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Snail promotes Cyr61 secretion to prime collective cell migration and form invasive tumor nests in squamous cell carcinoma

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ABSTRACT

We previously identified genes associated with Snail-mediated squamous cell carcinoma (SCC) invasiveness, in which we observed significant elevation of Cyr61 expression. In this study, SCC cell lines overexpressing Cyr61 exhibited constitutive activation of Rho A and upregulated invasiveness without the disruption of homophilic cell attachment. Humoral Cyr61 enhanced further production of endogenous Cyr61 by SCC cells, which stimulated collective cell migration and the development of an invasive tumor nest. We propose a Cyr61-dependent model for the development of invasive SCC nest, whereby a subset of tumor cells that highly produce Cyr61 may direct other tumor cells to undergo collective cell migration, resulting in a formation of primary SCC mass.

1. Introduction

Epithelial-to-mesenchymal transition (EMT) is the complex reprogramming process undergone by epithelial cells to enable cell motility and morphogenesis. It is widely considered to accelerate tumor aggressiveness, especially metastasis, in which tumor cells undergo EMT and infiltrate blood or lymphatic vessels [1]. Snail and other transcriptional repressors (Zeb-1, Zeb-2, Twist, and Slug) trigger EMT through direct repression of E-cadherin [2–5]. In addition to the downregulation of E-cadherin to evoke cell motility, EMT inducers control the expression of many target genes indispensable for the adaptation of tumor cells to changes in their microenvironment until the establishment of metastasis [5,6].

Squamous cell carcinomas (SCCs) commonly form cancer nests in a continuous state of expansion. Although cancer nests are mainly composed of tumor cells that express surface E-cadherin and maintain homophilic cell adhesion, it has been demonstrated that tumor cells at the invasive front of several SCCs have decreased membrane expression of E-cadherin [7–9]. Indeed a feature of tumors is heterogeneous assemblage and partial cellular dissociation [10], which naturally indicates the concomitance of non-EMT-phenotypic cell and EMT-phenotypic cell at the tumor invasive front. However, the distinct involvement and advantage of EMT-phenotypic cells in the local invasion of SCC has largely been unclear. In the present study, Cyr61 (cysteine rich 61, CCN1), a secreted protein upregulated by Snail [11] was investigated based on the hypothesis that secreted protein can become a paracrine mediator of local invasion.

Cyr61 is a heparin-binding, extracellular matrix-associated protein that belongs to the CCN protein family, and is implicated in diverse biological processes such as cell adhesion, proliferation, differentiation, and survival via its multivalent affinity to several factors including cell surface proteins, matrix proteins and extracellular cytokines [12–14]. Intriguingly, Cyr61 is also defined as a member of the matricellular protein group [15]. Secreted matricellular protein enables the modulation of cellular interactions with the extracellular matrix by its binding to structural matrix proteins, such as collagen, and its abrogation of focal adhesions, conferring a counter-adhesive effect on cells [16]. In cancer, overexpression of *Cyr61* was found in several cancers and resulted in tumor cell proliferation and migration [17]. Expression levels of Cyr61 were significantly correlated with the prognosis of patients with gastric adenocarcinomas [18]. Furthermore, Cyr61 is a paracrine regulator of angiogenesis, endothelial functions, and chemotactic intravascular invasion [19,20]. Here, we report a novel finding that Cyr61 acts as an activator of the collective cell migration of SCC cells. Snail-dependent production of Cyr61 in

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EMT-phenotypic cells could prime and promote dynamic cell migration of the invasive tumor nest in which the positive feedback of Cyr61 production emerges from SCC cells.

2. Materials and methods

2.1. Reagents and antibodies

Ammonium pyrrolidine dithiocarbamate, APDC, a chemical inhibitor of NFκB activation, was purchased from Sigma. TNF-α was obtained from R&D systems. RGD and RGE peptides were obtained from TaKaRa, Japan. Commercially available antibodies were as follows: anti-V5 tag (Invitrogen), anti-Cyr61 (Santa Cruz Biotechnology), anti-E-cadherin (Santa Cruz Biotechnology), anti-integrin αV (Millipore), anti-MMP-2 (Cell Signaling Technology), anti-IκBα (Cell Signaling Technology), anti-IκBε (Cell Signaling Technology), and anti-α-tubulin (Zymed Laboratories).

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells at 70–80% confluence with Trizol (Invitrogen). First-strand synthesis was performed with First-strand cDNA synthesis kit (Roche). Semi-quantitative RT-PCR reactions (20 μl) were amplified with 30 cycles of denaturing at 95 °C for 30 s, annealing for 30 s and extension at 72 °C for 1 min. Primers and annealing temperatures were follows:

ICAM-1, 5′-GCC AAC CAA TGT GCT ATT CA-3′ and 5′-CTC CCG TTT CAG CTC CTT CT-3′, 52 °C; XIAP, 5′-GAA GAC CCT TGG GAA CAA CA-3′ and 5′-CGC CTT AGC TGC TCT CTT CAG T-3′, 56 °C; Bcl-xL, 5′-GGC AAC CCA TCC TGG CAC CT-3′ and 5′-AGC GTT CCT GGC CCT TTC G-3′, 60 °C; RhoGAP1, 5′-CCC ATC AAC ACC TTC ACC AA-3′ and 5′-AGA GAG GCA GTG AGA AGT GT-3, 56 °C; RhoGAP8, 5′-TCG GGC TGA ATT TGA TCT GG-3′ and 5′-TGT GGG AGG TAG CTC GAA AT-3′, 56 °C; GEF-H1, 5′-ATG TCT CGG ATC GAA TCC CT-3′ and 5′-CGA AGA GAA ACG GAC TGC AA-3′, 56 °C; G3PDH, 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-CAG CCC CAG CGT CAA AGG TG-3′, 53 °C.

PCR products were analyzed by 1.8% agarose gel electrophoresis and sequenced to verify their identity.

2.3. Cell lines and cell culture

The human vulval epidermal cell line A431 and five human oral SCC cell lines, OM-1, HOC719-PE, HOC719-NE, TSU and HOC313 have been described previously [11,21,22]. Snail-overexpressing SCC cell lines, A431 Snail and OM-1 Snail, were generated previously [21]. Full-length *Cyr61* cDNA was amplified by RT-PCR with LATaq polymerase (TaKaRa) and cloned into the *Kpn* I and *Xho* I sites of pcDNA3.1-V5/His-tagged vector (Invitrogen). Primers for the amplification were 5′-AAA GGT ACC ATG AGC TCC CCG CAT CGC CA-3′ and 5′-TTT CTC GAG CGG GTC CCC TAA ATT TGT GAA TGT C-3′. The PCR product was verified by sequencing. *Cyr61* expression vector or empty pcDNA3.1-V5/His vector was transfected into A431 and OM-1 cells, and stable cell clones were established by G418 selection. All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air and maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (Sigma).

2.4. Immunoblotting

Cells were harvested and resuspended in RIPA buffer (Sigma). The protein concentration was determined using BSA protein assay kit (Pierce). Twenty-five μg of each sample protein extract was incubated with Laemmli's sample buffer (Sigma) and 50 mM dithiothreitol, and resolved by SDS–PAGE.

Conditioned medium was taken at 3 h after replacement of growth medium with serum-free medium. Fifty μl of total 0.5 ml conditioned medium that harvested from 5.0×10^5 cells in 6 well dish was analyzed by SDS–PAGE.

The EZ-Detect Rho Activation Kit (Pierce) and the EZ-Detect Rac1 Activation Kit (Pierce) were used for detection of GTP-bound Rho and GTP-bound Rac1 in immnoblotting, according to the manufacturer's protocols.

2.5. Luciferase reporter assays

The *Cyr61* promoter region, consisting of nucleotides (nt) −928 to +28 (construct I), was amplified from genomic DNA of normal human fibroblasts with LATaq polymerase (TaKaRa) using primers, as described previously [23]. Other fragments of the *Cyr61* promoter region were also amplified by PCR. The sense primer sequences for each fragment were follows:

nt −784 (construct II), 5'-AAA GGT ACC AGA CGA TGG GCA A-3';
nt −53 (construct III), 5'-AAG GTA CCG TCA CTG CAA CAC GC-3';
nt −28 (construct IV), 5'-AAG GTA CCA GAC CGC GAG CGA-3'.

Each PCR product was cloned into the *Kpn* I and *Xho* I sites of pGL3-basic vector (Promega). HEK293 cells were co-transfected with 4 μg of the *Cyr61* reporter construct and 1 ng of phRL-CMV (Promega) as an internal control for transfection efficiency with FuGENE6 (Roche). Either the empty vector pcDNA3.1-His/V5 or pcDNA3-mm SnailHA [21] was additionally transfected. At 48 h after transfection, cells were lysed with passive lysis buffer, and the promoter activity was measured with a Dual-Luciferase Reporter Assay kit (Promega) according to the manufacturer's protocol, as described previously [21]. Briefly, the firefly-derived luciferase activity of interest was measured by addition of firefly-specific substrate to the transfected cell extract. Subsequently, Renilla-derived luciferase activity was measured by further addition of the Renilla-specific substrate. Transfection efficiency was normalized to CMV-driven Renilla-derived luciferase activity. The firefly-derived luciferase activity of interest was normalized against transfection efficiency and presented as fold change toward normalized activity of construct I. The results correspond to the mean of at least three independent experiments.

2.6. Wound healing assays

Cells were cultured in dishes as confluent monolayers. The monolayers were wounded using a sterile 200 μl standard pipette tip. The area of cell-free wound was recorded at indicated time points, and the wound healing effect was calculated as the distance of the remaining cell-free area compared with the initial area of the original wound.

2.7. In vitro three-dimensional culture

Three-dimensional cultures of SCC cells and histological observation of sample section were performed according to previous reports [21,22]. pGFP vector (TaKaRa) was transfected into OM-1 cells to label cells with green fluorescent protein (GFP). Stable cell clones were established by G418 selection. Expression of GFP was detected by direct visualization and immunoblotting.

2.8. Matrigel invasion assays

Cell invasion activity was measured with BioCoat Matrigel Invasion Chamber kit (Becton Dickinson) according to the manufacturer's protocol and previous reports [21,22].

2.9. Immunocytochemistry

Cells were seeded into 24-well glass bottom dishes in DMEM for 1 day. The growth medium was then removed and cell monolayers were fixed with 4% paraformaldehyde for 15 min. Nonspecific sites were blocked with a buffer containing 3% TritonX-100 and 5% BSA. Cells were incubated with anti-E-cadherin antibody or anti-GFP antibody (MBL) in PBS containing 1% BSA at 4 °C overnight. Protein expression was revealed by incubation with an Alexa-Fluor dye-labeled anti-rabbit antibody for 1 h at room temperature, and further incubation with Alexa-Fluor Phalloidin reagent for 30 min. After mounting with DAPI containing Vectorshield (Vector Laboratories Inc., Burlingame, CA), the cells were subjected to fluorescent microscopy analysis using confocal laser scanning microscope, TCS SP8, Leica.

2.10. Immunohistochemistry

Primary oral SCC tissue samples were obtained at the time of surgery with informed consent from patients and approval from the institutional review board at Hiroshima University Hospital. Immunohistochemical staining was performed with standard methods. Briefly, 4 μm-thick sections were cut from each formalin-fixed paraffin-embedded tissue block. After deparaffinization and rehydration, antigen-retrieval treatment was carried out with microwave heating (500 W) for 10 min in sodium citrate buffer (pH6.0), followed by treatment with 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity. Nonspecific binding was blocked by treating the slides with PBS containing 5% normal horse serum for 10 min at room temperature. Slides were then incubated with primary antibody against Cyr61 antibody (diluted 1:50). Diaminobenzidine (DAB) substrate was used for color development. The series of slides were counterstained with 1% Mayer's hematoxylin.

2.11. Statistical analysis

Data were expressed as mean ± SD from at least three independent experiments. Statistical comparisons were made according to the Student's *t*-test. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Snail elevated Cyr61 expression in EMT-phenotypic SCC cell lines

Since mRNA expression of *Cyr61* was upregulated in several EMT-phenotypic SCC cell lines [12], we first assessed the correlation between the expression level of Cyr61 protein and the overexpression