Cyr61 increases matrix metalloproteinase-3 expression and cell motility in human oral squamous cell carcinoma cells

Jing-Yuan Chuang[1], Nuo-Yi Yu[2], I-Ping Chiang[3], Chih-Ho Lai[4,5], Chia-Der Lin[6], and Chih-Hsin Tang[7,5]*

[1]Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung Taiwan

[2]Graduate Institute of Biotechnology, National Chung Hsing University, Taichung, Taiwan

[3]Department of Pathology, China Medical University Hospital, Taichung, Taiwan

[4]Department of Microbiology, School of Medicine, China Medical University, Taichung, Taiwan

[5]Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan

[6]Department of Otolaryngology, China Medical University Hospital, Taichung, Taiwan

[7]Department of Pharmacology, School of Medicine, China Medical University, Taichung, Taiwan

***: Address correspondence to:**

Chih-Hsin, Tang PhD

Department of Pharmacology, School of Medicine, China Medical University.

No. 91, Hsueh-Shih Road, Taichung, Taiwan.

Tel: 886-4-22052121-7726; Fax: 886-4- 22333641

E-mail: chtang@mail.cmu.edu.tw

ABSTRACT

Oral squamous cell carcinoma (OSCC) has a striking tendency to migrate and metastasize. Cysteine-rich 61 (Cyr61), from the CCN gene family, is a secreted and matrix-associated protein, which is involved in many cellular activities such as growth and differentiation. However, the effects of Cyr61 on human OSCC cells are largely unknown. In this study, we found that Cyr61 increased the migration and the expression of matrix metalloproteinases-3 (MMP)-3 in human OSCC cells. αvβ5 or α6β1 monoclonal antibody (mAb), focal adhesion kinase (FAK) inhibitor, and mitogen-activated protein kinase (MEK) inhibitors (PD98059 and U0126) inhibited the Cyr61-induced increase of the migration and MMP-3 up-regulation of OSCC cells. Cyr61 stimulation increased the phosphorylation of FAK, MEK, and extracellular signal-regulated kinase (ERK). In addition, NF-κB inhibitors suppressed the cell migration and MMP-3 expression enhanced by Cyr61. Moreover, Cyr61 increased NF-κB luciferase activity and binding of p65 to the NF-κB element on the MMP-3 promoter. Taken together, our results indicate that Cyr61 enhances the migration of OSCC cells by increasing MMP-3 expression through the αvβ3 or α6β1 integrin receptor, FAK, MEK, ERK, and NF-κB signal transduction pathway.

Running title: Cyr61 increases the migration of OSCC cells

Key words: Cyr61; Migration; OSCC; FAK; NF-κB

INTRODUCTION

Oral squamous cell carcinoma (OSCC) represents 1–2% of all human malignancies. It is characterized by a high degree of local invasiveness and a high rate of metastasis to cervical lymph nodes. The migration of OSCC into maxillary and mandibular bones is a common clinical problem [Lyons and Jones, 2007]. Because OSCC cancer is a type of highly malignant tumor with a potent capacity to invade locally and metastasize distantly [Greenberg et al., 2003; Thomas and Speight, 2001], an approach that decreases its ability to invade and metastasize may facilitate the development of effective adjuvant therapy.

The invasion of tumor cells is a complex, multistage process. To facilitate the cell motility, invading cells need to change the cell-cell adhesion properties, rearrange the extracellular matrix (ECM) environment, suppress anoikis and reorganize their cytoskeletons [Roussos et al., 2011]. Matrix metalloproteinases (MMPs) have important roles in these processes because their proteolytic activities assist in degradation of ECM and basement membrane [Egeblad and Werb, 2002]. MMPs are a large family of structurally related calcium- and zinc-dependent proteolytic enzymes involved in the degradation of many different components of the extracellular matrix [Vincenti, 2001]. MMP-3 (stromelysin-1) is secreted as an inactive soluble pro-form which can be activated by a variety of proteases [Carmeliet et al., 1997]. Like most MMPs, MMP-3 is not expressed in normal tissue, but is rapidly induced in cases of tissue repair or during remodeling processes [Page-McCaw et al., 2007]. It has been also reported that MMP-3 plays a critical role in ECM turnover and cell-cell interactions, as well as tumor metastasis [Boonrao et al., 2010; Tang et al., 2010]

Cysteine-rich 61 (Cyr61) is the first cloned member of the CCN family [Lau and Nathans, 1985], which comprises Cyr61/CCN1, connective tissue growth factor (CTGF/CCN2), nephroblastoma overexpressed (Nov/CCN3), Wisp-1/elm1 (CCN4), Wisp-2/rCop1 (CCN5), and Wisp-3 (CCN6). Most members of the CCN family share a uniform modular structure and exhibit diverse cellular functions such as regulation of cell division, chemotaxis, apoptosis, adhesion, motility, and ion

transport [Dhar and Ray, 2010]. Cyr61 has been reported to mediate cell adhesion, stimulate chemostasis, augment growth factor–induced DNA synthesis, foster cell survival, and enhance angiogenesis [Grzeszkiewicz et al., 2002]. Elevated Cyr61 expression is associated with advanced breast adenocarcinoma pathogenesis, pancreatic cancer, and gliomas [Lin et al., 2004; Tsai et al., 2002]. Interestingly, down-regulated Cyr61 expression is also noted in prostate cancer, uterine leiomyoma, rhabdomyosarcoma, and non–small cell lung carcinoma [Croci et al., 2004; Sampath et al., 2001]. The contrasting expression of Cyr61 in different types of cancer suggests that Cyr61 may exert a sophisticated function depending on cellular context.

Previous studies have shown that Cyr61 modulates cell migration and invasion in human cancer cells [Lin et al., 2004; Tan et al., 2009]. Cyr61-mediated migration may involve activation of integrin receptors [Tan et al., 2009]. However, the effect of Cyr61 on MMPs expression and migration activity in human OSCC cells is mostly unknown. We hypothesized that Cyr61 might be capable of regulating OSCC cancer migration and MMPs expression. Here we found that Cyr61 increased the migration and the expression of MMP-3 in OSCC cells. In addition, αvβ3/α6β1 integrin receptor, focal adhesion kinase (FAK), MAPK kinase (MEK), extracellular signal-regulated kinase (ERK) and NF-κB signaling pathways may be involved in the increase of MMP-3 expression and cell migration by Cyr61.

MATERIALS and METHODS

Materials

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, p-FAK, FAK, p-ERK, ERK, p-IKK, IKK, p-IκB, IκB, p-p65, p65, MMP-3 and the small interfering RNAs (siRNAs) against MMP-3, FAK, and control (for experiments using targeted siRNA transfection; each consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz,

CA, USA). PD98059 and U0126 were purchased from Calbiochem (San Diego, CA, USA). Rabbit polyclonal antibodies specific for αvβ3, α5β1, and α6β1 integrin were purchased from Chemicon (Temecula, CA). The recombinant human Cyr61 was purchased from PeproTech (Rocky Hill, NJ). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA). The phosphorylation site mutant of FAK(Y397F) was a gift from Dr. J. A. Girault (Institut du Fer a` Moulin, Moulin, France). The ERK2 (K52R) mutant was a gift from Dr. M. Cobb (South-Western Medical Center, Dallas, TX). The MEK1 mutant was a gift from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). The IKKα(KM) and IKKβ(KM) mutants were gifts from Dr. H. Nakano (Juntendo University, Tokyo, Japan). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

The human OSCC cell line SCC4, SAS, and Cal-27 were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in DMEM supplemented with 20 mM HEPES and 10% heat-inactivated FCS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37℃ with 5% CO_2.

Migration Assay

The migration assay was performed using Transwell (Costar, NY; pore size, 8 μm) in 24-well dishes. Before the migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including the FAK inhibitor, MMP-3 inhibitor, U0126, PD98059, or vehicle control (0.1% DMSO). Approximately 1×10^4 cells in 100 μl of serum-free medium were placed in the upper chamber, and 300 μl of the same medium containing Cyr61 was placed in the lower chamber. The plates were incubated for 24 h at 37℃ in 5% CO_2, and then cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side

of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Each clone was plated in triplicate in each experiment, and each experiment was repeated at least three times. The number of migrating cells in each experiment was adjusted with a cell viability assay to correct for proliferation effects of Cyr61 (corrected migrating cell number = counted migrating cell number/percent of viable cells) [Lu et al., 2010].

Quantitative Real-Time PCR

Total RNA was extracted from OSCC cells using a TRIzol kit (Invitrogen Carlsbad, CA). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using oligo(dT) primer [Hsieh et al., 2003; Wang et al., 2003]. The quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). 2 μl of total cDNA mixtures were added per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (β-actin was used as internal control) (Applied Biosystems, CA). qPCR assays were carried out in triplicate (one independent RNA sample for each treatment) on an StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 ℃ followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ for 60 s. 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 (denoted C_T).

Western Blot Analysis

Cellular lysates were prepared as described [Huang et al., 2003; Tseng et al., 2003]. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 hr at room temperature and then probed with rabbit anti-human antibodies against

MMP-3, β-actin, FAK, p-FAK, p-ERK, p-IKK, IKK, p-IκBα, IκBα, p-p65, or p65 (1:1000) for 1 hr at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 hr at room temperature. The blots were visualized with enhanced chemiluminescence and Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Flow Cytometric Analysis

The human OSCC cells were plated in 6-well dishes. The cells were then washed with PBS and detached with trypsin at 37℃. The cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After rinsing in PBS, the cells were incubated with a mouse anti-human antibody against αvβ3 or α6β1 integrin (1:100) for 1 h at 4℃. The cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary IgG (1:100; Leinco Tec. Inc., St. Louis, MO) for 45 min and analyzed by flow cytometry using FACS Calibur and CellQuest software (BD Biosciences).

Reporter assay

The OSCC cells were transfected with reporter plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Twenty-Four hours after transfection, the cells were treated with inhibitors for 30 min and then Cyr61 or vehicle was added for 24 hr. Cell extracts were then prepared, and luciferase and β-galactosidase activities were measured.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation analysis was performed as described [Huang and Chen, 2005]. DNA immunoprecipitated with anti-p65 was purified and extracted with phenol-chloroform. The purified DNA pellet was subjected to PCR, and PCR products were resolved with 1.5% agarose gel electrophoresis and visualized with UV light. The primers 5'-AATTCACATCACTGCCACCA-3' and

5'-CTCTGTGGCAATAAGATCCC-3' were utilized to amplify across the MMP-3 promoter region (–392 to –207) [Souslova et al., 2010].

Statistics

The values given are means ± S.E.M. The significance of difference between the experimental groups and controls was assessed by Student's t-test. The difference was significant if the *p*-value was <0.05.

RESULTS

Cyr61 increases migration and MMP-3 expression in OSCC cells

Cyr61 has been reported regulates migration and invasion of human cancer cells [Lin et al., 2004; Tan et al., 2009]. However, the effect of Cyr61 on migration of OSCC cells is mostly unknown. The Cyr61 for OSCC cell migration was examined using the Transwell assay. Treatment of SCC4 cells with Cyr61 (10-100 ng/ml) increased cell migration (Fig. 1A). In addition, Cyr61 also dose-dependently induced other human OSCC cell migration (SAS and Cal-27 cells) (Fig. 1B). Previous study has shown a significant expression of MMPs in human OSCC cells [Sorsa et al., 2004]. We therefore, hypothesized that any of these MMPs may be involved in Cyr61-directed OSCC migration. Treatment of cells with Cyr61 induced the expression of MMP-3 but not other MMPs by using qPCR (Fig. 1C). Cyr61 also increased mRNA and protein expression of MMP-3 in a dose- and time-dependent manner (Fig. 1D-F). Pretreatment of cells with MMP-3 inhibitor reduced Cyr61-induced cell migration (Fig. 1G). Furthermore, transfection of cells with MMP-3 siRNA markedly inhibited the Cyr61-induced cell migration and MMP-3 expression (Fig. 1H). These data suggest that Cyr61-induced cancer migration may occur via up-regulation of the MMP-3.

Cyr61-directed OSCC migration through αvβ3/α6β1 integrin

Previous study has shown Cyr61 affects cells migration through binding to cell surface integrin receptors [Tan et al., 2009; Tsai et al., 2002]. We therefore, hypothesized that integrins signaling pathway may be involved in Cyr61-directed OSCC cancer migration. Pretreatment of SCC4 cells for 30 min with anti-αvβ3 or α6β1 monoclonal antibody (mAb) but not anti-α5β1 mAb markedly inhibited the Cyr61-induced cancer migration (Fig. 2A). In addition, αvβ3 or α6β1 mAb reduced Cyr61-enhanced MMP-3 expression (Fig. 2B). On the other hand, Cyr61 also increased cell surface αvβ3 and α6β1 expression (Fig. 2C). Therefore, the Cyr61 increased cell migration and MMP-3 expression in human OSCC cells via αvβ3/α6β1 integrin receptor.

FAK, MEK, and ERK signaling pathways are involved in the Cyr61-mediated cell migration of OSCC cells

FAK has been shown to be capable of regulating integrin-mediated signaling [Mitra and Schlaepfer, 2006; Schlaepfer and Hunter, 1998]. Phosphorylation of tyrosine 397 of FAK has been used as a marker of FAK activity. As shown in Fig. 3A, FAK phosphorylation decreased in a time-dependent manner in response to Cyr61 stimulation. Treatment of OSCC cells with FAK inhibitor blocked Cyr61-inhanced migration and MMP-3 expression (Fig. 3B&C). Transfection of cells with FAK(Y397F) mutant or FAK siRNA also reversed the Cyr61-induced cell migration and MMP-3 expression (Fig. 3D&E). MEK/ERK signaling pathway can be activated by a variety of growth factors [Zehorai et al., 2010]. We then examined whether Cyr61 stimulation also enhances the activation of the MEK/ERK pathway. Cyr61-induced the migration of SCC4 cells were greatly reduced by treatment with MEK inhibitors PD98059 and U0126 (Fig. 4B). The MEK inhibitors PD98095 and U0126 also inhibited the Cyr61-increased MMP-3 expression (Fig. 4C). In addition, treatment of cells with Cyr61 increased phosphorylation of ERK (Fig. 4A). Transfection of cells with MEK1 or ERK2 mutant reduced the Cyr61-mediated cell migration and MMP-3 expression (Fig. 4D&E). Taken together, these results indicate

that the αvβ3/α6β1 integrin, FAK, MEK, and ERK pathway is involved in Cyr61-induced migration and MMP-3 up-regulation in human OSCC cells.

Involvement of NF-κB in Cyr61-induced cell migration and MMP-3 expression

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human OSCC cells [Lu et al., 2011]. To examine whether NF-κB activation is involved in the signal transduction pathway caused by Cyr61 that leads to cell migration and MMP-3 expression, the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was used. Fig. 5A&B show that PDTC inhibited the enhancement of cell migration and MMP-3 expression induced by Cyr61. Furthermore, pretreatment of SCC4 cells with an IκB protease inhibitor [L-1-tosylamido-2-phenylenylethyl chloromethyl ketone (TPCK)] antagonized the potentiating action of cell migration and MMP-3 expression (Fig. 5A&B). These results indicated that NF-κB activation is important for Cyr61-induced cancer cell migration and the expression of MMP-3. We further examined the upstream molecules involved in Cyr61-induced NF-κB activation. Stimulation of cells with Cyr61 induced IKKα/β phosphorylation in a time-dependent manner (Fig. 5C). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited the Cyr61-induced cell migration and MMP-3 expression (Fig. 5D&E). These data suggest that IKKα/β activation is involved in Cyr61-induced the migration activity of human OSCC cells. Treatment with SCC4 cells with Cyr61 also caused IκBα and p65 phosphorylation in a time-dependent manner (Fig. 5C).

We next investigated whether p65 binds to the NF-κB element on the MMP-3 promoter after Cyr61 stimulation. The *in vivo* recruitment of p65 to the MMP-3 promoter (-392 to -207) was assessed by the chromatin immunoprecipitation assay [Souslova et al., 2010]. *In vivo* binding of p65 to the NF-κB element of the MMP-3 promoter occurred after Cyr61 stimulation (Fig. 6A). Binding of p65 to the NF-κB element and p65 phosphorylation by Cyr61 was attenuated by FAK inhibitor, U0126, or PD98059 (Fig. 6A&B). To directly determine NF-κB activation after Cyr61

treatment, SCC4 cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Fig. 6C, Cyr61 treatment of OSCC cells for 24 h caused increase in κB-luciferase activity. In addition, the Cyr61-induced increase in κB-luciferase activity was also inhibited by treatment with FAK inhibitor, PD98059, or U0126 (Fig. 6C). Co-transfection with FAK, ERK, IKKα, or IKKβ mutant also reduced Cyr61-increased NF-κB luciferase activity (Fig. 6D) Taken together, these data suggest that activation of FAK, MEK, and ERK are required for Cyr61-induced NF-κB activation in human OSCC cells.

DISCUSSION

The elucidation of the molecular biology of cancer cells in recent years has identified various molecular pathways that are altered in different cancers. This information is currently being exploited to develop potential therapies that target molecules in these pathways. To achieve metastasis, cancer cells must evade multiple barriers and overcome certain rules. Several discrete steps are discernible in the biological cascade leading to metastasis: loss of cellular adhesion, increased motility and invasiveness, entry and survival into the circulation, entrance into new tissue, and eventual colonization of a distant site [Gupta and Massague, 2006]. The mechanism of metastasis is a complicated and multistage process, however our study showed that Cyr61 induces cell migration and the expression of MMP-3 in human OSCC cells. Here, we provide evidence that MMP-3 acts as crucial transducers of cell signaling, regulating cell migration and Cyr61 acts as a critical mediator of the metastasis activity of cancer cells in the tumor microenvironment. However, we did not have tissue samples from OSCC patients to examine the expression of Cyr61 in clinic patients. Therefore, whether Cyr61 through paracrine or autocrine to increase cell motility are needed further examination.

Enzymatic degradation of ECM is one of the crucial steps in cancer invasion and metastasis. It has been reported that MMPs play important role in Cyr61-induced

metastasis in human cancer cells [Maeta et al., 2007; Tan et al., 2009]. In this study, we found that Cyr61 induced MMP-3 expression in human OSCC cells without significantly changing the expression of MMP-1, -2, -9, and -13 mRNAs. Treatment of cells with MMP-3 inhibitor reduced Cyr61-mediated cell migration. In addition, the inhibition of Cyr61-enhanced MMP-3 protein expression with siRNA significantly suppressed Cyr61-induced migration. Therefore, MMP-3 may be the Cyr61-responsive mediator, and it causes the degradation of ECM may lead to subsequent cancer migration and metastasis.

FAK, a potential candidate signaling molecule, has been shown to be capable of regulating integrin-mediated signaling [Miranti and Brugge, 2002]. We demonstrate that Cyr61 decreased phosphorylation of tyrosine 397 of FAK. Furthermore, the FAK inhibitor, mutant, or siRNA antagonized the Cyr61-induced migration activity and MMP-3 expression, suggesting that FAK inhibition is an obligatory event in Cyr61-induced migration in these cells. We also found that PD98059 and U0126 (MEK inhibitors) inhibited Cyr61-induced migration. Stimulation of cells with Cyr61 increased phosphorylation of ERK. The MEK1 and ERK2 mutant also reduced the Cyr61-mediated MMP-3 expression and cell migration. Therefore, our results provide evidence that Cyr61 increase migration and MMP-3 expression in OSCC cells through FAK/MEK/ERK signaling pathway.

NF-κB has been shown to control the induced transcription of MMP-3 in human cancer cells [Tang et al., 2010]. The results of this study show that NF-κB activation contributes to Cyr61-induced MMP-3 production and migration in human OSCC cells, and that the inhibitors of the NF-κB-dependent signaling pathway, including PDTC or TPCK inhibited Cyr61-induced MMP-3 expression and cancer migration. In an inactivated state, NF-κB is normally held in the cytoplasm by the inhibitor protein IκB. Upon stimulation, such as by TNF-α, IκB proteins become phosphorylated by the multisubunit IKK complex, which subsequently targets IκB for ubiquitination, and then are degraded by the 26S proteasome. Finally, the free NF-κB translocates to the nucleus, where it activates the responsive gene [Hatada et al., 2000]. In the present

study, we found that treatment of SCC4 cells with Cyr61 resulted in increases in IKKα/β phosphorylation and p65 binding to NF-κB element. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that Cyr61-induced an increase in NF-κB activity. These extracellular signals activate the IKK complex, which is comprised of catalytic subunits (IKKα and IKKβ) and a linker subunit (IKKγ/NEMO). This kinase complex phosphorylates IκBα at Ser^{32} and Ser^{36} and signals for ubiquitin-related degradation [Chen et al., 1995]. The released NF-κB is then translocated into the nucleus where it promotes NF-κB-dependent transcription. There is also a strong evidence that IKKα and IKKβ are themselves phosphorylated and activated by one or more upstream activating kinases [Hatada et al., 2000]. p65 is phosphorylated at Ser^{536} by a variety of kinases through various signaling pathways, and this enhances the p65 transactivation potential. The results of this study showed that Cyr61 increased the phosphorylation of IKK, IκBα, and p65. On the other hand, FAK inhibitor, U0126 and PD98059 reduced Cyr61-mediated NF-κB promoter activity. Our data indicated that FAK/MEK/ERK and NF-κB pathways might play important role in the expression of MMP-3 and cell migration of human OSCC cells. Furthermore, pretreatment of cells with αvβ3 or α6β1 integrin mAb reduced Cyr61-mediated FAK, ERK, and p65 phosphorylation (Supplementary Fig. S1). Therefore, Cyr61 induced FAK, ERK, and NF-κB activation through αvβ3/α6β1 integrin receptor.

In conclusion, we present here a molecular mechanism of Cyr61-induced migration of human OSCC cells by up-regulation of MMP-3. Cyr61 increases MMP-3 expression through αvβ3/α6β1 integrin, FAK, MEK, ERK, and NF-κB signaling pathway and induces tumor metastasis.

Acknowledgments

This work was supported by grants from the National Science Council of Taiwan (NSC 100-2320-B-039-028-MY3); China Medical University (CMU98-OC-06); Taiwan Department of Health, China Medical University Hospital Cancer Research of Excellence (DOH100-TD-C-111-005; DOH101-TD-C-111-005); We thank Dr. J. A. Girault for providing the FAK(Y397F) mutant; Dr. W. M. Fu for providing MEK1 mutant. Dr. M. Cobb for providing ERK2 mutant; Dr. H. Nakano for providing IKKα and IKKβ mutants.

FIGURE LEGENDS

Fig. 1 Cyr61 induces cell migration and MMP-3 expression in human OSCC cells

(A&B) Cells were incubated with various concentrations of Cyr61, and *in vitro* migration activities measured with the Transwell after 24 hr. (C) SCC4 cells were incubated with Cyr61 (100 ng/ml) for 24 hr, and the mRNA expression of MMPs was examined by qPCR. (D&E) SCC4 cells were incubated with Cyr61 for 24 hr, and the mRNA and protein expression of MMP-3 was examined by qPCR and Western blotting. (F) SCC4 cells were incubated with Cyr61 for indicated time intervals, and MMP-3 expression was examined by Western blotting. (G&H) SCC4 cells were pretreated with MMP-3 inhibitor (40 μM) or transfected with MMP-3 and control siRNA for 24 hr followed by stimulation with Cyr61, and *in vitro* migration was measured with the Transwell after 24 hr. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with Cyr61-treated group.

Fig. 2 Cyr61 increases OSCC cell migration through αvβ3/α6β1 integrin.

(A&B) SCC4 cells were pretreated with αvβ3, α5β1, or α6β1 monoclonal antibody (10 μg/ml) for 30 min followed by stimulation with Cyr61. The *in vitro* migration activity and MMP-3 expression were measured with Transwell and qPCR. (C) SCC4 cells were treated with Cyr61 for 24 hr, and cell surface αvβ3 and α6β1 integrin expression was examined by flow cytometry. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with Cyr61-treated group.

Fig. 3 Involvement of FAK signaling pathway in response to Cyr61 in OSCC cells.

(A) SCC4 cells were incubated with Cyr61 for indicated time intervals, and p-FAK expression was determined by Western blotting. (B&C) SCC4 cells were pretreated with FAK inhibitor (200 nM) for 30 min followed by

stimulation with Cyr61. The *in vitro* migration activity and MMP-3 expression were measured with Transwell and qPCR. (D&E) SCC4 cells were transfected with FAK mutant or siRNA for 24 hr followed by stimulation with Cyr61. The *in vitro* migration activity and MMP-3 expression were measured with Transwell and qPCR. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with Cyr61-treated group.

Fig. 4 MEK/ERK pathway is involved in Cyr61-mediated migration in human OSCC cells.

(A) SCC4 cells were incubated with Cyr61 for indicated time intervals, and p-ERK expression was determined by Western blotting. (B&C) SCC4 cells were pretreated with PD98059 (30 μg/ml) and U0126 (10 μM) for 30 min followed by stimulation with Cyr61. The *in vitro* migration activity and MMP-3 expression were measured with Transwell and qPCR. (D&E) SCC4 cells were transfected with MEK or ERK mutant for 24 hr followed by stimulation with Cyr61. The *in vitro* migration activity and MMP-3 expression were measured with Transwell and qPCR. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with Cyr61-treated group.

Fig. 5 NF-κB is involved in Cyr61-mediated migration in human OSCC cells.

(A&B) SCC4 cells were pretreated with PDTC (10 μM) or TPCK (3 μM) for 30 min followed by stimulation with Cyr61. The *in vitro* migration activity and MMP-3 expression were measured with Transwell and qPCR. (C) SCC4 cells were incubated with Cyr61 for indicated time intervals, and p-IKK, p-IκB, and p-p65 expression was determined by Western blotting. (D&E) SCC4 cells were transfected with IKKα or IKKβ mutant for 24 hr followed by stimulation with Cyr61. The *in vitro* migration activity and MMP-3

expression were measured with Transwell and qPCR. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with Cyr61-treated group.

Fig. 6 FAK/MEK/ERK pathway is mediated Cyr61-induced NF-κB activation.

(A) SCC4 cells were pretreated with FAK inhibitor, or U0126 then stimulated with Cyr61 for 120 min, and the chromatin immunoprecipitation assay was then performed. Chromatin was immunoprecipitated with anti-p65. One percentage of the precipitated chromatin was assayed to verify equal loading (input). (B) SCC4 cells were pretreated with FAK inhibitor, PD98059, or U0126 then stimulated with Cyr61 for 120 min, and the p-p65 expression was examined by Western blotting. SCC4 cells were pretreated with FAK inhibitor, PD98059 and U0126 for 30 min (C) or transfected with mutant of FAK, ERK, IKKα and IKKβ mutant (D) before exposure to Cyr61. NF-κB luciferase activity was measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with Cyr61-treated group. (E) Schematic presentation of the signaling pathways involved in Cyr61-induced MMP-3 expression in human OSCC cells. Cyr61 acts through αvβ3/α6β1 integrin receptor to activate FAK, MEK, and ERK, leading to the activation of p65, resulting in the activation of NF-κB element on the human MMP-3 promoter and the initiation of MMP-3 expression. This MMP-3 induction increases the cell migration of human OSCC cells.

REFERENCES

Boonrao M, Yodkeeree S, Ampasavate C, Anuchapreeda S, Limtrakul P. 2010. The inhibitory effect of turmeric curcuminoids on matrix metalloproteinase-3 secretion in human invasive breast carcinoma cells. Archives of pharmacal research 33:989-98.

Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F, Collen D. 1997. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. Nat Genet 17:439-44.

Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, Maniatis T. 1995. Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. Genes Dev 9:1586-97.

Croci S, Landuzzi L, Astolfi A, Nicoletti G, Rosolen A, Sartori F, Follo MY, Oliver N, De Giovanni C, Nanni P, Lollini PL. 2004. Inhibition of connective tissue growth factor (CTGF/CCN2) expression decreases the survival and myogenic differentiation of human rhabdomyosarcoma cells. Cancer research 64:1730-6.

Dhar A, Ray A. 2010. The CCN family proteins in carcinogenesis. Experimental oncology 32:2-9.

Egeblad M, Werb Z. 2002. New functions for the matrix metalloproteinases in cancer progression. Nature reviews. Cancer 2:161-74.

Greenberg JS, El Naggar AK, Mo V, Roberts D, Myers JN. 2003. Disparity in pathologic and clinical lymph node staging in oral tongue carcinoma. Implication for therapeutic decision making. Cancer 98:508-15.

Grzeszkiewicz TM, Lindner V, Chen N, Lam SC, Lau LF. 2002. The angiogenic factor cysteine-rich 61 (CYR61, CCN1) supports vascular smooth muscle cell adhesion and stimulates chemotaxis through integrin alpha(6)beta(1) and cell surface heparan sulfate proteoglycans. Endocrinology 143:1441-50.

Gupta GP, Massague J. 2006. Cancer metastasis: building a framework. Cell 127:679-95.

Hatada EN, Krappmann D, Scheidereit C. 2000. NF-kappaB and the innate immune response. Curr Opin Immunol 12:52-8.

Hsieh MT, Hsieh CL, Lin LW, Wu CR, Huang GS. 2003. Differential gene expression of scopolamine-treated rat hippocampus-application of cDNA microarray technology. Life sciences 73:1007-16.

Huang HC, Shi GY, Jiang SJ, Shi CS, Wu CM, Yang HY, Wu HL. 2003. Thrombomodulin-mediated cell adhesion: involvement of its lectin-like domain. The Journal of biological chemistry 278:46750-9.

Huang WC, Chen CC. 2005. Akt phosphorylation of p300 at Ser-1834 is essential for its histone acetyltransferase and transcriptional activity. Mol Cell Biol 25:6592-602.

Lau LF, Nathans D. 1985. Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. The EMBO journal 4:3145-51.
Lin MT, Chang CC, Chen ST, Chang HL, Su JL, Chau YP, Kuo ML. 2004. Cyr61 expression confers resistance to apoptosis in breast cancer MCF-7 cells by a mechanism of NF-kappaB-dependent XIAP up-regulation. The Journal of biological chemistry 279:24015-23.
Lu DY, Leung YM, Cheung CW, Chen YR, Wong KL. 2010. Glial cell line-derived neurotrophic factor induces cell migration and matrix metalloproteinase-13 expression in glioma cells. Biochemical pharmacology 80:1201-9.
Lu KW, Chen JC, Lai TY, Yang JS, Weng SW, Ma YS, Lu PJ, Weng JR, Chueh FS, Wood WG, Chung JG. 2011. Gypenosides inhibits migration and invasion of human oral cancer SAS cells through the inhibition of matrix metalloproteinase-2 -9 and urokinase-plasminogen by ERK1/2 and NF-kappa B signaling pathways. Human & experimental toxicology 30:406-15.
Lyons AJ, Jones J. 2007. Cell adhesion molecules, the extracellular matrix and oral squamous carcinoma. Int J Oral Maxillofac Surg 36:671-9.
Maeta N, Osaki M, Shomori K, Inaba A, Kidani K, Ikeguchi M, Ito H. 2007. CYR61 downregulation correlates with tumor progression by promoting MMP-7 expression in human gastric carcinoma. Oncology 73:118-26.
Miranti CK, Brugge JS. 2002. Sensing the environment: a historical perspective on integrin signal transduction. Nat Cell Biol 4:E83-90.
Mitra SK, Schlaepfer DD. 2006. Integrin-regulated FAK-Src signaling in normal and cancer cells. Curr Opin Cell Biol 18:516-23.
Page-McCaw A, Ewald AJ, Werb Z. 2007. Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 8:221-33.
Roussos ET, Condeelis JS, Patsialou A. 2011. Chemotaxis in cancer. Nature reviews. Cancer 11:573-87.
Sampath D, Zhu Y, Winneker RC, Zhang Z. 2001. Aberrant expression of Cyr61, a member of the CCN (CTGF/Cyr61/Cef10/NOVH) family, and dysregulation by 17 beta-estradiol and basic fibroblast growth factor in human uterine leiomyomas. The Journal of clinical endocrinology and metabolism 86:1707-15.
Schlaepfer DD, Hunter T. 1998. Integrin signalling and tyrosine phosphorylation: just the FAKs? Trends Cell Biol 8:151-7.
Sorsa T, Tjaderhane L, Salo T. 2004. Matrix metalloproteinases (MMPs) in oral diseases. Oral diseases 10:311-8.
Souslova V, Townsend PA, Mann J, van der Loos CM, Motterle A, D'Acquisto F, Mann DA, Ye S. 2010. Allele-specific regulation of matrix metalloproteinase-3 gene by transcription factor NFkappaB. PloS one 5:e9902.

Tan TW, Yang WH, Lin YT, Hsu SF, Li TM, Kao ST, Chen WC, Fong YC, Tang CH. 2009. Cyr61 increases migration and MMP-13 expression via alphavbeta3 integrin, FAK, ERK and AP-1-dependent pathway in human chondrosarcoma cells. Carcinogenesis 30:258-68.

Tang CH, Yamamoto A, Lin YT, Fong YC, Tan TW. 2010. Involvement of matrix metalloproteinase-3 in CCL5/CCR5 pathway of chondrosarcomas metastasis. Biochemical pharmacology 79:209-17.

Thomas GJ, Speight PM. 2001. Cell adhesion molecules and oral cancer. Crit Rev Oral Biol Med 12:479-98.

Tsai MS, Bogart DF, Castaneda JM, Li P, Lupu R. 2002. Cyr61 promotes breast tumorigenesis and cancer progression. Oncogene 21:8178-85.

Tseng CP, Huang CL, Huang CH, Cheng JC, Stern A, Tseng CH, Chiu DT. 2003. Disabled-2 small interfering RNA modulates cellular adhesive function and MAPK activity during megakaryocytic differentiation of K562 cells. FEBS letters 541:21-7.

Vincenti MP. 2001. The matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) genes. Transcriptional and posttranscriptional regulation, signal transduction and cell-type-specific expression. Methods Mol Biol 151:121-48.

Wang YC, Lee PJ, Shih CM, Chen HY, Lee CC, Chang YY, Hsu YT, Liang YJ, Wang LY, Han WH. 2003. Damage formation and repair efficiency in the p53 gene of cell lines and blood lymphocytes assayed by multiplex long quantitative polymerase chain reaction. Analytical biochemistry 319:206-15.

Zehorai E, Yao Z, Plotnikov A, Seger R. 2010. The subcellular localization of MEK and ERK--a novel nuclear translocation signal (NTS) paves a way to the nucleus. Molecular and cellular endocrinology 314:213-20.