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Original Article

CYR61 promotes colorectal carcinoma progression via activating epithelial-mesenchymal transition

Shelan Rasool[1], Qais AL Ismaeel[1], Sardar H Arif[2]

[1]Department of Anatomy, Biology and Histology, College of Medicine, University of Duhok, Duhok 42001, Kurdistan Region of Iraq; [2]Department of Surgery, College of Medicine, University of Duhok, Duhok 42001, Kurdistan Region of Iraq

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Abstract: Colorectal carcinoma is the third most common type of cancer. Although the role of matricellular proteins and their association with tumor progression is well documented, limited data are available concerning their involvement in colorectal cancer. The current study investigated the expression pattern of matricellular proteins SPARC and CYR61 with epithelial-mesenchymal transition proteins in human CRC tissues and unleashed their association with colorectal cancer progression. The expression of these proteins was associated with advancement in tumor staging, nodal metastasis, and vascular invasion. Elevated CYR61 protein levels were also consistent with higher mesenchymal markers ZEB1 and Vimentin in collected biopsies and CRC cells. Moreover, expression of CYR61 promoted CRC cell migration, invasion, proliferation, and apoptosis. Our findings conclusively revealed the significant involvement of CYR61 in CRC progression through activating epithelial-mesenchymal transition. This discovery holds great promise for advancing therapeutic approaches in the treatment of CRC.

Keywords: Colorectal cancer, matricellular proteins, epithelial-mesenchymal transition, proliferation, metastasis, apoptosis

Introduction

Recent epidemiological data indicated that colorectal carcinoma (CRC) is the third most common cancer worldwide with an estimated 1.9 million new cases and 930,000 cancer-related deaths in 2020 alone [1]. The highest morbidity and mortality rates were observed in Australia and Europe, whereas the lowest rates were recorded in the African and South Asian regions. The CRC burden is expected to rise to 3.2 million new cases and 1.6 million deaths by 2040 [2]. The majority of cases are localized with or without lymph node metastases. Approximately 20% of patients are diagnosed with metastatic form, particularly in the liver. Surgical recession is still considered the golden standard for patients with localized tumors whereas chemotherapy remains the main line of treatment for advanced metastatic conditions [3]. Around 80% of CRC cases are curable when diagnosed at early stages, however, the determination of the pathologic stage is considered the most important indicator of prognosis following the surgical resection of early cancer [4].

The extracellular matrix (ECM) is a diverse network of proteins, growth factors, and other secretory components, which are classified into adhesive and matricellular (MCP) [5]. Physiologically, ECM has been recognized as a structural framework of cells in tissues and organs. However, many cells express surface receptors for specific matrix proteins and their ligands alter signal transduction pathways that control cell shape, motility, and survival [6].

SPARC (secreted protein, acidic and rich in cysteine or osteonectin) and CYR61 (Cysteine-rich angiogenic inducer 61) are multifunctional matricellular proteins that belong to a group of matrix-associated factors that mediate cell-matrix interaction. They play essential roles during embryonic development, regulation of inflammation, wound healing, tissue remodeling, and fibrogenesis [7, 8]. Despite their favorable physiological functions, both proteins have

been implicated in a variety of pathological conditions. High expression of SPARC in cancer, diabetes, and arthritis has been well documented. Accordingly, CYR61 was shown to promote cell proliferation, survival, and angiogenesis through binding to corresponding integrin [9]. One of the hallmarks of cancer is the formation of the tumor microenvironment that is established through a complex interaction between MCP with endothelial cells, adipocytes, and cancer-associated fibroblasts. MCPs exert a major role in tumor initiation, growth, invasion, metastasis, and even the development of chemotherapeutics resistance [10]. These data suggest that MCPs are of great value owing to their diverse biological activities in health and cancers. Therefore, understanding the biology of these proteins may provide new insights into the development of therapeutic interventions that target cancer in general and colorectal cancer in particular.

The initiation of tumors and the subsequent invasion of tumor cells into the surrounding stroma are crucial stages in the development of metastases. This transformation is referred to as epithelial-mesenchymal transition (EMT), a distinct morphological alteration in which tumor cells switch from a well-differentiated epithelial state to a more invasive mesenchymal state [11]. The detachment of tumor cells from the primary tumor necessitates a gradual loss of cellular interaction at adherence junctions. This process is facilitated by the downregulation of epithelial proteins like E-cadherin and, concurrently increasing cell motility through the upregulation of mesenchymal proteins such as Vimentin and N-cadherin [11].

The induction of EMT can be triggered by several growth factors and matricellular proteins that act via developmental transcription factors such as ZEB1, SNAIL, and TWIST. MCPs can induce EMT by activating multiple receptor-mediated signaling pathways that have been demonstrated to play essential roles in tumor cell migration and invasion [12].

Because there is inadequate solid evidence concerning the involvement of MCPs in colorectal cancer, this study aims to evaluate the role of matricellular proteins particularly CYR61 and SPARC in colorectal cancer progression and their association with the epithelial-mesenchymal transition markers in both human histopathological specimens and CRC cell-based experiments.

Materials and methods

Study participants and specimens

After obtaining informed consent and approval from the research ethics committee of the College of Medicine, University of Duhok, a total of 96 patients diagnosed with CRC were included in the study. All patients underwent surgery at the Surgery Department of Vajeen Hospital between 2017 and 2021. Following surgical resection, the tissue specimens were preserved in 10% neutral buffered formalin and then embedded in paraffin blocks. The tissue sections were stained with Hematoxylin and Eosin (H&E) for further analysis. Meanwhile, 42 para-cancerous matched normal tissues were collected from the foresaid 96 patients.

Immunohistochemistry (IHC)

Immunohistochemical staining was performed on tissue blocks by using 4-μm-thick sections. The paraffin sections were de-paraffinized in xylene and then gradually rehydrated in different concentrations of ethanol. To retrieve the antigens, microwave-induced antigen retrieval was employed in a 0.01 M citrate buffer with a pH of 6.0. To block any endogenous peroxidase activity, the sections were treated with 0.3% hydrogen peroxide in methanol for 15 minutes. The sections were then incubated at room temperature with specific primary antibodies at specified dilutions as indicated in (Supplementary Table 1). For immunohistochemical staining, the DAKO Kit system (DAKO, Denmark) and a Peroxidase/DAB Kit (DAKO) were utilized. Following staining, the sections were counterstained with Hematoxylin, dehydrated, and finally mounted.

Immunohistochemistry evaluation

Protein expression was evaluated by two independent pathologists using a semi-quantitative scoring system that assessed the intensity of staining and the distribution of positive cells. The positive reaction's intensity was graded on a scale of negative (0), weak (1), moderate (2), and strong (3). The reactivity was determined by the percentage of positively stained cells,

with categories ranging from 0-6% (0), 7-26% (1), 27-51% (2), 52-76 (3), and 77-100% (4).

The staining index score ranging from 0-12 was determined by multiplying the staining intensity score and the proportion of positive cells. A staining index value of 0-6 showed low protein expression, whereas a staining index score of 6-12 indicated strong protein expression [13-15].

Cell culture

Human colorectal cancer cell lines SW480, HT29, and HCT116 were purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. The cell cultures were maintained in a saturated humidity incubator at a temperature of 37°C with 5% CO_2.

Transient transfection

For the transient overexpression of CYR6, The CYR61 coding gene was sub-cloned into the pcDNA3.1 + vector (Invitrogen, San Diego, CA) and transfected into the HT29 cell line. For silencing CYR61 expression in SW480, CYR61 siRNA and Non-targeting siRNA (Thermo Fisher Scientific/USA) were used. All transfection experiments were performed using lines using Ingenio® Electroporation solution (MirusBio/USA). Briefly, the cells were grown to 80% confluence re-suspended with fresh media, and spun down at 1000 rpm for 5 mins. The cell plate was mixed with 60 µl of Ingenio® Electroporation solution. Then, either 1 µg/ml of plasmid DNA or siRNA was added to the mixture. The resulting suspension was transferred into a 4 mm cuvette. Electroporation was carried out using the Gene PulserX cell electroporator (Bio-Rad/USA), which was configured with a voltage of 250 V and a capacitance of 250 µF. Following electroporation, the transfected cells were resuspended in 1 ml of fresh growth media. Cells were then maintained in the growth media and prepared for the required experiment.

MMT assay

To assess the effect of CYR61 expression on CRC cell viability, an MTT colorimetric assay was conducted using HT29 and SW480 cells 3 days after transfection. The cells were distributed separately into 96-well plates, with each well containing 2000 cells. The plates were then kept at 37°C for 1-3 days. Every day, 20 µl of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Thermo Fisher Scientific/USA) solution with a concentration of 5 mg/ml was added to each well and incubated for 4 hours. Subsequently, a solution containing acidic isopropanol (consisting of 10% SDS, 5% isopropanol, and 0.01 M HCl) was added at a volume of 100 µl per well and incubated overnight at 37°C. The absorbance (optical density OD) was measured at 570 nm using a microplate reader (Bio-Rad/US).

Wound-healing assay

The migration rate of SW480 and HT29 cells transfected with plasmid or siRNA was assessed using a wound-healing assay. For this purpose, 1.5×10^6 cells/well were placed in 6-well plates and allowed to grow overnight until they reached 90% confluence. A straight scratch was made using a sterile pipette tip. The detached cells were then gently rinsed off with PBS three times, and the cells were cultured in the medium for an additional 24 hours. The movement of cells was observed and captured using a digital camera (Leica/Germany) at both 0 hours and 24 hours.

Transwell migration and invasion assay

The transwell migration and invasion assay were conducted using a 24-well transwell system with an 8 µm pore size (Corning, USA). For migration assay, 5×10^4 cells were suspended in 300-µl serum-free medium and seeded into the upper chamber. The lower chamber was filled with 800 µl of medium containing 10% FBS. After a 48-hour incubation period, the chamber was fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Cells in the upper chamber were removed using a cotton swab. The cells in four randomly selected microscopic fields were counted and photographed. For the invasion assay, the same steps were taken as above, except that 1×10^5 cells were put into an upper chamber that had already been coated with matrigel (BD Biosciences, USA). Each experiment was performed in triplicate.

Table 1. Correlations between the expression of matricellular proteins (CYR61 and SPARC) and the clinicopathological features of CRC

Parameters		SPARC		*P*-value	CYR61		*P*-value
		Low	High		Low	High	
		No. (%)	No. (%)		No. (%)	No. (%)	
Age	≤50	24 (32)	6 (28.6)	0.76	9 (20)	21 (39.6)	0.049
	>50	51 (68)	15 (71.4)		34 (79)	32 (60.4)	
Sex	Male	40 (53.3)	10 (47.6)	0.64	22 (51.2)	28 (52.8)	0.87
	Female	35 (46.7)	11 (52.4)		21 (48.8)	25 (47.2)	
Histological type	Adenocarcinoma	65 (86.7)	20 (95.2)	0.276	39 (90.7)	46 (86.8)	0.55
	Mucinous Adenocarcinoma	10 (13.3)	1 (4.8)		4 (9.3)	7 (13.2)	
Size of tumor	≤5	18 (24)	3 (14.3)	0.341	7 (16.3)	14 (26.4)	0.23
	>5	57 (76)	18 (85.7)		36 (83.7)	39 (73.6)	
Grade	1	5 (6.7)	2 (9.5)	0.78	6 (14.0)	1 (1.9)	0.06
	2	59 (78.7)	15 (71.4)		32 (74.4)	42 (79.2)	
	3	11 (14.7)	4 (19.0)		5 (11.6)	10 (18.9)	
TNM Stage	1	13 (17.3)	6 (28.6)	0.36	17 (39.5)	2 (3.8)	<0.0001
	2	26 (34.7)	8 (38.1)		22 (51.2)	12 (22.6)	
	3	29 (38.7)	7 (33.3)		3 (7)	33 (62.3)	
	4	7 (9.3)	0 (0)		1 (2.3)	6 (11.3)	
Depth of invasion	1	5 (6.7)	0 (0)	0.21	4 (9.3)	1 (1.9)	0.08
	2	17 (22.7)	9 (42.9)		14 (32.6)	12 (22.6)	
	3	41 (54.7)	10 (47.6)		22 (51.2)	29 (54.7)	
	4	12 (16)	2 (9.5)		3 (7)	11 (20.8)	
Nodal metastasis	0	43 (57.3)	13 (61.9)	0.83	37 (86)	19 (35.8)	<0.0001
	1	19 (25.3)	4 (19)		2 (4.7)	21 (39.6)	
	2	13 (17.3)	4 (19)		4 (9.3)	13 (24.5)	
Distant metastasis	0	68 (90.7)	21 (100)	0.14	42 (97.7)	47 (88.7)	0.09
	1	7 (9.3)	0 (0)		1 (2.3)	6 (11.3)	
Vascular invasion	Negative	25 (33.3)	11 (52.4)	0.11	24 (55.8)	12 (22.6)	0.001
	Positive	50 (66.7)	10 (47.6)		19 (44.2)	41 (77.4)	
Preneural infiltration	Negative	37 (49.3)	11 (52.4)	0.80	26 (60.5)	22 (41.5)	0.65
	Positive	38 (50.7)	10 (47.6)		17 (39.5)	31 (58.5)	
	Total	75 (100)	21 (100)		43 (100)	53 (100)	

Caspase activity assay

Caspase 3 and 9 activity Assay Kits (Elabscience Biotechnology In, USA) were used to assess caspase 3 and 9 activity in infected cells according to the manufacturer's protocols. Briefly, 1×10^5 Cells infected with CYR61 siRNA and Non-targeting siRNA as a control were cultured in 96 well plates and incubated for 24 h. Cells then were detached and centrifuged at 2000 rpm for 5 min. Then 55 μl working solution buffer containing (50 μl 2× Reaction working solution and 5 μl Ac-DEVD-pNA) was added into cells and the cell plate was placed on an oscillating shaker for 30 minutes. The optical density at 405 was measured using a microplate reader (Bio-Rad).

Western blot

Cells were lysed with Laemmli buffer (Thermo Fisher Scientific/USA) and centrifuged at 12,000 g for 10 minutes. The supernatant containing the proteins was collected and equal amounts of protein from each experimental group were loaded onto SDS-PAGE. The proteins were then transferred to a PVDF membrane (Millipore, MA, USA) and incubated overnight at 4°C with the primary antibodies as indicated in **Table 1**. The membranes were then

washed with PBS and incubated with a species-specific secondary antibody conjugated to horseradish peroxidase (**Table 1**) for 2 hours at room temperature. Finally, the membranes were visualized using an enhanced chemiluminescence kit (Millipore, MA, USA).

RNA extraction, cDNA synthesis, and quantitative PCR

Cells were subjected to RNA extraction using an RNeasy RNA isolation kit (Germantown, MD, USA), followed by reverse transcription into cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific/USA). Quantitative PCR was conducted in triplicate utilizing SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). The quantitative data were normalized to the GAPDH internal control, and the conventional ΔΔCT method was employed for data analysis. The primer sequences employed in this study can be found in (Supplementary Table 2).

Statistical analysis

All in vitro experiments were performed at least twice to avoid discrepancy. One-way ANOVA and t-test were used to compare data between study groups using GraphPad Prism 7.1 software. Concerning immunohistochemical experiments, all statistical analyses were performed using the Statistical Package for Social Sciences version 20.0 (SPSS, Chicago, Illinois, USA). To explore the associations between protein expression and clinicopathological factors, or between cancer and normal tissues, Fisher's exact or chi-square tests were conducted. All analyses were considered statistically significant if the *p*-value was less than 0.05.

Results

Demographic data of CRC patients

Among the 96 collected specimens, 52.1% (N=50) were male and 47.9% (N=46) were female. Concerning TNM staging, the majority of CRC patients fell into stages 2 and 3 (35.4%, N=34 and 37.5%, N=36) respectively. Patients with nodal metastasis displayed 41.7% compared to 7.3% (N=7) with distant metastasis. Patients with vascular invasion represented a higher percentage (62.5%, N=60), however, 50% were positive for perineural infiltration.

Matricellular proteins (CYR61 and SPARC) and EMT-related markers (ZEB1, Vimentin, and E-cadherin) expression is associated with the clinical outcome of CRC

The correlation between the expression of matricellular proteins (CYR61 and SPARC) and clinicopathological features of CRC patients are shown in **Table 1**. CYR61 displayed a higher expression percentage that was consistent with progression in TNM staging (P<0.0001). Moreover, expression of the same protein was statistically significantly higher in patients with nodal metastasis and vascular invasion respectively (P<0.0001 and P=0.001). However, the SPARC expression did not show a significant correlation with the clinicopathological characteristics of the patients. Concerning EMT-related markers expression (**Table 2**), a significant association was observed between increased levels of ZEB1 expression and TNM stage (P=0.012), nodal metastasis (P=0.017), and vascular invasion (P=0.027). Similar findings were observed with Vimentin, where its elevated expression displayed a significant correlation with TNM staging (P=0.016) and nodal metastasis (P=0.004) respectively. Concerning E-cadherin, a significant correlation was observed between reduced expression and TNM stage (P<0.001), nodal metastasis (P=0.007), vascular invasion (P=0.006), and distance metastasis (P=0.023).

Additionally, differences in the expression level of matricellular proteins and EMT-associated markers between cancerous and para-cancerous matched normal tissues are illustrated in **Table 3**. CYR61 and ZEB1 expression rates were higher in cancerous tissues (P<0.0001; P=0.008 respectively), while SPARC and E-cadherin expression rates were lower than in para-cancerous matched normal tissues (P=0.001; P<0.0001).

CYR61 is positively correlated with mesenchymal-related markers in CRC tissues

The correlation between the expression of matricellular proteins (CYR61 and SPARC) and EMT-related markers (ZEB1, Vimentin, and E-cadherin) was investigated (**Table 4**). The elevated expression of CYR61 protein was significantly correlated with the high expression of ZEB1 (P=0.002) and Vimentin (P=0.023). In contrast, the high CYR61 expression group had

Table 2. Correlations between the expression of EMT-related proteins (ZEB1, Vimentin and E-cadherin) and the clinicopathological features of CRC

Parameters		ZEB1 expression		*P*-value	Vimentin expression		*P*-value	E-cadherin expression		*P*-value
		Low	High		Low	High		Low	High	
		No. (%)	No. (%)		No. (%)	No. (%)		No. (%)	No. (%)	
Age	≤50	15 (30.0)	15 (32.6)	0.783	24 (39.3)	6 (17.1)	0.02	19 (33.3)	11 (28.2)	0.59
	>50	35 (70)	31 (67.4)		37 (60.7)	29 (82.9)		38 (66.7)	28 (71.8)	
Sex	Male	28 (56.0)	22 (47.8)	0.43	38 (62.3)	12 (34.3)	0.008	28 (49.1)	22 (56.4)	0.48
	Female	22 (44)	24 (52.2)		23 (37.7)	23(65.7)		29 (50.9)	17 (43.6)	
Histological type	Adeno	44 (88)	41 (89.1)	0.86	52 (85.2)	33 (94.3)	0.18	49 (86)	36 (92.3)	0.33
	Mucinous	6 (12)	5 (10.9)		9 (14.8)	2 (5.7)		8 (14)	3 (7.7)	
Size of tumor	≤5	8 (16)	13 (28.3)	0.14	12 (19.7)	9 (25.7)	0.49	12 (21.1)	9 (23.1)	0.81
	>5	42 (84)	33 (71.7)		49 (80.3)	26 (74.3)		45 (78.9)	30 (76.9)	
Grade	1	4 (8)	3 (6.5)	0.95	5 (8.2)	2 (5.7)	0.85	3 (5.3)	4 (10.3)	0.35
	2	38 (76)	36 (78.3)		46 (75.4)	28 (80)		43 (75.4)	31 (79.5)	
	3	8 (16)	7 (15.2)		10 (16.4)	5 (14.3)		11 (19.3)	4 (10.3)	
TNM_Stage	1	16 (32)	3 (6.5)	0.012	4 (14.8)	4 (28.6)	0.016	6 (10.5)	13 (33.3)	<0.001
	2	17 (34)	17 (37)		4 (31.1)	4 (42.9)		15 (26.3)	19 (48.7)	
	3	15 (30)	21 (45.7)		4 (49.2)	4 (17.1)		29 (50.9)	7 (17.9)	
	4	2 (4)	5 (10.9)		4 (4.9)	4 (11.4)		7 (12.3)	0 (0)	
Depth of invasion	1	4 (8)	1 (2.2)	0.32	2 (3.3)	3 (8.6)	0.65	2 (3.5)	3 (7.7)	0.12
	2	16 (32)	10 (21.7)		17 (27.9)	9 (25.7)		13 (22.8)	13 (33.3)	
	3	24 (48)	27 (58.7)		32 (52.5)	19 (54.3)		30 (52.6)	21 (53.8)	
	4	6 (12)	8 (17.4)		10 (16.4)	4 (11.4)		12 (21.1)	2 (5.1)	
Nodal metastasis	0	35 (70)	21 (45.7)	0.017	29 (47.5)	27 (77.1)	0.004	26 (45.6)	30 (76.9)	0.007
	1	11 (22)	12 (26.1)		21 (34.4)	2 (5.7)		19 (33.3)	4 (10.3)	
	2	4 (8)	13 (28.3)		11 (18)	6 (17.1)		12 (21.1)	5 (12.8)	
Distant metastasis	0	48 (96)	41 (89.1)	0.16	58 (95.1)	31 (88.6)	0.23	50 (87.7)	39 (100)	0.023
	1	2 (4)	5 (10.9)		3 (4.9)	4 (11.4)		7 (12.3)	0 (0)	
Vascular invasion	Negative	24 (48)	12 (26.1)	0.027	20 (32.8)	16 (45.7)	0.20	15 (26.3)	21 (53.8)	0.006
	Positive	26 (52)	34 (73.9)		41 (67.2)	19 (54.3)		42 (73.7)	18 (46.2)	
Perineural infiltration	Negative	28 (56)	20 (43.5)	0.22	30 (49.2)	18 (51.4)	0.83	25 (43.9)	23 (59)	0.14
	Positive	22 (44)	26 (56.5)		31 (50.8)	17 (48.6)		32 (56.1)	16 (41)	
	Total	50 (100)	46 (100)		61 (100)	35 (100)		57 (100)	39 (100)	

a lower E-cadherin expression rate than the low CYR61 expression group (P≤0.001). However, there was no correlation between the expression of SPARC and EMT-related markers.

Expression of matricellular proteins (SPARC and CYR61) in colorectal cancer cell lines

The mRNA and protein levels of SPARC and CYR61 expression were determined in several human CRC cell lines including HT29, HCT116, and SW480 using quantitative real-time PCR and western blot. These cells were well-characterized from less aggressive (HT29) to Mild (HCT116) and highly aggressive cell lines (SW480).

Compared to HT29 cells, moderate to high expression levels of CYR61 mRNA were observed in the HCT116 and SW480 cell lines respectively. The significance was assessed by t-test (P=0.009; P<0.0001) (**Figure 1A** and **1B**). Western blot analysis also revealed high expression of CYR61 proteins in HCT116 and SW480, compared to less aggressive (HT29) cells (**Figure 1C**). The expression of SPARC protein is almost negligible among all cell lines and couldn't be specifically associated with particular characteristics. Among the three selected CRC cell lines, the expression of CYR61 mRNA and protein was higher in SW480 and lower in the HT29 cell line. Therefore, we chose to use

Table 3. Differences in matricelluar and EMT-related proteins expression between cancerous and cancerous-adjacent normal tissues

Parameters		Non cancer Tissue	Cancer Tissue	*P*-value
		No. (%)	No. (%)	
ZEB1 Expression	Low	32 (76.2)	50 (52.1)	0.008
	High	10 (23.8)	46 (47.9)	
Vimentin Expression	Low	31 (73.8)	61 (63.5)	0.238
	High	11 (26.2)	35 (36.5)	
E-cadherin Expression	Low	10 (23.8)	57 (59.4)	<0.001
	High	32 (76.2)	39 (40.6)	
SPARC Expression	Low	21 (50)	75 (78.1)	0.001
	High	21 (50)	21 (21.9)	
CYR61 Expression	Low	33 (78.6)	43 (44.8)	<0.001
	High	9 (21.4)	53 (55.2)	

Table 4. Correlation between matricellular proteins expression and EMT-related proteins in CRC

Parameters		SPARC Expression		P	CYR61 Expression		P
		Low	High		Low	High	
		No. (%)	No. (%)		No. (%)	No. (%)	
ZEB1	Low	35 (46.7)	12 (57.1)	0.07	30 (69.8)	20 (37.7)	0.002
	High	40 (53.3)	9 (42.8)		13 (30.2)	33 (62.3)	
Vimentin	Low	51 (68)	10 (47.6)	0.086	22 (51.2)	39 (73.6)	0.023
	High	24 (32)	11 (52.4)		21 (48.8)	14 (26.4)	
E-cadherin	Low	45 (60)	12 (57.1)	0.814	12 (27.9)	45 (84.9)	<0.001
	High	30 (40)	9 (42.9)		31 (72.1)	8 (15.1)	

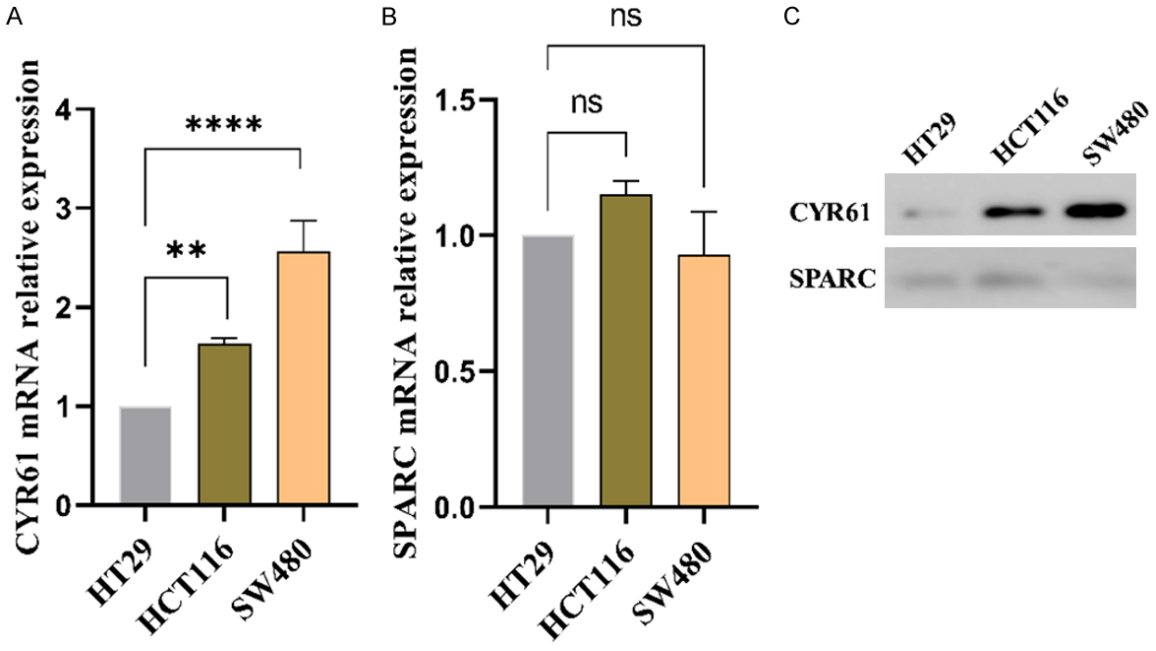

Figure 1. Expression of SPARC and CYR61 in different human colorectal cancer cell lines. A, B. The bar graph represents the quantitative relative expression of SPARC and CYR61 in various CRC cell lines by real-time PCR. The relative mRNA expression level was evaluated using the $2^{-\Delta\Delta CT}$ approach, and the mRNA level was normalized to control gene GAPDH. Delta CT value is represented by bar charts with standard errors of the mean (**P=0.009 and ****P<0.0001). The results of three representative experiments are shown. C. Western blot analysis was performed to verify the expression of SPARC and CYR61 in CRC cell lines.

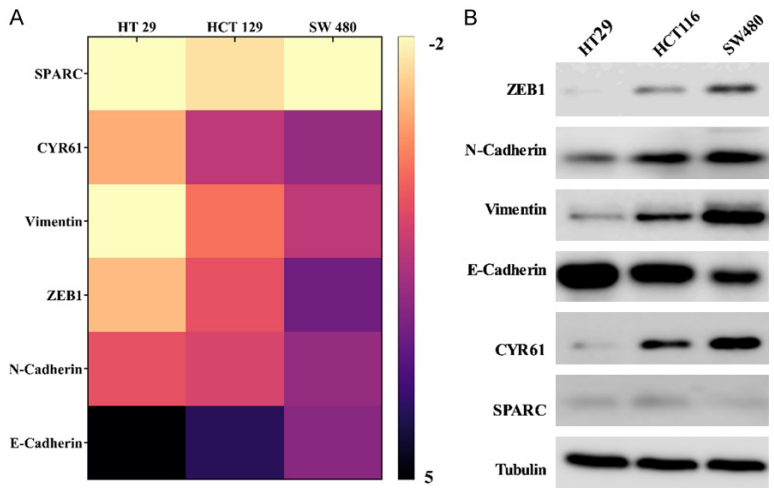

Figure 2. The Expression of matricellular proteins SPARC and CYR61 is associated with the activation of the EMT program. A. mRNA expression level of matricellular proteins (SPARC and CYR61) and EMT-associated genes was quantified as ΔCt, which was calculated by normalizing the expression level of the gene of interest to a reference gene GAPDH. The heap map was drawn by blotting ΔCt-value in Prism Graph9. The color saturation scale reflects to the level of gene expression; yellow indicates a low expression, whereas black indicates a maximum of expression. B. Western blot was performed to verify the results.

the HT29 and SW480 for functional experiments.

Expression of CYR61 is associated with activation of EMT markers

Real-time PCR data, represented as a heat map in **Figure 2A**, showed that mRNA expression levels of CYR 61 genes were elevated in both HCT116 and SW480 compared to the HT29 cell line, and this expression was associated positively with the mesenchymal associated genes, ZEB1, Vimentin, and N-cadherin. Expression of CYR61, meanwhile, was found to be downregulated in less aggressive HT29 cells, in parallel with the activation of the epithelial genes, E-cadherin.

In contrast, no significant correlation was observed in SPARC expression in association with EMT-related markers. At the protein level, expression of CYR61 is associated with the EMT program further verified by Western blot analysis and revealed that protein levels for selected proteins were consistent with the level of mRNA (**Figure 2B**). Consequently, it seems that the expression of CYR61 is associated with the activation of mesenchymal markers.

CYR61 expression is linked to EMT induction in CRC cell lines

To determine whether CYR61 is involved in the regulation of the EMT program, CYR61 was silenced in SW480 and overexpressed in HT29 cells for functional experiments. For silencing, SW480 cells were transfected with CYR61 siRNA or non-targeting siRNA as a control. On the other hand, HT29 cells were transfected with CYR61- pcDNA3.1 + to upregulate the expression of CYR61 or pcDNA3.1 + empty vector as a control.

RT-PCR analysis demonstrated that there was a significant reduction of ZEB1, Vimentin, and N-Cadherin in SW480 cells transfected with CYR61 siRNA (P=0.009; P=0.004; P=0.008), respectively, compared to control cells. Moreover, epithelial-related gene E-cadherin expression levels were upregulated (P=0.001), indicating that the EMT process was suppressed following CYR61 knockdown (**Figure 3A**). In contrast to CYR61 knockdown, the overexpression of CYR61 in HT29 cells displayed a significant activation of mesenchymal markers (ZEB1, N-Cadherin, and Vimentin) (P=0.001; P=0.004; P=0.0001 respectively) accompanied by downregulation of the epithelial marker E-cadherin gene (P=0.001) (**Figure 3B**).

Very similar findings were detected for the protein levels with CYR61 and EMT-associated markers (**Figure 3C** and **3D**), suggesting that CYR61 may play a crucial regulatory function in the process of EMT in CRC cells.

CYR61 promotes the migratory and invasive ability of CRC cells

It is now well established that over-expression of CYR61 and EMT induction are among many genetic events involved in CRC progression (18). Since CYR61 expression is involved in the activation of the EMT program, it is possible to hypothesize that CYR61 may be associated

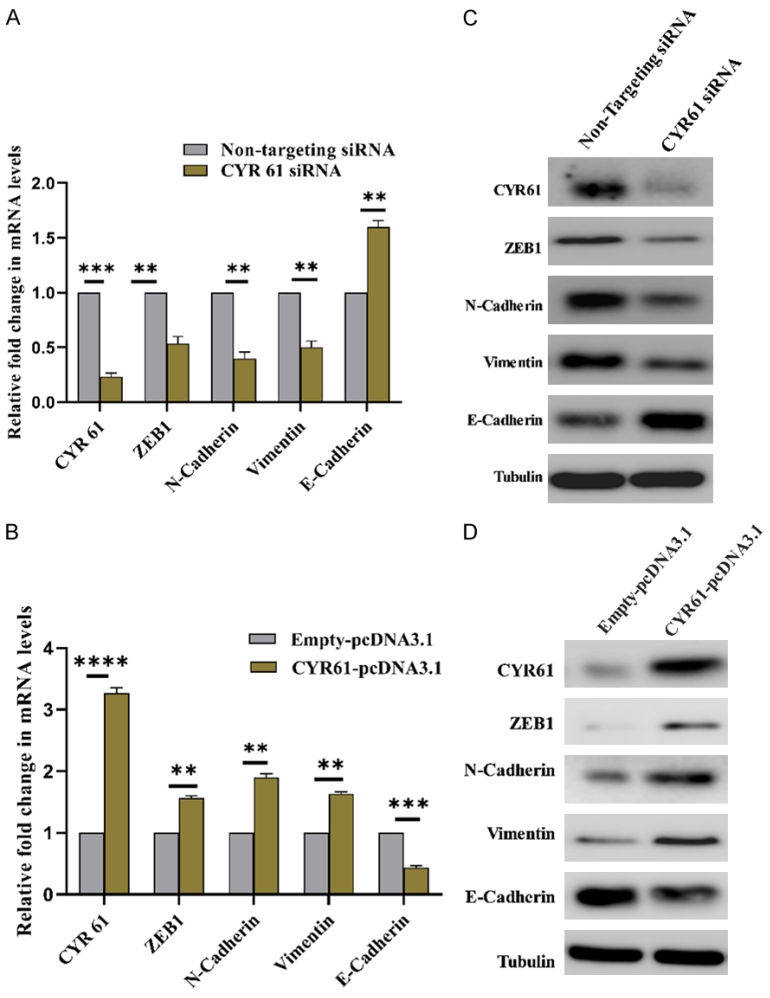

Figure 3. CYR61 expression induced EMT program in CRC cell lines. A and B. Real-time PCR was performed to analyze mRNA expression levels of CYR61 and EMT-associated genes. The relative mRNA level was quantified by the $2^{-\Delta\Delta CT}$ method, and the d mRNA level was normalized to the housekeeping gene GAPDH. The bar chart with standard errors of the mean represents delta CT value (**P≤0.009; ***P=0.0001). C and D. Western blot was performed to verify the results.

with EMT in the enhancement of cancer cell invasion and migration. To determine the functional role of CYR61 in metastasis, the effects of CYR61 on the migrated and invasive ability of CRC cells were assessed by Transwell and wound healing assays. The findings from the Transwell assay demonstrated a significant reduction in both migratory and invasive (**Figure 4A-C**) capacities of CYR61 siRNA-SW480 transfected cells (P=0.002 and P=0.008) respectively, compared to the control group.

Conversely, overexpressed CYR61 cells exhibited significantly enhanced migratory and invasive abilities compared to the control group (P=0.001 and P=0.008) (**Figure 4D-F**). The results of the wound healing assay revealed that CYR61 knockdown significantly inhibited gap closure in SW480 cells (P=0.001) (**Figure 4G** and **4H**), while CYR6161 overexpression promoted gap closure compared with the control group (P=0.004) (**Figure 4I** and **4J**). Overexpression and silencing of CYR61 protein were further verified by Western blot analysis, which revealed a significant upregulation and downregulation of protein expression following transfection (**Figure 4K** and **4L**). Collectively, our findings provided evidence that CYR-61 protein was implicated in enhancing the migratory and invasive phenotypes of CRC cells.

CYR61 expression promotes the proliferation of colorectal cancer cells in vitro

We next investigate the functional role of CYR61 in CRC cell proliferation. To this end, expression of CYR61 was knocked down in SW480 cells and overexpressed in HT29 cells. The MTT assay was then performed to evaluate cell proliferation in transfected cells. Based on the MTT results, it was observed that the knockdown of CYR1 significantly suppressed SW480 cell *proliferation* at 1, 2, and 3 days compared with that in the control group (P=0.02; P=0.009; P=0.008) (**Figure 5A**). Besides, when CYR61 was overexpressed in HT29 cells, the results were reversed, where the proliferation of HT29 cells was significantly increased in response to CYR61 overexpression (P=0.02; P=0.005; P=0.001) (**Figure 5B**). The efficiency of transfection was confirmed by Real-Time PCR and western blot. Collectively, findings indicate that CYR61 promotes the proliferative ability of CRC cells (**Figure 5C-F**).

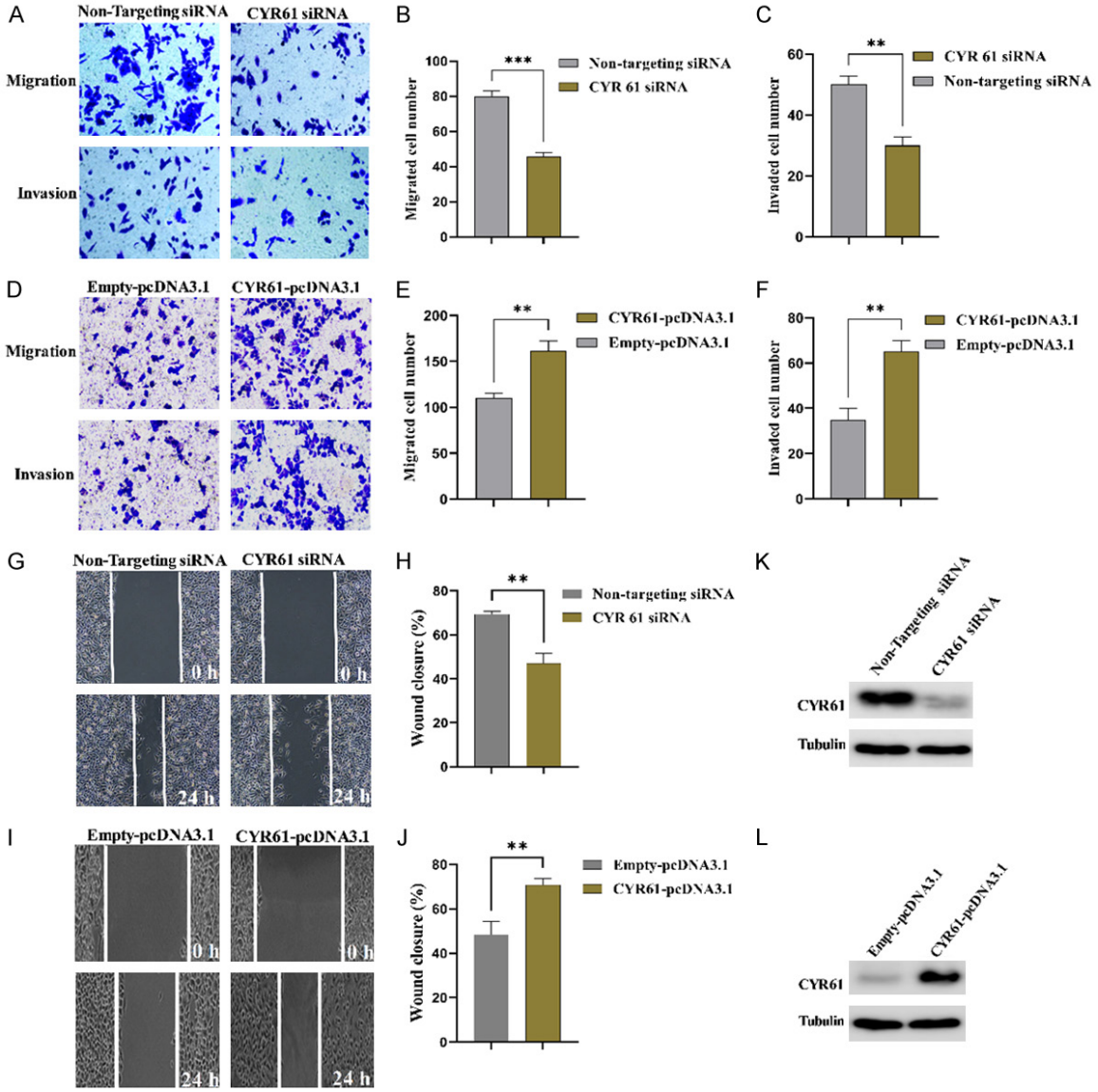

Figure 4. CYR61 promotes CRC cell migration and invasion. CYR61 was silenced in SW480 and overexpressed in HT29 cells. For the purpose of silencing, SW480 cells were transfected with CYR61 siRNA or non-targeting siRNA as a control. Whereas HT29 cells were transfected with CYR61- pcDNA3.1 + to upregulate the expression of CYR61 or pcDNA3.1 + empty vector as a control. A-C. Transwell cell migration and invasion assays (Magnification; 100×) revealed that knockdown of CYR61 significantly suppressed the migratory and invasive ability of SW480 cells. D-F. Conversely, overexpressed CYR61 cells exhibited significantly enhanced migratory and invasive abilities of HT29 cells. G and H. The results of the wound healing assay (Scale; 100 μm) revealed that CYR61 knockdown significantly inhibited gap closure in SW480 cells. I and J. Whereas CYR61 overexpression promoted gap closure compared with the control group. The scale bar was 100 μm. Data are presented as standard errors of the mean (**P≤0.008; ***P<0.0008). K and L. Silencing and overexpression of CYR61 was confirmed by Western blot. The results of three representative experiments are shown.

Knockdown of CYR61 induced SW480 cells apoptosis

Since knockdown of CYR61 inhibited SW480 cell proliferation, the caspase 3 and 9 activity assay were performed to determine the apoptotic function of this protein on SW480 cell apoptosis. As described previously, SW480 cells were transfected with CYR61 siRNA and Non-targeting siRNA as a control for 24 hours. The transfected cells were then subjected to a caspase activity assay. The results showed that the downregulation of CYR61 significantly increased the activity of caspase 3 and 9 of

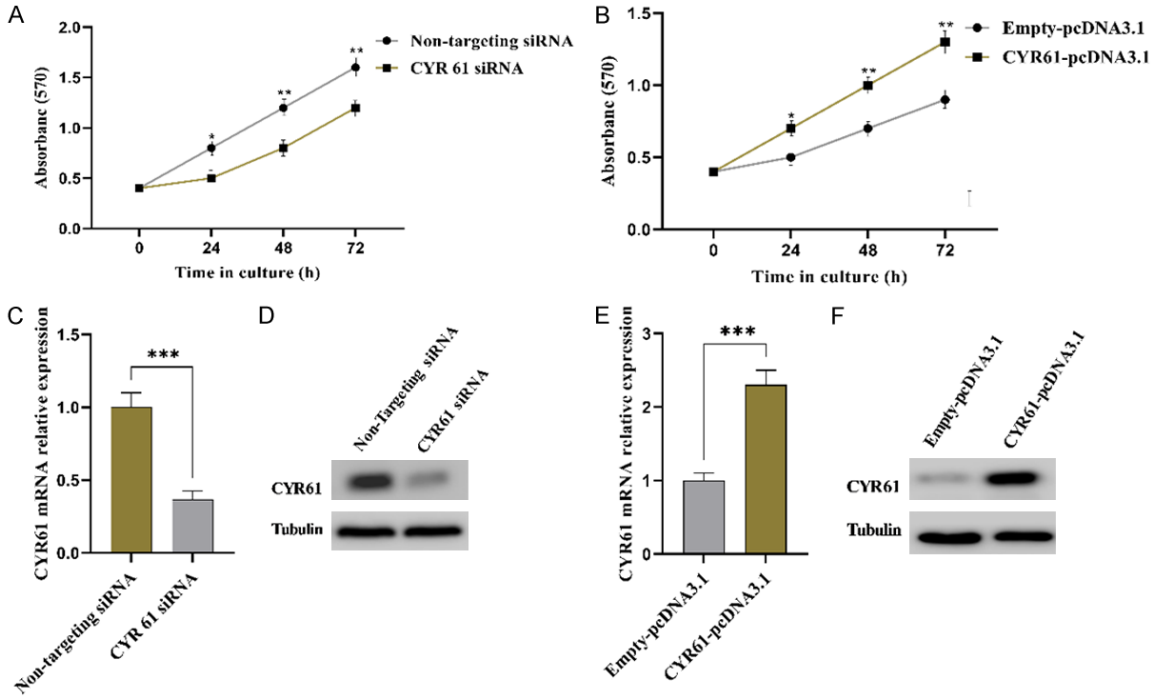

Figure 5. CYR61 expression promotes colorectal cancer cell proliferation. To downregulate or upregulate the expression of CYR61 in CRC cells, SW480 cells were transfected either with CYR61 siRNA or Non-targeting siRNA as a control group, whereas HT 29 cells were transfected with CYR61- pcDNA3.1 + or pcDNA3.1 + empty vector. Proliferation of transfected cells was measured using MTT assay and the absorbance read at 570 nm. A. Knockdown of CYR1 significantly suppressed SW480 cell proliferation. B. The proliferation of HT29 cells was significantly increased in response to CYR61 overexpression. C-F. Transfection efficiency was confirmed by RT-PCR and Western blot analysis. Data are presented as standard errors of the mean (*P<0.02; **P≤0.009; and ***P=0.0001). The results of three representative experiments are shown.

CYR61 siRNA transfected cells compared to the control group (P=0.005; P=0.001 respectively) (**Figure 6A**). Subsequently, the cell lysate was collected 24 hours post-transfection and the western blot was conducted to detect the expression levels of apoptosis-associated proteins. Compared to the control cells, the expression level of proapoptotic proteins (Bax, cleaved caspase 3, and cleaved caspase 9) was significantly increased in CYR61 knockdown cells (P=0.006; P=0.0002; P=0.0005) whereas the expression levels of the antiapoptotic protein Bcl-2 were significantly decreased (P<0.0001). However, none of the groups displayed a significant change in caspase3 or caspase9 expression (**Figure 6B** and **6C**). This data indicates that CYR61 may regulate the apoptotic program in CRC cells.

Discussion

Matricellular proteins are expressed in low to undetectable levels in normal tissues and cells [16]. Overexpression is commonly observed in certain cellular events associated with tissue remodeling, wound healing, and embryonic development. It should be highlighted that overexpression of such proteins has also been identified in different types of cancer such as brain, breast, pancreatic, and prostate [17]. It is believed that the tumor cells with the surrounding activated stromal cells aberrantly secrete MCPs into the tumor microenvironment thus enhancing cancer development [18].

The present study aims to evaluate the potential role of matricellular proteins SPARC and CYR61 in colorectal cancer progression and to elucidate their possible association with the EMT cascade. Despite the availability of abundant research linking MCPs to cancer, little literature investigated their contribution to CRC progression.

Firstly, we explored the expression pattern of SPARC and CYR61 in paraffin-embedded biop-

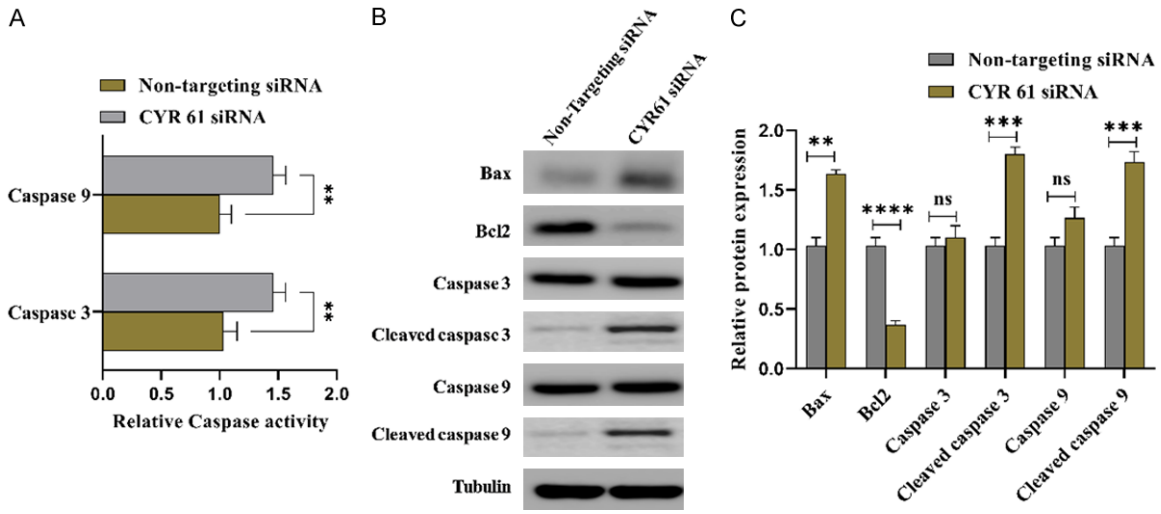

Figure 6. Effect of CYR61 silencing on SW480 cell apoptosis. caspase 3 and 9 activities in SW480 transfected cells were measured by spectrophotometry. The activity of caspases in control cells was designated as 1, and the activity values of the study group were standardized against it. A. CYR61 siRNA cells showed a significant increase in the activity of both caspase-3 and caspase-9. B. The expression level of apoptosis-related proteins was detected by western blot. C. The relative expression of proteins was quantified using Image J software by normalizing the intensity of protein bands to tubulin. Standard errors of the mean for three experiments are represented by bar graphs (**P≤0.006; ***P≤0.0005 and ****P<0.0001).

sies collected from 96 colorectal cancer patients with different pathological stages to unleash their correlation with the clinicopathological parameters. Our findings are consistent with previous studies indicating that the overexpression is associated with the severity of the clinicopathological features. The duration of survival rate was significantly lower in patients with high expression of CYR61 [19]. Moreover, a single report by Ladwa et al. revealed that CYR61 was expressed in 82% of CRC tissue samples and was associated with more advanced stages [20]. Because of its high sensitivity, this protein has potential as a serum biomarker for the diagnosis of CRC [21].

Similarly to CYR61, SPARC expression in 332 cases of colorectal cancer correlates with disease progression and may be used as a prognostic marker for CRC [22]. While the elevated SPARC expression in CRC has been documented, there is also a contradictory notion. The low SPARC expression in 847 immunohistochemically stained samples was shown to be associated with the poor prognosis and aggressive clinicopathological features of CRC [23].

It should be highlighted that non-significant changes in SPARC in our study could be attributed to its weak expression in tumor tissues compared to the stromal cells surrounding tumors. Moreover, the stromal cells may express SPARC to provide tumor cells with invasive capability [24]. The lymphovascular invasion of CRC was correlated to SPARC expression in the tumor stromal microenvironment. Therefore, future studies should consider the MCP expression in stromal cells to find out their association with tumor invasion [25].

We also investigated the expression of EMT-related markers in human CRC tissues and examined their association with CYR61 and SPARC. Our findings indicated that the elevated CYR61 was consistent with overexpression of mesenchymal markers Vimentin and ZEB1 and correlated negatively with the epithelial E-cadherin. This pattern was also associated with nodal metastasis and vascular invasion. These findings are consistent with previous studies concluding that ZEB1 overexpression and E-cadherin downregulation may be positively associated with CRC metastasis [26]. Moreover, ZEB1 was positively correlated with Vimentin and negatively associated with E-cadherin [27]. ZEB1 was also shown to play a crucial role in tumor progression, chemoresistance, and radioresistance.

To further validate MCP expression, in vitro studies were performed using a set of human adherent colorectal cancer cell lines as

described previously. Western blot and qPCR confirmed that CYR61 displayed a higher expression in more aggressive SW480 and HCT119 cell lines compared to HT29.

Regarding CYR61 expression, our findings are in concordance with earlier studies confirming that CRC tissues and cell lines expressed a higher level of CYR61 compared to normal colon mucosa and were positively correlated with CRC progression, cell proliferation, and poor prognosis [28]. The upregulation of CYR61 protein and genes was detected in the early but not advanced stage, suggesting its essential role in the early stage of tumor development. No significant correlation between CYR61 and the clinicopathological features was noticed [29]. A study that used microRNA (miRNA) to investigate the therapeutic potential of CYR61 revealed that silencing this particular protein inhibited EMT in CRC mice. Furthermore, cell proliferation, invasion, and metastasis were significantly suppressed in CRC cells upon silencing [30].

It was previously documented that SPARC is prominently expressed in metastatic tumors such as glioblastoma, melanoma, breast, and prostate cancer, while its expression is low to undetectable levels in less metastatic types such as neuroblastoma, ovarian and gastric cancers [31]. The tissue microarray analysis of 292 CRC cases revealed higher expression of SPARC in stromal cells surrounding the tumor [32]. DNA microarray analyses of human CRC samples confirmed that relatively high levels of SPARC are associated with poor outcomes in adenocarcinoma of gastrointestinal tumors mainly the esophagus, colon, and rectum [33].

Based on the above explanation, it can be concluded that high levels of SPARC and CYR61 in the tumor microenvironment may indicate the gradual loss of normal tissue homeostasis. This could be due to the capability of these proteins to alter the microenvironment by influencing cell shape, proliferation, and migration.

Since EMT is often linked to tumor progression, invasion, and metastasis [34], we attempted to determine if the high expression of SPARC and CYR61 is associated with the EMT program. For that purpose, qPCR and Western blot were performed to validate this hypothesis. As expected, mRNA expression levels of SPARC and CYR61 genes were significantly elevated in both HCT116 and SW480 cell lines, and this expression was associated positively with the mesenchymal associated genes, ZEB1, Vimentin, and N-cadherin. The same findings were detected at protein levels suggesting that the expression of these proteins may result in the activation of the mesenchymal-related EMT markers.

It is essential to highlight that E-cadherin maintains cell-cell adhesion therefore, its downregulation may result in the loss of intracellular cellular integrity and subsequently promote cell migration [35]. Accordingly, with the development of mesenchymal phenotypes, cells rapidly proliferate and develop micro-metastases. The reason behind the loss of E-cadherin expression seems to be due to the activation of several inhibitor proteins mainly Snail, ZEB, and E47 that combine with E-cadherin promoter and thus inhibit its expression [36]. Moreover, altered microRNA expression may also contribute to the loss of E-cadherin expression and subsequently promote CRC progression [37]. The tumor suppressor genes regulate E-cadherin expression via controlling its feedback loops including miR-200a (b-catenin) and miR-200c related expression by ZEB [38]. The loss of membranous E-cadherin expression was associated with higher N stage, T stage, and worse survival rate in a major study conducted on 1197 CRC human tissue samples [39].

Upon confirming the association between CYR61 expression and the EMT, CYR61 knockdown was performed on the SW480 cell line using CYR61 siRNA. As described previously, CYR61 knockdown suppressed the expression of EMT-related markers, particularly N-cadherin and Vimentin. To further validate this finding, we investigated the effects of CYR61 overexpression in HT29 cell line with EMT markers. As a result, the mesenchymal markers (ZEB1, N-cadherin, and Vimentin) displayed a significant upregulation in cells transfected with CYR61- pcDNA upon overexpression compared to controls. Accordingly, CYR61 expression activates the EMT program, particularly the mesenchymal markers.

To explore the contribution of CYR61 in CRC progression, the effects of its knockdown on cell proliferation, migration, and invasion were investigated. The proliferation, migration, and

invasion in the SW480 cell line displayed a significant suppression upon CYR61 knockdown. On the other hand, CYR61 overexpression promoted migration and invasion in HT29 cells. These findings suggest that CYR61 promotes CRC proliferation, migration, and invasion by enhancing mesenchymal-related markers.

Despite limited available data concerning the role of CYR61 in CRC progression, its involvement in other forms of cancer is clearly understood [40-46]. CYR61 was able to promote the upregulation of mesenchymal markers of EMT (TWIST-1 and N-cadherin) in osteosarcoma cells via the Raf-1 signaling pathway. Consistently, it induced cell migration ability of osteosarcoma cells. However, knockdown of CYR61 expression inhibited cell migration and suppressed mesenchymal phenotypes [40]. Higher expression of CYR61 mRNA and protein levels was detected in more aggressive pancreatic cancer cell lines such as Panc-1-side-population (SP) cells. Upon silencing, the cell migration was substantially inhibited [41]. The CYR61 expression was closely associated with the activation of Vimentin, LSCC invasion, and lymph node metastasis in laryngeal squamous cell carcinoma (LSCC) tissues [42]. With respect to breast cancer, CYR61 inhibition significantly suppressed migration and invasion of MDA-MB231 human breast cancer cells [43]. In addition, CYR61 expression was higher in the metastatic form of breast cancer confirmed by immunohistochemical analysis of 239 tissue samples [44]. The higher CYR61 expression levels enhanced the migratory abilities of lung cancer H460 and TE-7 cells [45]. Knockdown of CYR61 in the esophageal squamous cell carcinoma cell line (EC109) significantly suppressed migration and invasion [46].

The possible explanation behind the notable association between CYR61 expression and EMT activation could be due to CYR61's ability to induce angiogenesis, which is essential for supplying cancer cells with necessary oxygen and nutrients and thus enhancing cell proliferation and invasion [47, 48].

In conclusion, the results of the current study suggest that the expression of matricellular proteins CYR61 and SPARC in both tissue samples and cancer cell lines plays a crucial role in CRC progression represented by enhancing cell proliferation, migration, and invasion. These results in subsequent upregulation of the EMT program specifically the mesenchymal-related markers. To the best of our knowledge, this study is considered the first of its kind to investigate the association between matricellular proteins and EMT with CRC progression at both clinical and in vitro levels. The current work suggests that targeting MCP proteins precisely CYR61 could be used as a therapeutic intervention and should be further validated by additional studies.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qais AL Ismaeel, Department of Anatomy, Biology and Histology, College of Medicine, University of Duhok, Duhok 42001, Kurdistan Region of Iraq. Tel: +964-750-767-0444; E-mail: qaisibraheem@uod.ac

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Supplementary Table 1. Description of antibodies used for Western blotting and Immunohistochemistry

Antibody	Species	Clone	Dilution	Sources
E-Cadherin	Mouse	Monoclonal	WB 1:1000 IHC 1:500	Abcam, USA
N-Cadherin	Mouse	Monoclonal	WB 1:1000	Abcam, USA
Tubulin	Mouse	Monoclonal	WB 1:30000	Sigma-Aldrich, UK
Vimentin	Mouse	Monoclonal	WB 1:2000 IHC 1:1000	Santa cruze, USA
ZEB1	Mouse	Monoclonal	WB 1:500 IHC 1:200	Abcam, USA
SPARC	Mouse	Monoclonal	WB 1:500 IHC 1:100	Invitrogen, USA
CYR61	Rabbit	Polyclonal	WB 1:1000 IHC 1:200	Abcam, USA
Bax	Mouse	Monoclonal	WB 1:3000	Proteintech, USA
Bcl2	Mouse	Monoclonal	WB 1:1000	Thermo fisher, USA
Caspase 3	Mouse	Monoclonal	WB 1:1000	Thermo fisher, USA
Cleaved caspase 3	Rabbit	Polyclonal	WB 1:1000	Thermo fisher, USA
Caspase 3	Mouse	Monoclonal	WB 1:1000	Thermo fisher, USA
Cleaved caspase 3	Rabbit	Polyclonal	WB 1:1000	Thermo fisher, USA
Anti-Goat IgG/HRP	Donkey	Polyclonal	WB 1:2000	Santa Cruz, USA
Anti-Mouse IgG/HRP	Goat	Polyclonal	WB 1:3000	Dako, Denmark
Anti-Rabbit IgG/HRP	Goat	Polyclonal	WB 1:3000	Dako, Denmark

Supplementary Table 2. Primer sequences used for RT-PCR

Primer name	Primer sequences	
	Forward Primer	Reverse Primer
ZEB1	GATGACCTGCCAACAGACCA	CTTTCACTGCTCCTCCCTGG
E-Cadherin	GCCTCCTGAAAAGAGAGTGGAAG	TGGCAGTGTCTCTCCAAATCCG
N-Cadherin	CCTCCAGAGTTTACTGCCATGAC	GTAGGATCTCCGCCACTGATTC
Vimentin	CTCTGGCACGTCTTGACCTT	GCCATCAACCTCTTCGTGGA
GAPDH	GTCAAGGCTGAGAACGGGAA	CAGCCACGAACACGATGAAC
CYR61	CGAGGTGGAGTTGACGAGAAAC	AGGACTGGATCATCATGACGTTCT
SPARC	GTGAAGGCAACATGAGGGTGCA	GTTGGAGGACAAGTCACTGGATC