Involvement of Cyr61 in the growth, invasiveness and adhesion of esophageal squamous cell carcinoma cells

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Abstract. Cysteine-rich 61 (Cyr61), a secreted protein which belongs to the CCN family, has been found to be differentially expressed in many cancers and to be involved in tumor progression. The expression of Cyr61 in esophageal squamous cell carcinoma (ESCC) has only recently been described, but the roles of Cyr61 in ESCC cells still remained unclear. In this study, we have shown that there are high levels of Cyr61 in ESCC cell lines. Furthermore, using RNA interference (RNAi), we stably silenced the expression of Cyr61 in EC109 cells, an ESCC cell line. The colony formation, MTT, cell migration, cell invasiveness and cell adhesion assays were employed to address the roles of Cyr61 in the growth, migration and adhesion of ESCC cells. The results have shown that Cyr61 knockdown by RNAi leads to a significant reduction of colony formation and cell growth. The migration and invasiveness ability of EC109 cells were also suppressed with the Cyr61 down-regulation. Furthermore, the adhesion of the EC109 cells was decreased in the Cyr61 knockdown cells compared to the control cells. Taken together, our data suggest that Cyr61 may play crucial roles in regulating neoplasm progression of ESCC.

Introduction

Cysteine-rich 61 (Cyr61/CCN1) belongs to the Cyr61/CTGF/Nov (CCN) protein family, which includes Cyr61/CCN1, connective tissue growth factor (CTGF/CCN2), nephroblastoma-overexpressed (Nov/CCN3), Wnt-1 induced secreted protein 1 (Wisp-1/CCN4), Wisp-2/CCN5, and Wisp-3/CCN6 (1,2). Encoded by a growth factor-inducible immediate early gene, Cyr61 is a 40 kDa protein which is extremely cysteine-rich. This heparin-binding protein shares a 40-50% amino acid homology with the other CCN family members. An important structural feature of CCN proteins is that they contain four conserved modules which exhibit similarities to the insulin-like growth factor-binding proteins (IGFBPs), the von Willebrand factor type C (VWC), the thrombospondin type 1 (TSP1) and the carboxyl terminus of several extra-cellular mosaic proteins (CT) (3).

The CCN proteins mediate a variety of biological processes such as cell adhesion, stimulation of chemostasis, enhancement of growth factor-induced DNA synthesis, cell survival, and angiogenesis (4-6). With respect to tumorigenesis, Cyr61 has been shown to be involved in the development of several kinds of tumors. Cyr61 overexpression has been associated with progression and formation of larger tumors in breast cancer (7). Cyr61 has also been reported to stimulate the growth of gastric adenocarcinoma (8). High expression level of Cyr61 have been found in rhabdomyosarcomas, malignant melanomas, colon adenocarcinomas, and bladder papillomas (8,9)as well as in malignant gliomas (10). Cyr61 has been reported to enhance the tumorigenicity through the integrin-linked kinase signaling pathway (10). However, in non-small cell lung cancer and prostate carcinoma, Cyr61 was found to be down-regulated (11,12). These findings indicate that Cyr61 has variable biological functions which are dependent on the cellular contexts.

In human esophageal squamous cell carcinoma (ESCC), the expression of Cyr61 has only recently been described. Our previous study has shown that Cyr61 is overexpressed in ESCC and that Cyr61 might be a new molecular marker to predict the prognosis of ESCC patients (13,14). Moreover, we have also revealed that Cyr61 is involved in the fascin-mediated alteration of cell proliferation and invasiveness in ESCC (15). However, the roles of Cyr61 in ESCC remain unclear.

To investigate the role of Cyr61 in ESCC, we have used the pSUPER RNA interference (RNAi) system to stably suppress the expression of the *Cyr61* gene in EC109, an ESCC cell line in which Cyr61 is highly expressed and we have analyzed its effects on cell growth, invasive behavior and cell adhesion.

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Materials and methods

Cell lines and cell culture. Human ESCC cell lines (EC109 and EC8712) and SHEE cells (a kind of immortal embryonic esophageal epithelium cell line established in our laboratory) were maintained in Medium 199 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) (16). The other three ESCC cell lines, KYSE150, KYSE180 and KYSE510, were maintained in DMEM medium (Invitrogen) containing 10% FCS. All cells were cultured in an atmosphere of 5% CO_2 at 37˚C.

RNAi. The mammalian expression vector, pSUPER.neo circular (OligoEngine, Seattle, WA, USA), was used for small interfering RNA (siRNA) expression in EC109 cells. Briefly, two siRNA pairs were synthesized; one pair encoded *Cyr61* nucleotides 521-539 (siRNA1, CTGTGAATATAACTCCAGA) and the second pair encoded nucleotides 513-531 (siRNA2, GGCAGACCCTGTGAATATA). The pSUPER.neo vector of the non-specific siRNA was used as a negative control (Vector). The siRNA expression plasmids were transfected into EC109 cells using SuperFect reagent (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. G418 (400 μg/ml) (Calbiochem, Darmstadt, Germany) was added to the culture medium after 24 h. Stable G418-resistant clones were obtained in 7-9 days. The expanded cells were then used for subsequent studies.

Western blotting. Total cell lysates were prepared in RIPA buffer, separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated in blocking buffer and then incubated with the indicated antibody. Finally, immunoreactive bands were revealed using luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Photography and quantitative analyses were performed using the FluorChem™ IS-8900 (Alpha Innotech Co., San Leandro, CA). The following antibodies were used: mouse anti-Cyr61, mouse anti-β-tubulin and mouse anti-β-actin (Sigma, St. Louis, MO, USA).

Immunofluorescence staining. The staining procedure was carried out as described (17). After being fixed with 100% methanol at -10˚C for 15 min, cells were incubated with blocking buffer (goat serum) for 20 min, and incubated with primary antibody overnight at 4˚C followed by incubation with FITC-labeled secondary antibody for 30 min at 37˚C. Finally, the nuclei were counterstained using propidium iodide (PI) (Sigma), mounted in glycerol, and viewed with a fluorescence microscope.

Cell growth study. The MTT and colony formation assays were used to evaluate cell growth. The MTT test was performed as previously described with some modifications (18). In brief, cells were seeded in 96-well plates ($5x10^3$ cells/well), and after incubation for 24, 48, 72 or 96 h, MTT solution (5 mg/ml) was added to the medium. The formazan crystals that formed were dissolved, and absorption was measured at 490 nm with an automatic ELISA reader. In the colony formation assay, cells were plated at a density of 50 cells/well in 6-well plates. They were then moved to a cell incubator. After 30 days, the number of colony-forming cells (>50 cells) was calculated under a microscope. The data were expressed as mean ± SD.

Chamber migration assay. Migration was evaluated using a modified Boyden chamber assay. Cell culture inserts containing polyethylene tetrephthalate were placed within a 24-well chamber containing 0.6 ml of Medium 199 with 10% FCS. Cells ($1x10^5$) were seeded onto the inserts suspended in 0.2 ml of serum-free Medium 199. Non-migratory cells were removed from the upper surface of the filter after incubation for 24 h. Migrated cells were fixed and stained with Giemsa reagent. Migrating cells were quantified based on the procedure as described above.

Cell invasiveness assay. The invasiveness was determined by an invasiveness chamber assay. Cells ($1x10^5$) were seeded onto the top chamber of a 24-well matrigel (BD Sciences, Franklin Lakes, NJ, USA) coated micropore membrane filter with 8-μm pores (Millipore), and the bottom chamber was filled with 0.6 ml of Medium 199 with 10% FCS as a chemoattractant. The membranes were fixed and stained by Giemsa reagent, and the cells on the upper surface were carefully removed with a cotton swab after 24 h. Invasiveness was quantified by counting 10 random fields under a light microscope (x400). Data obtained from three separate chambers are shown as mean values.

Cell adhesion assay. The cell adhesion assay was performed as described (19). Wells of a 96-well plate were coated at room temperature overnight with 5 μg matrigel in a final volume of 50 μl. Additional uncoated wells were incubated to serve as a negative control. Cells were trypsinized from the dish, resuspended in serum-free culture medium, and $5x10^3$ cells/100 μl were added to each well. The plates were incubated for 15, 30, 45 or 60 min at 37˚C. For quantification, the attached cells were treated with MTT solution and absorption was measured at 490 nm/630 nm with an automatic ELISA reader.

Statistical analysis. All data are expressed as the mean ± SD and were analyzed with the SPSS statistics software (SPSS 13.0 by SPSS Inc. Chicago, IL, USA). Comparisons between data sets were performed using the χ^2 test and t-test when appropriate. $P<0.05$ was considered statistically significant.

Results

Expression of Cyr61 in ESCC cell lines. We first examined the levels of Cyr61 expression in several ESCC cell lines and SHEE cells, an immortalized esophageal epithelial cell line by Western blotting. Cyr61 was detected in all cell lines evaluated, with SHEE cells expressing the lowest level (Fig. 1A). High levels of Cyr61 were detected in EC109 cells and these cells were selected as the model for the subsequent function studies. The distribution of Cyr61 in EC109 cells was also explored. Immunofluorescence showed that Cyr61 was localized intensely in the cytoplasm (Fig. 1B).

Silencing of Cyr61 expression. To study the function of the Cyr61 gene in EC109 cells, the pSUPER system was adopted to stably suppress Cyr61 expression. G418-screened

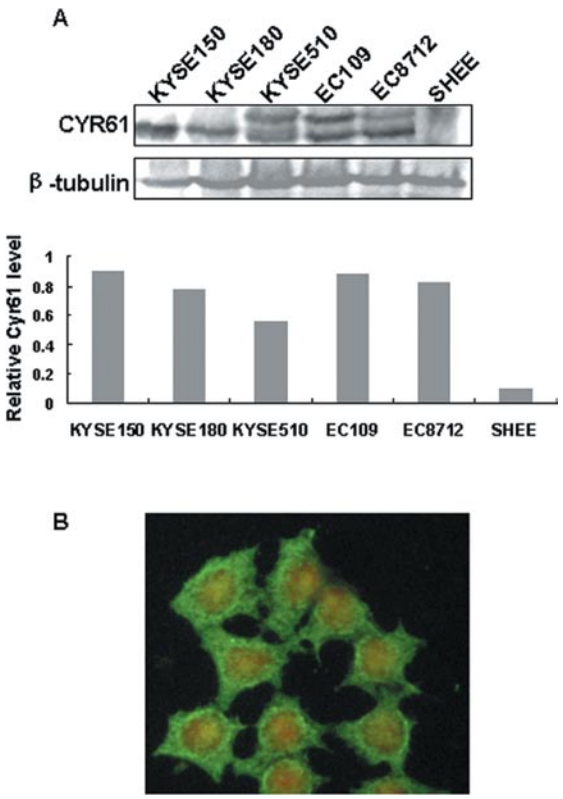

Figure 1. Expression of cysteine-rich 61 (Cyr61) in esophageal squamous cell carcinoma (ESCC) cell lines and SHEE cells. (A) Cyr61 levels in whole-cell extracts were determined in various ESCC cell lines and SHEE cells by Western blot analysis. β-tubulin served as a loading control. Signal intensity for the expression of Cyr61 was quantified by densitometric scanning and normalized to the internal control (β-tubulin). (B) Immunofluorescence distribution of Cyr61 was examined in EC109 cells. The green fluorescence (FITC-labeled) corresponds to Cyr61 (x400).

EC109 cells were used for analysis of the silencing effect. Western blotting and immunofluorescence staining showed that Cyr61 expression decreased markedly in treated cells compared with the control. Cyr61 expression level was efficiently reduced by 85% in siRNA1-treated cells and 90% in siRNA2-treated cells, whereas transfection of the vector of non-specific siRNA (Vector) did not reduce Cyr61 expression level (Fig. 2).

Altered growth of EC109 cells. The colony formation assay was used to evaluate the growth of the cells in which Cyr61 was silenced. After culturing in G418-containing media for 30 days, much fewer colonies were formed in the siRNA1 or siRNA2 cells, whereas colony formation was still obvious in the control groups (Fig. 3A). To further test the negative effect of Cyr61 knockdown on EC109 cell growth, an MTT assay was performed and growth curves were generated (Fig. 3B). As shown by the curves, both siRNA1 and siRNA2 cells proliferated more slowly than vector cells and untreated EC109 cells during the first 96 h after the cells were plated. The reduction of colony formation and growth of Cyr61-silenced cells suggests that Cyr61 suppression may negatively regulate EC109 cell growth.

Altered migration and invasiveness of EC109 cells. The motility of siRNA1, siRNA2 and two control cells was examined by determining their migration through a poly-ethylene filter in the absence of matrigel. Migration rates of the siRNA1 and siRNA2 cells were greatly decreased as compared to the control (Fig. 4A). Cyr61 knockdown also significantly reduced cell invasive properties as compared to parental cells (Fig. 4B). Cell migration rates of siRNA1 and

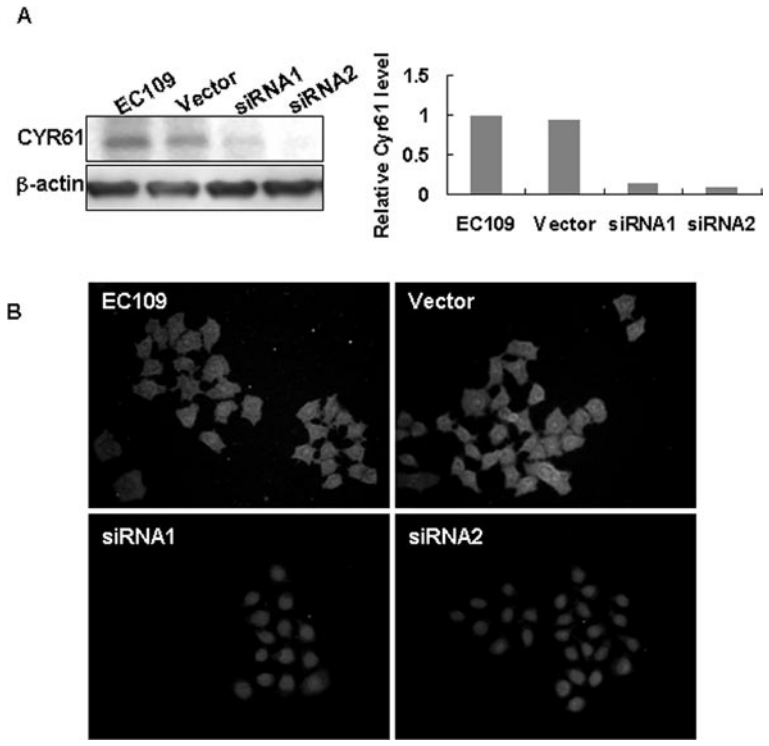

Figure 2. Cysteine-rich 61 (Cyr61) silencing in EC109 cells by siRNAs. (A) Cyr61 silencing in EC109 cells was evaluated by Western blot analysis. EC109 cells were transfected with Cyr61 siRNA-expressing plasmid (siRNA) or empty vector (Vector). β-actin served as a loading control. (B) Immunofluorescence analysis of Cyr61 silencing by siRNAs (x400).

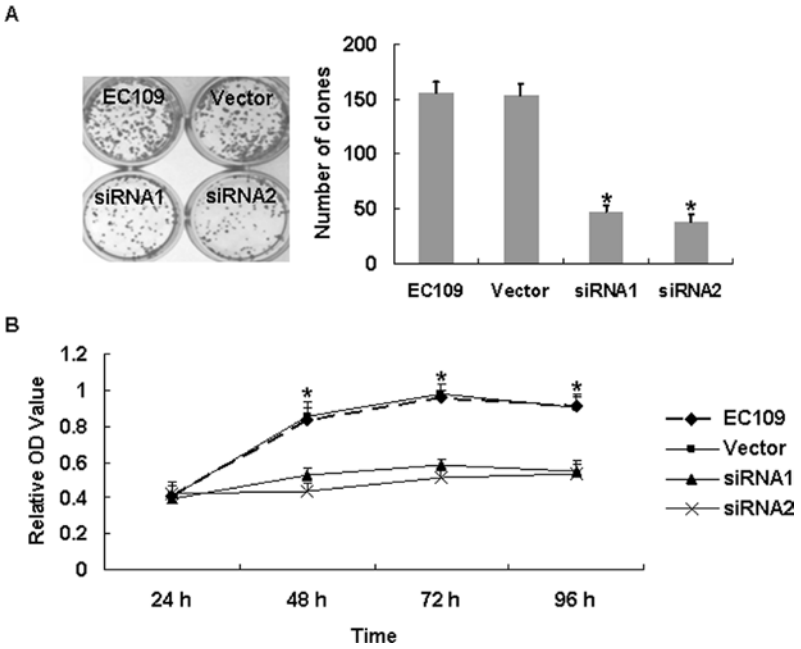

Figure 3. Effect of cysteine-rich 61 (Cyr61) knockdown on cell growth. (A) The colony formation assay and (B) the MTT assay were used to evaluate the proliferation of the Cyr61 knockdown cells. *P<0.05.

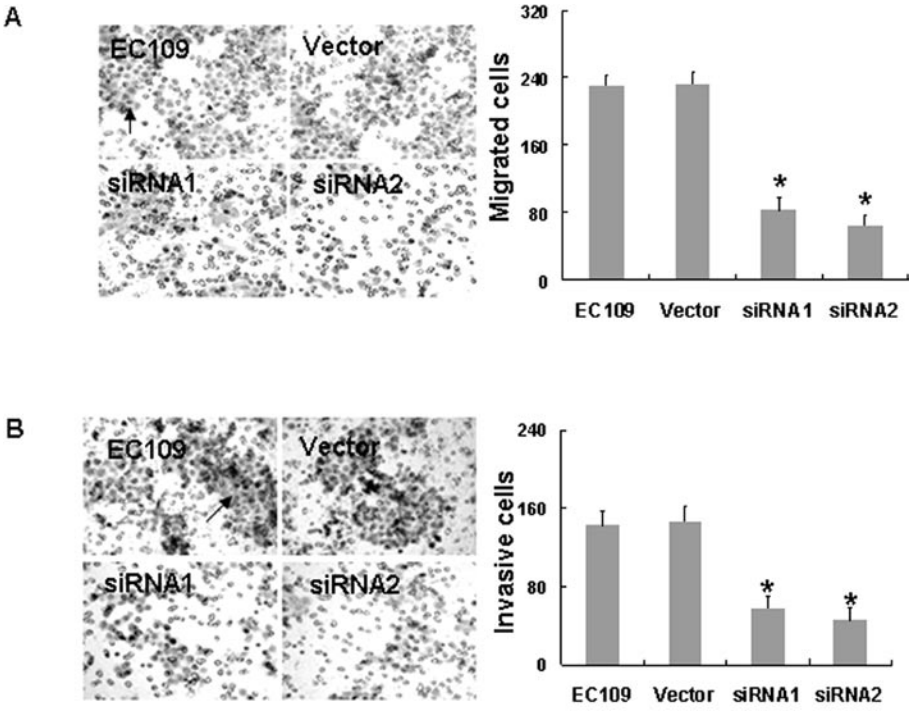

Figure 4. Role of cysteine-rich 61 (Cyr61) in cell migration and invasiveness. (A) Cell migration was assessed with modified Boyden chamber inserts. (B) Cell invasiveness was examined with matrigel-coated chambers. Migrated and invasive cells were fixed and stained, and representative fields were photographed. For quantification, the cells were counted in 10 random fields under a light microscope (x400). Arrows demonstrate cells that have migrated or invaded through the membrane. *P<0.05.

siRNA2 cells were decreased by 66.7 and 62%, and their cell invasiveness decreased by 57 and 68%, respectively.

Altered adhesion of EC109 cells. Finally, we studied the adhesion alteration in the Cyr61 knockdown cells by using an adhesion assay. The attached cells were quantified 15, 30, 45 or 60 min after seeding in the matrigel-coated plate. In the first 15 min, no differences were observed between the siRNA-treated cells and the control cells. However, at the other three time points, the adhesion ability of the EC109 cells decreased with Cyr61 knockdown as compared to the controls. The suppression rate reached the highest at 45 min, with 53% and 55% decreases with siRNA1 and siRNA2 cells, respectively (Fig. 5).

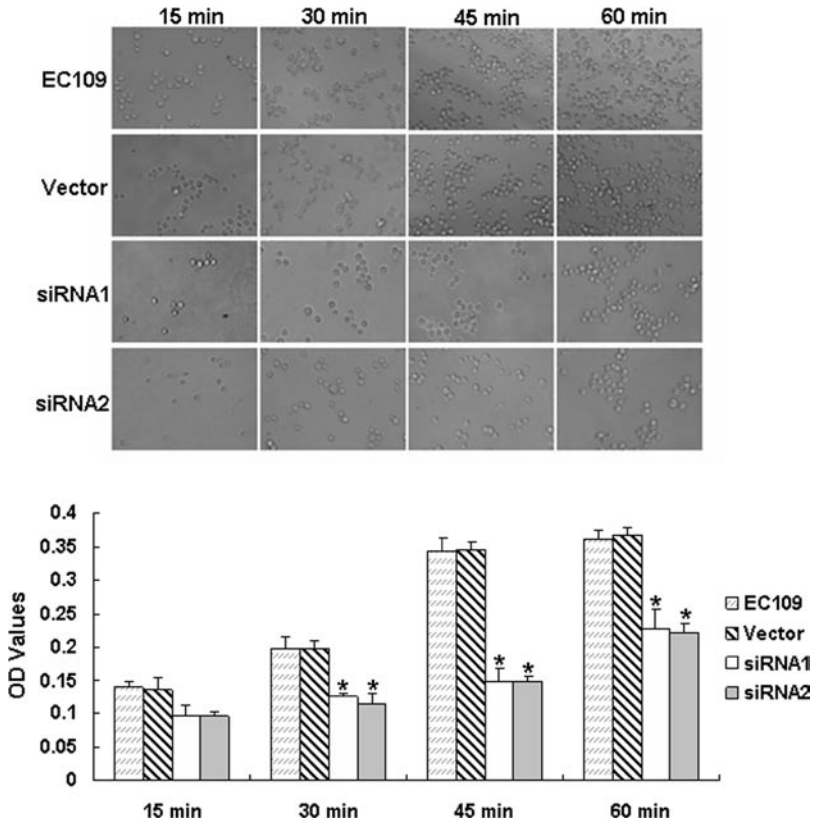

Figure 5. Role of cysteine-rich 61 (Cyr61) in cell adhesion. Cell adhesion was determined by cell adhesion assay. Adhesive cells of representative fields were photographed before treatment with MTT (x400). *P<0.05.

Discussion

As the first identified member of the novel CCN family of growth regulators, Cyr61 has been shown not only to influence a myriad of cellular functions but also to be actively involved in the pathogenesis of various diseases, especially in the development of tumors from diverse origins (5,20). Overexpression of Cyr61 had been observed in many cancers including ESCC (13,14). But the roles still remained unclear. For a better understanding of the function of Cyr61, in the present study we employed the pSUPER system to stably silence the Cyr61 gene in EC109 cells and found that Cyr61 facilitated the growth, migration, invasiveness and adhesion of the tumor cells.

Our previous studies had shown that Cyr61 is up-regulated in ESCC and that overexpression of Cyr61 is related to poor survival of ESCC patients (13,15). Here, we further reveal that Cyr61 is involved in the growth, invasiveness and adhesion of ESCC cells. Dysregulation of cell proliferation and invasiveness properties are hallmarks of cancer cells. Our data suggest that Cyr61 might play important roles in the progression of ESCC. Increasing evidence demonstrates that aberrant expression of Cyr61 is linked to tumorigenesis. Earlier reports have revealed that elevated Cyr61 level is associated with advanced breast cancer and gliomas (7,10). Overexpressed Cyr61 in normal breast cells could induce tumor formation in nude mice (21). Moreover, overexpressed Cyr61 in breast cancer cells has been shown to promote the invasion of these cells when being transplanted into mice (22). Up-regulation of Cyr61 has also been detected in rhabdomyosarcoma, colon adenosarcoma, papilloma of the bladder, glioblastoma multiforme and several types of pediatric tumors such as malignant fibrous histiocytoma, infantile myofibromatosis, and malignant hemangiopericytoma (23). On the other hand, down-regulation of Cyr61 expression has frequently been noted in prostate cancer, endometrial cancer, uterine leiomyoma, and non-small cell lung carcinoma (11,12,24,25). The paradoxical expression of Cyr61 in different types of tumors suggests that Cyr61 might play important and disparate functions in carcinogenesis depending on origin of the tissue and the cellular context.

As an ECM component, Cyr61 had been shown to exert a range of diverse functions, by binding to the cell surface at least through five integrins, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_M\beta_2$, and heparan sulfate proteoglycans (HSPGs) (26). Responding to the alteration of the extracellular environment, for example, in ligand engagement, integrins can elicit intracellular signaling pathways, such as the Wnt, PI3K/Akt and MAPK pathways, to regulate cell survival, proliferation, gene transcription, adhesion to ECM, migration and invasiveness (27). Some integrins, for example ITGB1 (integrin β1), have been shown to be differentially expressed in ESCC cells (28). It is possible that the effect of Cyr61 in EC109 cells might also be mediated through integrins. In addition, a possible interpretation for the varying effects of Cyr61 is that in different cell contexts, Cyr61 might interact with different ligands and sequentially trigger different signaling pathways. As a result, the overall effects of Cyr61 are dependent on the presence or absence of different ligands.

Integrins relay signals bi-directionally across the cell membrane between ECM and the cell-surface ligands on one

side and cytoskeletal and signaling effectors on the other (29). We recently showed that Cyr61 was down-regulated with the knockdown of ezrin or fascin, two proteins which have been revealed to promote cancer cell invasiveness and proliferation (15,30). Fascin is an actin-bundling protein and ezrin is a linker between the actin cytoskeleton and the plasma membrane. Both of them play important roles in the cytoskeletal reorganization (31,32). Cytoskeletal reorganization is a key step for cells to migrate and invade surrounding tissues (33). Positioned at the inner side of the cell membrane, some cytoskeleton related proteins such as ezrin and fascin are the intermediates for the interaction between the cytoskeleton and the ECM. Therefore, we speculated that changes in the cytoskeleton might be involved in the Cyr61 effect on cell proliferation and invasiveness. In conclusion, by using siRNA technology we successfully silenced the Cyr61 gene in EC109 cells and found that knockdown of Cyr61 suppressed cell growth and decreased cell invasiveness and adhesion, suggesting that Cyr61 might act as an oncoprotein in ESCC.

Acknowledgements

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