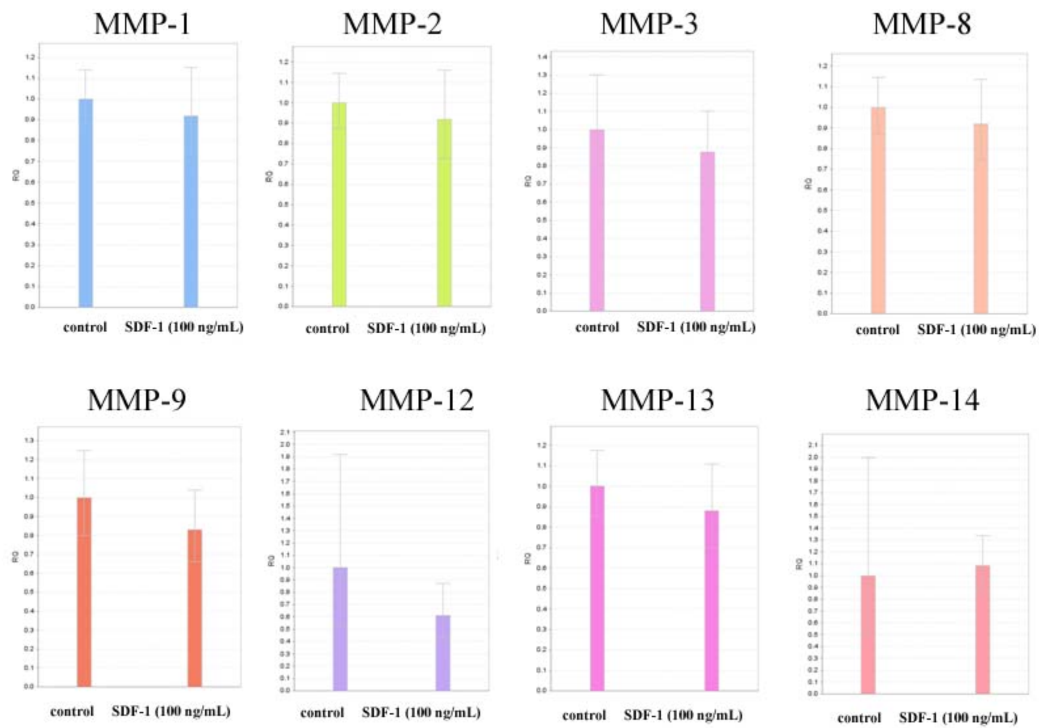


Figure 2. Effect of SDF-1 (100 ng/mL) on MMPs mRNA levels on OECM-1 cell. OECM-1 cells were treated with SDF-1 (100 ng/mL) for 2 h and then subjected to real-time PCR to analyze the mRNA expression of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-12, MMP-13 and MMP-14. The values represented the means ± SD of at least three independent experiments.

Figure 3

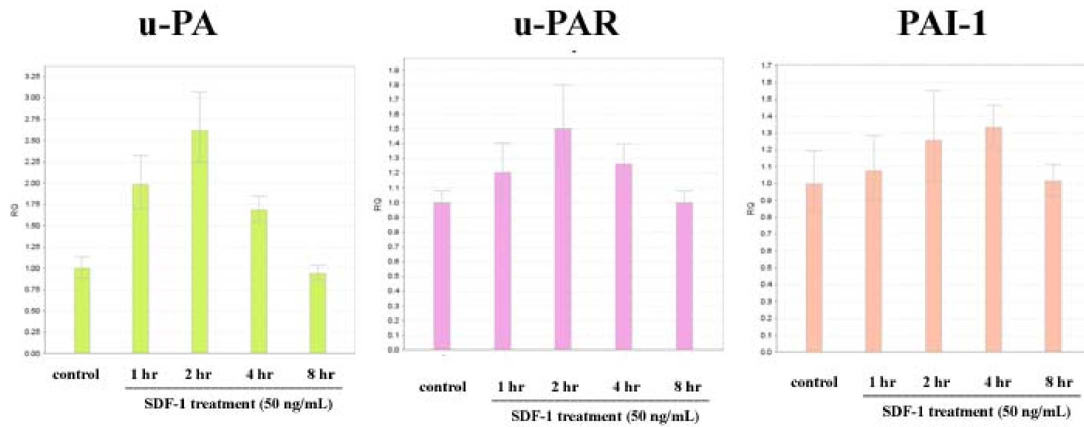
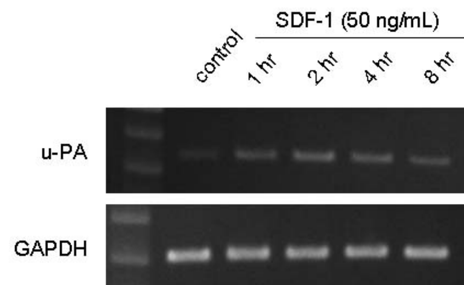


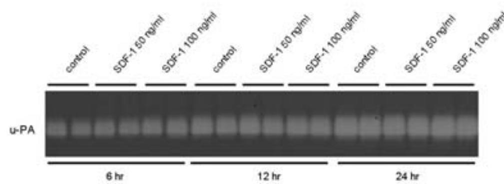
Figure 3. Effect of SDF-1 (100 ng/mL) on u-PA system mRNA levels on OECM-1 cell. OECM-1 cells were treated with SDF-1 (50 ng/mL) for 1, 2, 4, and 8 h and then subjected to real-time PCR to analyze the mRNA expression of u-PA, u-PAR and PAI-1. The values represented the means ± SD of at least three independent experiments.

Figure 4

(A)



(B)



(C)

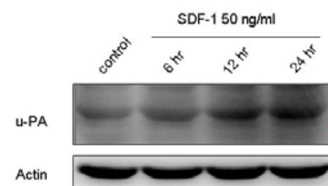


Figure 4. Effects of SDF-1 on the mRNA ,activity and protein level of u-PA. (A) OECM-1 cells were treated with SDF-1 (50 ng/mL) for 1, 2, 4, and 8 h and then subjected to RT-PCR to analyze the mRNA expression of u-PA. OECM-1 cells were treated with SDF-1 (50 or 100 ng/mL) for 6, 12, and 24 h and then subjected to casein zymography to analyze the activity of u-PA (B) or Western blotting to analyze the protein levels of u-PA (C). Quantitative results of u-PA protein levels, which were adjusted with β-actin protein level.

Figure 5

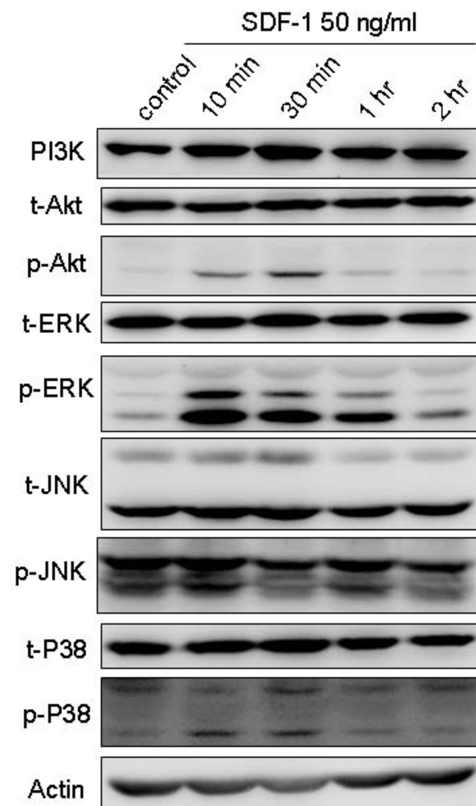


Figure 5. Effect of SDF-1 on MAPKs and PI3K/Akt pathway. The effect of SDF-1 on the phosphorylation of ERK1/2, p38, JNK1/2, PI3k and Akt was investigated by Western blot analysis. SDF-1 induced ERK1/2 and Akt phosphorylation in OECM-1 cells.

Figure 6

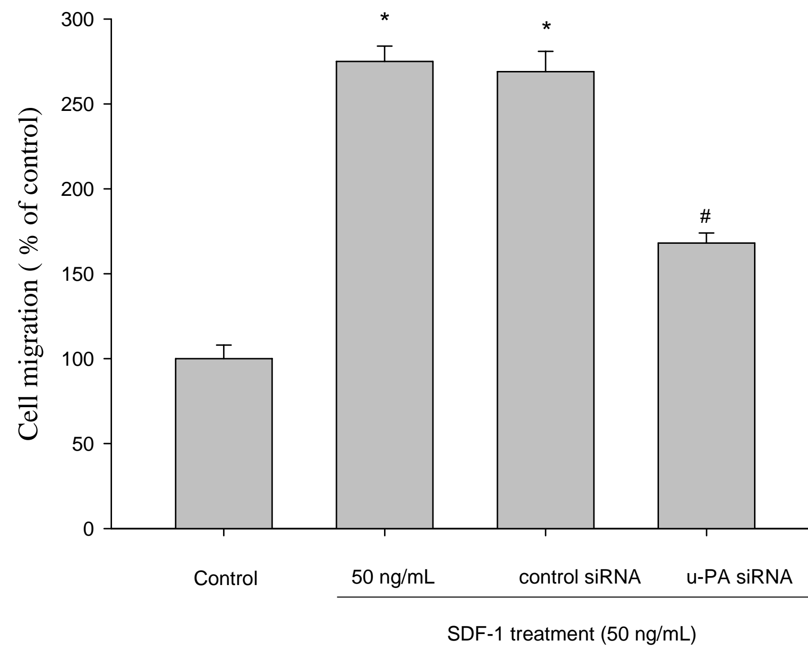


Figure 6. Critical role of u-PA in SDF-1-induced migration in OECM-1 cells. OECM-1 cells were transiently transfected with au-PA-specific siRNA or control siRNA for 7 h. The migration abilities of OECM-1 cells were quantified by counting the number of cells that invaded to the underside of the porous polycarbonate as described in the *Materials and Methods* section. The values represented the means ± SD of at least three independent experiments. $^{*}p < 0.05$ as compared with the control group; $^{\#}p < 0.05$ as compared with the SDF-1 treatment group.

Figure 7

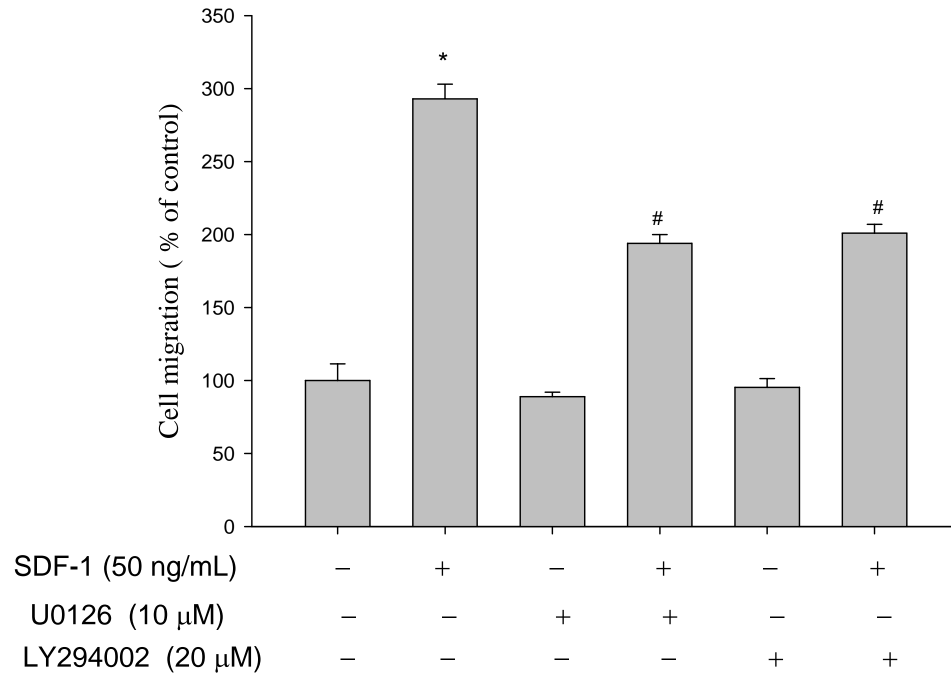


Figure 7. Effects of MAPK inhibitors on SDF-1-induced migration in OECM-1 cells. OECM-1 cells were treated with SDF-1 for 24 h in the presence or absence of 10 uM U0126 and 20 uM LY294002. The migration abilities of OECM-1 cells were quantified by counting the number of cells that invaded to the underside of the porous polycarbonate as described in the *Materials and Methods* section. The values represented the means ± SD of at least three independent experiments. $^{*}p < 0.05$ as compared with the control group; $^{\#}p < 0.05$ as compared with the SDF-1 treatment group.

國科會補助計畫衍生研發成果推廣資料表

日期:2011/10/31

國科會補助計畫	計畫名稱: 基質細胞衍生因子(SDF-1)及其受體(CXCR4)在口腔黏膜下纖維化及口腔癌的表現及其機制探討
	計畫主持人: 楊順發
	計畫編號: 97-2314-B-040-025-MY3 學門領域: 牙醫學

無研發成果推廣資料

97 年度專題研究計畫研究成果彙整表

計畫主持人：楊順發	計畫編號：97-2314-B-040-025-MY3
計畫名稱：基質細胞衍生因子(SDF-1)及其受體(CXCR4)在口腔黏膜下纖維化及口腔癌的表現及其機制探討	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
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		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
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國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

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2. 研究成果在學術期刊發表或申請專利等情形：
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專利：□已獲得 □申請中 ■無
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3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）
基質細胞衍生因子(stromal-derived factor-1；SDF-1)又稱為 CXCL12，為一趨化激素(chemokines)，可與其受體蛋白 CXCR4 結合，進而進行其訊息傳遞，並與腫瘤的生長、遷移、吸附與血管新生的癌化發生過程有關。而尿型胞漿素原活化劑 urokinase-plasminogen activator； u-PA)和基底膜的降解有關，因此被認定與癌細胞的侵入與轉移有關。但是 SDF-1 與 u-PA 的相關性仍不清楚。因此，本實驗發現，加入 SDF-1 會誘導口腔癌細胞其 u-PA 的表現並促使癌細胞的轉移。我們更進一步利用 siRNA 來抑制 u-PA 的表現，發現也會抑制由 SDF-1 所誘導的轉移能力。另外，我們也發現 SDF-1 誘導癌症的轉移是透過 Erk 1/2 及 PI3K/Akt 等路徑。而使用 Erk 1/2 及 PI3K/Akt 的專一性抑制劑的確也可以抑制由 SDF-1 所誘導的轉移能力。總而言之，我們發現 SDF-1 所誘導口腔癌的轉移是透過 u-PA 的表現並經由 MAPK pathway 所調控的。了解了口腔癌轉移的詳細機制後，也許可以利用相關抑制劑來當作抗口腔癌轉移的標的。
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