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The matrix protein CCN1/CYR61 is required for $\alpha_v\beta_5$ -mediated cancer cell migration

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Abstract

CYR61 is one of the six proteins of the CCN family of proteins known to play diverse roles in angiogenesis, cellular proliferation, survival, migration and wound healing. However, the specific function of CYR61 in cancer is unclear and the literature remains controversial. We used quantitative real-time PCR (qRT-PCR) to establish the expression profile of CYR61 and integrin $\alpha_v\beta_5$ in three non-small cell lung cancer (NSCLCA), five colorectal cancer (CRCA), one breast cancer (BCA) and one esophageal squamous carcinoma (ESCA) cell lines. We showed that the levels of CYR61 were significantly increased in ESCA cell line along with the enhanced levels of $\alpha_v\beta_5$ integrin. Further, we investigated whether tumor cell-secreted CYR61 can facilitate cell migration by interacting with the $\alpha_v\beta_5$ integrin. Using tumor cell lines with low, intermediate and high CYR61 expression and their isogenic variants as a cellular model, we determined that integrin $\alpha_v\beta_5$ expressed on these tumor cells is required for cell migration. Moreover, we showed that modulation of expression levels of CYR61 in these cancer cells affected their capacity for migration. These results represent an advance to the understanding of the role of CYR61 and $\alpha_v\beta_5$ integrin as proteins that cooperate to mediate cancer cell migration.

Keywords

CYR61; stable over-expression; stable knock down; integrin $\alpha_v\beta_5$; migration

Introduction

The angiogenic factor cysteine-rich angiogenic protein 61 (CYR61, CCN1) is a member of the highly conserved CCN family which includes CTGF (connective tissue growth factor, CCN2), Nov (nephroblastoma over-expressed protein, CCN3), WISP-1 (Wnt-1-induced secreted protein 1, CCN4), WISP-2 (CCN5) and WISP-3 (CCN6)¹. All CCN proteins share four conserved domains with sequence homology to insulin-like growth factor binding

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

protein, von Willebrand factor type C repeat, thrombospondin type 1 and growth factor cysteine knots². The four modular domains of CYR61 may explain the diverse functions of the protein. Indeed, CYR61 has been reported to mediate cell adhesion, including cell migration, stimulation of chemotaxis, enhancement of growth factor-induced DNA synthesis, cell survival and angiogenesis^{3,4}. Previous studies have suggested that CYR61 may be a marker for more aggressive phenotypes⁵ probably occurring through the ability of CCN proteins to bind and activate cell surface integrins¹. Moreover, CYR61 has been found to be differentially expressed in many cancers and to be involved in the development and progression of various cancers⁶. The level of CYR61 expression has been found to be increased in rhabdomyosarcomas, malignant melanomas, colon adenocarcinomas, bladder papillomas, and pancreatic cancer⁷⁻⁹. Over-expressed CYR61 stimulates the progression of breast cancers^{10,11}. High levels of CYR61 were found in malignant gliomas and CYR61 was shown to enhance the tumorigenicity through the integrin-linked kinase signaling pathway¹². Moreover, up-regulation and involvement of CYR61 expression was recognized in prostate cancer cells¹³ and recently in esophageal squamous cell carcinoma¹⁴. Paradoxically, CYR61 is down-regulated in lung cancers and forced expression of CYR61 inhibits tumorigenicity of lung cancer cells¹⁵. Finally, it was also reported that CYR61 inhibits the growth of endometrial cancer¹⁶ and leiomyomas¹⁷.

CCN proteins, like some other extracellular matrix and matricellular proteins, regulate cell adhesion, migration, differentiation, proliferation, and survival through their interactions with cell adhesion receptors, including integrins¹⁸. Indeed, the CCN-integrin connection was first demonstrated by the direct binding of CYR61 to integrin $\alpha_v\beta_3$ to mediate endothelial cell adhesion¹⁹. CCNs utilize distinct integrins depending on the target cell types and the activities mediated. For example, in fibroblasts, CYR61 stimulates cell adhesion, migration, and DNA synthesis through $\alpha_6\beta_1$, $\alpha_v\beta_5$, and $\alpha_v\beta_3$, respectively¹⁸. By contrast, CYR61 stimulates cell migration in endothelial cells and vascular smooth muscle cells through binding to $\alpha_v\beta_3$ and $\alpha_6\beta_1$, respectively²⁰. Moreover, CYR61 mutants that disrupt its $\alpha_6\beta_1$ binding sites specifically abrogate $\alpha_6\beta_1$ -dependent CYR61 activities without affecting $\alpha_v\beta_3$ -mediated angiogenic functions²¹. Similarly, mutation that impairs the CYR61 $\alpha_v\beta_3$ binding site abolishes $\alpha_v\beta_3$ - but not $\alpha_6\beta_1$ -mediated functions, further establishing that these distinct integrin binding sites and their cognate signaling pathways can act independently of one another²². On the other hand, CYR61 must interact with both $\alpha_6\beta_1$ and $\alpha_v\beta_5$ to mediate its synergism with TNF α , indicating that different integrin pathways can also function in concert to elicit distinct CCN activities²³. The interaction of CCNs with multiple receptors may contribute to their unique activities and functions.

The specific functions of CYR61 and its interactions with integrin $\alpha_v\beta_5$ in cancer are still unclear and the literature remains controversial. In our studies we have evaluated the expression levels of CYR61 and integrin subunits α_v and β_5 in colorectal adenocarcinoma, lung carcinoma, breast carcinoma, esophageal squamous cell carcinoma and squamous cell adenocarcinoma cancer cell lines. Our data indicate that there are low expression levels of CYR61 in the colorectal adenocarcinoma SW620, negligible levels in the lung carcinoma H460 and high expression levels in the esophageal squamous cell carcinoma TE-7 cell line as determined using quantitative real-time PCR and Western blotting techniques. Furthermore, CYR61 as a part of the extracellular matrix has been shown to be a secreted ligand of several integrins. Thus, we examined the integrin expression in these three cancer cell lines using DNA microarray analysis²⁴ and confirmed that these three tumor cell lines express both subunits of the CYR61 integrin receptor, α_v and β_5 . Interestingly, the colorectal adenocarcinoma cells express the integrin $\alpha_v\beta_5$ subunits but not CYR61 and do not show invasion through matrigel²⁴. These observations strongly suggest that CYR61 may be involved in the mechanism of tumor cell migration.

Materials and Methods

Cell Culture

Colorectal cancer cells (SW837, HT-29, HCA-7 and HCT116), lung carcinoma cells (H1155 and H2122), breast cancer cells (MCF-7) and esophageal squamous cell carcinoma TE-7 cells were all obtained from ATCC. SW620 cells were originally obtained as BIC-1 esophageal adenocarcinoma cell line and H460 as SEG-1 esophageal adenocarcinoma cell line from Dr. David Beer, University of Michigan. However, Boonstra et al., 2010 recently verified the authenticity of all available esophageal adenocarcinoma cell lines and genotyping by short tandem repeat profiling revealed that BIC-1 is not esophageal adenocarcinoma as it was considered for decades but SW620 colorectal adenocarcinoma cell line, SEG-1 is lung carcinoma cell line H460²⁵.

SW620, SW480 and SW837 cell lines were maintained in a 37°C, non-CO₂ equilibrated environment in Leibovitz's L-15 medium (L-15; Mediatech, Inc., Manassas, VA) containing 10% FBS and 1% penicillin/streptomycin. HCT116 cell line was maintained at 37°C in the presence of 5% CO₂ in Iwakata and Grace modified medium (McCoy's 5A; Mediatech, Inc., Manassas, VA) containing 10% FBS and 1% penicillin/streptomycin. All non-small cell lung cancer cell lines were incubated at 37°C in 5% CO₂ and cultured with RPMI-1640 medium (RPMI-1640; Mediatech, Inc., Manassas, VA) containing 10% FBS and 1% penicillin/streptomycin. Remaining cell lines were maintained in Dulbecco's modified essential medium (DMEM; Gibco-BRL, Rockville, MD) containing 10% FBS and 1% penicillin/streptomycin and cultured at 37°C in the presence of 5% CO₂.

Lentiviral Particle and Stable Cell Line Generation

To generate stable CYR61 over-expressing cell lines, full length CYR61 cDNA clone along with PCR primers for amplification and modification of the resulting product for TOPO directional cloning were obtained from the ATCC and Biosynthesis. The CYR61 cDNA was stably transduced into SW620, H460 and TE-7 cancer cell lines using ViraPower Lentiviral Expression kit (Invitrogen, Carlsbad, CA). CYR61 cDNA was PCR amplified from the original ATCC vector with *Pfx* polymerase to generate blunt-end PCR products for directional cloning into the expression pLenti6/V5-D-TOPO vector (Invitrogen, Carlsbad, CA) which was designed to facilitate rapid, directional TOPO cloning and high level expression of PCR products in mammalian cells.

For CYR61 expression knock-down, two different shRNAs directed against the CYR61 mRNA were designed using Invitrogen's proprietary design software. shRNAs were stably transduced by using the Block-iT Lentiviral RNAi Expression kit (Invitrogen, Carlsbad, CA). Two strands of shRNA sequences targeting CYR61 mRNA were synthesized (CYR61GS-1 targeting sequence: 5' – GCCACACGAGTTACCAATGATT – 3'; CYR61GS-2 targeting sequence: 5' – GCATCCTATACAACCCCTTTAC – 3'), annealed and cloned into the entry pENTR/U6 vector (Invitrogen, Carlsbad, CA) which contains *attL* sites to facilitate transfer of the U6 RNAi cassette into the destination pLenti6/BLOCK-iT-DEST vector to generate an expression clone. To obtain pLenti6/BLOCK-iT expression clone, the LR clonase reaction between entry and destination construct was performed.

Lentiviral particles were generated by individually transfecting 293FT cells with the CYR61 expression constructs pLenti6/V5 for over-expression or pLenti6-GW/U6 for silencing and the ViraPower Packaging Mix with Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Stable CYR61-expressing/silencing cells were generated by transduction with lentiviral particles at a 1:100 dilution in growth media with 6 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO). Selection of stably expressing/silencing CYR61 cell lines was conducted with 2µg/ml blasticidin for H460 and TE-7 cell lines and

10µg/ml for SW620 cell line (Invitrogen, Carlsbad, CA). pLenti6/V5-GW/lacZ (Invitrogen, Carlsbad, CA) was used as a positive expression control vector for CYR61 over-expression and pLenti6-GW/U6-lamin^{shRNA} plasmid (Invitrogen, Carlsbad, CA) was used as a positive control for CYR61 gene silencing.

RNA Extraction and Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA extraction from all isogenic variants was performed using RNeasy kit (Qiagen, Valencia, CA). Human CYR61 (Hs00155479_A1), integrin alpha V (Hs00233808_m1), integrin beta 5 (Hs00174435_m1) and GAPDH (Hs99999905_A1) primer/probes were obtained from ABI (Applied Biosystems, Branchburg, NJ). cDNA was synthesized from 500 ng of total RNA in a 50µl reaction with master mix containing 10×RT buffer, 5.5 mM MgCl₂, 2 mM dNTPs, 2.5 µM random hexamers, 2 Units of RNase Inhibitor and 62.5 Units of Multi Scribe Reverse Transcriptase. All MasterMix reagents were purchased from ABI (Applied Biosystems, Branchburg, NJ). Reactions were performed in MJ Thermocycler PTC-200 (MJ Research, Inc., Watertown, Massachusetts) followed by these conditions: 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5 minutes. 2µl of 5ng/µl cDNA was then used as a template from which to amplify the human CYR61 sequence. The conditions for PCR reactions were: 10 minutes at 95°C followed by 15 seconds at 95°C, 1 minute at 60°C for 40 cycles by using ABI7000. GAPDH amplification was included as a reference for a relative quantification of the target genes. Non-template controls were included on each PCR plate. CYR61, integrin alpha V and integrin beta 5 levels were normalized to the GAPDH control. Amplification plots were generated and the C_t value (cycle number at which fluorescence reaches threshold) recorded.

Western Blot Analysis

Cell lysates were prepared from all cell variants using the radio-immunoprecipitation assay lysis buffer (RIPA) containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton N-100, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate and protease inhibitor mixture (Leupeptin, Aprotinin, PMSF). 30µg of total protein was separated by 7.5% SDS-polyacrylamide gel electrophoresis followed by transfer to PVDF membranes (Bio-Rad, Hercules, CA). Rabbit polyclonal anti-CYR61 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti-actin clone AC-40 antibody was obtained from Sigma Aldrich (St. Louis, MO). Goat anti-rabbit (for CYR61) and goat anti-mouse (for β-actin) HRP secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used for detection. Blots were incubated with ECL Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ) and were exposed to BioMax XAR film (Kodak, Rochester, NY). The same blot was used for both, CYR61 and β-actin detection. In details, after the CYR61 analysis was performed, the stripping buffer (2% SDS, 62.5 mM TRIS pH6.8, 100 mM β-mercaptoethanol) was used to strip the original western blot by incubating the membrane in 60°C for 30 minutes. Then the stripping buffer was poured off and the membrane was washed 2 times in PBST (3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween 20, pH 7.4) for 5 minutes and 2 times with PBST/milk for 5 minutes. Then the membrane was re-probed to detect β-actin.

Conditioned Media Preparation

Untreated or CYR61 transfected cells were grown to around 80% confluence and medium was replaced with serum-free medium for a further 48-hours. 10 ml of conditioned media was centrifuged at 3,000×g for 10 min and supernatant was stored at -80°C. Conditioned media were incubated with rabbit polyclonal anti-CYR61 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Protein A/G PLUS Agarose beads (Santa

Cruz Biotechnology, Santa Cruz, CA) were then added to the suspension and incubated at 4°C for 1 hour to remove CYR61 from the conditioned media. Beads with bound CYR61 were washed 3 times with cold PBS. 2× Tris-Glycine SDS Sample Loading buffer (125 mM Tris HCl, 20% Glycerol, 4% SDS, 0.001% Bromophenol Blue, 0.2% β-mercaptoethanol, pH 6.8) was added to each bead pellet. To release the CYR61 from the beads to the loading dye, samples were denatured at 95°C for 5 minutes and then 10 μl of each sample was separated by 7.5% SDS-polyacrylamide gel and subsequently the western blot analysis was performed as described above. The equal volumes were used for immunoprecipitation and western blot analysis.

Epithelial Cell Transwell Migration Assay

Migration assays were performed using isogenic variants of the H460, TE-7, and SW620 cell lines. 8.0 μm pore size, 1.0×10^5 pore density translucent transwell migration chambers (BD Biosciences, Bedford, MA) in 24-well plate were used for migration analysis. Briefly, 600 μl of invasion buffer (DMEM media with 0.5% FBS, 0.1% BSA and without antibiotics) was added to each well. 5×10^4 of esophageal squamous carcinoma TE-7 cells or 1.5×10^5 of colorectal adenocarcinoma SW620 and lung carcinoma H460 cells re-suspended in 100 μl of invasion buffer were seeded on the top of the membrane. After overnight incubation (18 hours) at 37°C, 5% CO₂, the membranes were stained with 0.5% crystal violet (Sigma Aldrich, St. Louis, MO) in 20% methanol for 1 minute. The stain was rinsed off thoroughly with water, cells remaining in the top of the migration chamber were removed by lightly swabbing, and stained cells adhering to the bottom of the chamber were counted.

Cell Migration Assay using functional blocking antibodies

All isogenic cell line variants with altered CYR61 expression were used for the migration-blocking assays. To block cell migration with antibodies, the cells were treated with 50 μg/ml of specified blocking anti-α_vβ₅ monoclonal antibody, clone P1F6 (Millipore, Billerica, MA) for 1 hour at 4°C before the cells were seeded on the top of the membrane.

Image Analysis of transwell migrations

The 24 well BD Falcon Cell Culture Companion Plates for Inserts (BD Biosciences, San Diego, CA) were analyzed for migration of cells through the translucent transwell membrane (8.0 μm pore size, 1.0×10^5 pore density). Images of 14 fields per transwell were captured from each well using a 10X objective in combination with a 1.5X optivar. The image capture was performed with an Olympus IMT-2 microscope (Olympus America Inc., Center Valley, PA), a Hamamatsu ORCA-100 greyscale CCD camera (Hamamatsu USA, Bridgewater, NJ), and a Ludl motorized XY stage (Ludl Electronic Products, Hawthorne, NY). The camera and stage was computer controlled by SimplePCI software (ver 6.2, Compix Inc., Sewickley, PA). After the images were captured, the SimplePCI software performed an intensity threshold, and size exclusion to avoid measuring the membrane pores, and the remaining binary image was measured for area.

Statistical analysis

Data were collected and analyzed to obtain the mean and S.E.M. for three independent experiments. Statistical significance between any two groups was determined by the two-tailed Student's *t*-test using Microsoft Excel, *P* values less than 0.05 were considered to be significant.

Results

Expression levels of CYR61 in cancer cells

Real time PCR analysis (Fig. 1A) confirmed expression levels of CYR61 in several cancer cell lines. Overall the data indicated that CYR61 is expressed at almost undetectable levels in SW620, intermediate in H460 and high in TE-7 cell lines (Fig. 2A). The expression levels of CYR61 in H460 cells were 20 times higher and in TE-7 cells 250 times higher compared to the levels of CYR61 in SW620 cells.

Next, we modeled the isogenic variants of all three cancer cell lines with stably over-expressed and stably knocked-down CYR61 expression. We transduced the cells using the ViraPower Lentiviral Expression System, which allowed creation of a replication-incompetent, HIV-1-based lentivirus that was used to deliver and express the gene of interest in these cancer cell lines. To evaluate the efficiency of stable transfection, mRNA expression levels of CYR61 were then measured by quantitative real time PCR (Fig. 2B). Stable over-expression of CYR61 was obtained in all three cancer cell lines. The expression levels of CYR61 in SW620 (95-fold), H460 (25-fold) and TE-7 (7-fold) cell lines were increased significantly after the stable over-expression. Similarly, lentiviral particles containing the expression clone with shRNA targeted against CYR61 mRNA were used to stably knockdown Cyr61 expression in the H460 and TE-7 cancer cell lines. Quantitative real time PCR was used to measure the levels of stable CYR61 knock down in both cancer cell lines and revealed that there was more than 70% inhibition of CYR61 expression in both cell lines using two independent shRNA sequences against CYR61 (Fig. 2C). CYR61 protein expression levels in parental SW620, H460 and TE-7 cell lines were assessed also by Western blot analysis (Fig. 3A). As predicted by the DNA microarray²⁴ (Supplemental tab. 1) and RT-PCR results, CYR61 expression was undetectable in SW620 cells, intermediate in H460 cells and high in TE-7 cells. Western blot analysis also confirmed the forced stable over-expression (OE) of CYR61 or the CYR61 gene silencing (GS) in each cancer cell line (Fig. 3B). Finally, to confirm that CYR61 is a secreted protein, the amount of CYR61 protein in conditioned media from untreated lung carcinoma H460 cells and H460 cells over-expressing CYR61 was analyzed by immuno-precipitation and Western blot analysis (Fig. 3C). The results showed there was higher level of CYR61 protein in conditioned medium collected from H460 cells over-expressing CYR61 in comparison with parental H460 cells, and confirmed that CYR61 is a secreted protein.

Role of CYR61 in cancer cell migration

CYR61 has been shown to be a secreted protein that acts as a ligand of several integrins¹⁸. Thus, we examined the integrin expression levels in all three cancer cell lines. Data obtained from qRT-PCR (Fig. 1B and C) confirmed that the three tumor cell lines expressed both subunits of the CYR61 integrin receptor, α_V as well as β_5 . The highest expression levels of integrin α_V and β_5 subunits were detected in esophageal squamous cell carcinoma TE-7 cell line. These cells also showed the highest migration ability through the transwell membrane. Interestingly, SW620 cells express the integrin $\alpha_V\beta_5$ subunits but not CYR61 (Fig. 4) and do not show migration through the uncoated membrane. Fig. 4 shows the migration of all three cancer cell lines under the optimal experimental conditions, where TE-7 cells had to be seeded at much lower concentration (50,000 cells per insert) than both SW620 and H460 cells, which were seeded at higher concentration of 150,000 cells per insert. These observations suggested that CYR61 may be involved in the mechanism of cancer cell migration through the specific interaction with integrin $\alpha_V\beta_5$. To test this hypothesis, we measured the migratory capabilities of H460 and TE-7 cancer cell lines and their isogenic variants with altered CYR61 expression to determine if the expression of integrin $\alpha_V\beta_5$ was required for the cell migration on CYR61. Both H460 and TE-7 cancer cell lines exhibited

significantly reduced (30–50% respectively) migration capabilities through uncoated transwell filters after stable knockdown of CYR61. H460 and TE-7 cancer cells showed increased migration (25–30% respectively) through the uncoated transwell insert after stable over-expression of CYR61 (Fig. 5).

Migration of cancer cells is CYR61 and $\alpha_v\beta_5$ dependent

To test the functional role of integrin $\alpha_v\beta_5$ in mediating the pro-migratory effects of CYR61, we performed transwell migration assays using functional blocking antibody against the integrin $\alpha_v\beta_5$. As documented in Fig. 6, blocking antibody against integrin $\alpha_v\beta_5$ was able to diminish migration of all CYR61 isogenic variants of H460 and TE-7 cancer cell lines. The migration were reduced about 80% in all isogenic variants of H460 cells and about 60% in all TE-7 isogenic variants after 1 hour pre-treatment with integrin $\alpha_v\beta_5$ blocking antibody.

Discussion

In this study, we explored the functions of CYR61 in various cancer cell lines and have determined that CYR61 facilitates the migration of these cells through the integrin $\alpha_v\beta_5$. Our study confirmed that both CYR61 and integrin $\alpha_v\beta_5$ and their interactions are required for the migration of cancer cells, which has never been shown before.

CYR61 is a secreted, heparin-binding and extracellular matrix-associated angiogenic inducer. It regulates cell adhesion, migration, proliferation and is involved in tumor growth. CYR61 has been found to be differentially expressed in many cancers and is involved in the development and progression of various cancers⁶. Using SW620, H460 and TE-7 cancer cell lines and their isogenic variants with altered expression levels of CYR61 we determined that suppressing/increasing expression and secretion of CYR61 in various tumor cells affects their capacity for migration. We have found that the higher the CYR61 expression levels within these cancer cells, the more advanced the migratory abilities observed. We showed slightly increased migratory capabilities of H460 and TE-7 cells with CYR61 over-expression, as well as, significantly decreased migratory capabilities after stable knockdown of CYR61. Previously, it was shown that CYR61 is expressed in about 30% of invasive breast carcinomas²⁶. Recently, Haque et al. published that CYR61 is consistently found to be over-expressed in pancreatic cancer and the expression intensifies with disease progression²⁷. They showed that aggressive pancreatic ductal adenocarcinoma cell lines exhibited higher levels of CYR61 compared to less aggressive types and that CYR61 plays an important regulatory role in migration of these cells²⁷. Moreover, recent findings observed high levels of CYR61 in EC109 esophageal squamous cell carcinoma cell lines and it was shown that CYR61 knockdown by RNAi leads to a significant suppression of migration, invasiveness, adhesion, formation of colonies and cell growth¹⁴. These observations confirm our findings of decreased migratory abilities of esophageal squamous cell carcinoma TE-7 cells with stably down-regulated expression of CYR61. On the other hand, lower expression levels of CYR61 were reported in non-small cell lung cancers²⁸. Nevertheless, Tong et al. observed that the expression levels of CYR61 can alter dramatically among various lung cancer cell lines. They found high expression levels of CYR61 in three and none in five lung cancer cell lines, including H460 non-small cell lung carcinoma line²⁹. However, we have found intermediate levels of CYR61 in H460 cells what is most likely due to using more sensitive and accurate quantitative RTPCR analysis while Tong et al. used Northern blot analysis which provides only qualitative or semi-quantitative information of mRNA levels. Moreover, they showed that H460 cells with over-expressed CYR61 formed fewer colonies and had decreased proliferation rates in comparison to control cells transfected with empty vector²⁹. Their findings suggesting a

tumor suppressor role for CYR61 in non-small cell lung cancer cells is contradictory to our results identifying CYR61 as a promoter of migration in H460 cells.

CYR61 is a well-established ligand for several integrins^{18,19}, including $\alpha_v\beta_5$. From the diverse CYR61 functions, integrin binding has been demonstrated to affect invasion and survival^{4,26}. Examination of integrin expression in SW620, H460 and TE-7 cancer cell lines using microarrays confirmed that these tumor cell lines express both members of the CYR61 receptor, α_v and β_5 . Interestingly, the colorectal adenocarcinoma SW620 cells express both α_v and β_5 integrin subunits but do not express CYR61 and do not show migration through uncoated membranes, in compliance with findings of Watts et al. where no invasion of these cells through the matrigel was obtained²⁴. The negative migration of SW620 cells could be explained by the presence of high levels of integrin subunits α_6 and β_4 , the major pro-adhesion integrin combination³⁰. TE-7 squamous cell carcinoma cell line showed the highest expression levels of CYR61 and integrin $\alpha_v\beta_5$ and demonstrated the strongest migration capabilities. In previous studies it was reported that the cell line SEG-1 (aka H460 cells²⁵) showed strong invasiveness through matrigel, while the BIC-1 cell line (aka SW620²⁵) did not invade at all^{24,31}. We have reproduced these results and further demonstrate the importance of CYR61 within this process.

Integrin $\alpha_v\beta_5$ was shown to mediate CYR61-stimulated fibroblast migration³², promote resistance to apoptosis in MCF-7 cells⁴ and is essential for CYR61-induced metastasis of tumors growing within a pre-irradiated field³³. In our experiments when a functional blocking antibody against integrin $\alpha_v\beta_5$ was included, the migration of H460 and TE-7 cells was significantly inhibited. These results confirm for the first time that tumor cell-secreted CYR61 may facilitate cancer cell migration by interacting with the $\alpha_v\beta_5$ integrin in cells. The data presented in this study suggest that suppressing/increasing expression and secretion of CYR61 in cancer cells affects their capacity for migration and that there is a requirement of CYR61 for cancer cell migration by interactions with integrin $\alpha_v\beta_5$ expressed on tumor cells.

It had been demonstrated that downstream effectors of $\alpha_v\beta_5$ integrin as integrin-linked kinase (ILK) and small GTPase (RhoB) might be promising candidate targets for improving the efficiency of radiotherapy³⁴. Recently, it was reported that CCN3-stimulated migration of chondrosarcoma cells is mediated through the $\alpha_v\beta_3/\alpha_v\beta_5$ integrin receptor, FAK, PI3K, Akt, p65, and NF- κ B signal transduction pathway³⁵. Also, it was shown that integrin $\alpha_v\beta_5$ directly interacts with PAK4 (serine/threonine p-21 activating kinase) and regulates carcinoma cell motility in an integrin-specific manner³⁶. In breast cancer cells, integrin $\alpha_v\beta_5$ mediates activation of PAK4 after the cells are attached to vitronectin³⁷. Thus, besides of CYR61 as a possible target for cancer therapy, further investigation of signaling pathways that are activated downstream of integrin $\alpha_v\beta_5$ might also bring us new possible targets for therapeutic interventions and improving the efficiency of treatment of various cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. CYR61, ITGAV and ITGB5 mRNA expression levels in cancer cell lines measured by quantitative real-time PCR

Bar graphs showing mRNA expression levels of **A) CYR61** **B) ITGAV** and **C) ITGB5** in three lung, five colorectal, one breast and one esophageal squamous carcinoma cell lines. The relative expression levels of all genes were calculated by normalization to GAPDH. Data were obtained from three independent experiments are shown as mean $\pm$ SEM (each experiment was done in triplicate parallels).

Figure 2. CYR61 mRNA expression levels in colorectal adenocarcinoma SW620, lung carcinoma H460 and esophageal squamous cell carcinoma TE-7 cells measured by quantitative real-time PCR

A) Bar graph showing relative CYR61 mRNA expression levels for untreated cell lines. Asterisks indicate significantly higher expression of CYR61 in H460 and TE-7 cells compared to SW620 cells. **B)** Bar graph showing relative CYR61 mRNA expression levels for all three cell lines after stable over-expression where LacZ was used as a control. Asterisks indicate significant increase in expression of CYR61 in all three cell lines after their stable CYR61 over-expression compared to their LacZ controls. **C)** Bar graph showing relative CYR61 mRNA expression levels for H460 and TE-7 cell lines after CYR61 was stably knocked down using two independent shRNA sequences, Laminin A/C was used as a control. Asterisks indicate significant decrease in expression of CYR61 in both cell lines after their CYR61 knockdown compared to Lamin A/C controls. The relative CYR61 expression levels were calculated by normalization to GAPDH. Data were obtained from three independent experiments (three independent RNA samples from three individual clones of cells were used within each experiment) are shown as a mean $\pm$ SEM (* $p < 0.05$, ** $p < 0.0005$, *** $p < 0.005$).

Figure 3. Western blot analysis showing CYR61 protein expression levels in colorectal adenocarcinoma SW620, lung carcinoma H460 and esophageal squamous cell carcinoma TE-7 cell lines

A) CYR61 protein expression in untreated cell lines. As predicted by the microarray results, CYR61 expression was undetectable in SW620 cells, immediate in H460 cells and high in TE-7 cells. β -actin was used as a loading control. **B)** WB analysis as a confirmation of high/low expression levels after stably forced CYR61 over-expression/knock-down for all three cell lines. Treatment lanes: untreated cells; cells over-expressing CYR61 (OE), cells after stable transfection with two independent shRNAs directed against CYR61 mRNA (GS-1, GS-2). **C)** Immuno-precipitation and WB analysis of CYR61 expression in conditioned medium as a confirmation that CYR61 is secreted into the media. Conditioned medium was collected from untreated H460 and H460 cells over-expressing CYR61 after they were grown in serum-free medium for 48 hours. Experiments were performed at least three times using freshly isolated protein samples from independent cell lines.

Figure 4. CYR61 plays a role in cancer cell migration

Migration assay through uncoated transwell inserts for parental SW620, H460 and TE-7 cancer cell lines. SW620 cells did not show any migration through the untreated transwell inserts as compared to H460 and TE-7 cell lines. Microscopic pictures of random fields of transwell insert at 20× magnification (purple - migrated cells, white - pores of the membrane).

Figure 5. Transwell migration assay for all CYR61 isogenic variants of lung carcinoma H460 and esophageal squamous cell carcinoma TE-7 cell lines

A) Migration assay with H460 cells or **B)** TE-7 cells exhibiting significantly reduced migration through uncoated membrane after stable transfection with 2 independent shRNAs directed against CYR61 mRNA (GS-1 and GS-2) or a control shRNA targeting LacZ. There is also shown slightly increased migration of H460 and TE-7 cells stably over-expressing CYR61 (OE). All results are compared to LacZ control. Representative microscopic pictures are shown as random fields of transwell insert at 20× magnification. Three membrane filters were used for each condition within one experiment. Data were obtained from triplicate independent experiments and are shown as a mean $\pm$ SEM (* $p < 0.05$). Asterisk indicates statistical difference in migration between control LacZ cells and cells with forced stable CYR61 over-expression or knock down.

Figure 6. Blocking integrin $\alpha v \beta 5$ antibody diminished migration capabilities of all CYR61 isogenic variants of lung carcinoma H460 and esophageal squamous cell carcinoma TE-7 cell lines

A) H460 cells or B) TE-7 cells were treated for 1 h at 4°C with specified monoclonal (50 µg/ml) anti- $\alpha v \beta 5$ antibody before plating on the top of the membrane. Transwell inserts were then incubated for 18 hours at 37°C, 5% CO₂. Data were obtained from four independent experiments (three inserts for three individual clones of cells were used within each experiment) are shown as a mean $\pm$ SEM ($p < 0.05$). *Asterisk indicates statistical difference in migration between untreated control LacZ cells and untreated cells with forced stable CYR61 over-expression or knockdown. **Asterisks indicate statistical differences in

migration between control LacZ cells and cells with forced stable CYR61 over-expression or knock down after the cells were pre-treated with $\alpha\text{v}\beta 5$ blocking antibody.

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