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Research article

# CYR61/CCN1 expression in resected pancreatic ductal adenocarcinoma: A retrospective pilot study of the interaction between the tumors and their surrounding microenvironment

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ABSTRACT

*Background:* CCN1 is an extracellular matrix-associated protein thought to be implicated in tumor-stromal interaction in several solid tumors. The aim of our pilot study was to evaluate the correlation between CCN1 expression in stromal cells, pancreatic intraepithelial neoplasia (PanIN) and pancreatic ductal adenocarcinoma cells in resected pancreatic ductal adenocarcinoma (PDAC) specimens, and correlate that clinically.
*Methods:* A total of 42 paraffin-embedded PDAC tumor specimens were stained for CCN1 and evaluated via immunohistochemical (IHC) analysis. Statistical analysis was performed to correlate between CCN1 expression profiles in tumor tissues and clinicopathological parameters of patients.
*Results:* Our results showed *CCN1 (CYR61)* gene was highly expressed in PDAC tissues relative to other organ specific tumor tissues. Also, moderate and overexpression of CCN1 in PanIN was associated with PanIN grade 3 tissues. A statistically significant association was found between PanIN CCN1 scores on one hand and cancer stage, cancer grade, and CCN1 expression among ductal tumor cells and adjacent stromal cells on the other hand.
*Discussion:* The associations demonstrated suggest that CCN1 might be contributing to a substantial role in the interaction between the pancreatic tumors on one hand and their surrounding microenvironment and their precursors on the other hand; hence, it might serve as a potential therapeutic target for PDAC.

## 1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains an aggressive disease with a 5-year survival rate as low as 6% that has traditionally demonstrated resistance to systemic therapies [1]. Studies from patients with PDAC have demonstrated that many patients have multifocal, microscopic pancreatic intraepithelial neoplasms (PanINs) surrounding the tumor [2, 3, 4, 5]. Those PanINs are asymptomatic non-invasive microscopic flat or papillary lesions that arise within small intralobular pancreatic ducts and are classified into three grades: *PanIN-1A* (flat) and *PanIN-1B* (papillary) representing low-grade lesions, *PanIN-2* representing intermediate-grade PanIN, and *PanIN-3* - also referred to as *"carcinoma in situ"* - demonstrating high-grade PanINs.

Resistance to therapies in PDAC has been attributed, in part, to the potential "tumor-protecting" extensive fibrous (desmoplastic) stroma that surrounds malignant cells [6]. Studies have emphasized on the pivotal role of pancreatic tumor microenvironment in expediting the initiation and progression of pancreatic cancer, through complex bidirectional signaling pathways between the stroma and tumor cells [7]. In fact, pancreatic cancer is characterized by extensive fibrosis termed desmoplasia, which is

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documented in the pathology of PDAC [8]. High levels of collagen and hyaluronan within the extracellular matrix (ECM) in primary tumors were shown to instigate metastatic disease and poor prognosis among PDAC patients [9]. Therefore, studying tumor microenvironment players is preponderant on prognosis by affecting the efficacy of different anti-cancer therapies, and targeting tumor microenvironment proteins via new therapeutic strategies [10].

The CCN family is a complex group of secreted extracellular matrix (ECM)-associated proteins containing six multifunctional members designated CCN1 to CCN6 [11,12]. Historically, the CCN acronym was generated from the names of the first three discovered molecules: CYR61 (cysteine-rich protein 61), CTGF (connective tissue growth factor) and NOV (nephroblastoma overexpressed gene) [13], first described by Bork *et al.* in 1993 [11]. At the molecular level, these proteins belong to key signaling and regulatory network involved in fundamental biological functions, from wound healing to cell proliferation, differentiation, angiogenesis and tumorigenesis [14]. Of further interest, functions of both CCN1 (CYR61) and CCN2 appear particularly induced by growth factors, including fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β), leading to enhanced angiogenesis through interactions with angiogenic integrins αvβ3 and α6β1 [15,16]. Specifically, CCN1 has been shown to control retinal angiogenesis by targeting VEGF, Src homology 2 domain phosphatase-1 and Notch signaling [17], and it has a key role in maintaining and enhancing the malignant phenotype in breast cancer [18].

CCN1, formerly referred to as CYR61, has been also implicated in many human malignancies and according to many reports, its overexpression might serve as a potential prognostic tool. For example, molecular evidence has revealed that CCN1 contributes to glioma progression and overexpression correlates with aggressive behavior [19]. Additionally, CCN1 overexpression was significantly linked to poor prognosis in muscle-invasive bladder cancer [20] and lung cancer [21]. Collectively, these data imply that CCN1 might constitute a viable diagnostic marker and/or a clinical therapeutic target. Yet, patterns of CCN1/CYR61 expression and significance in human PDAC tissue specimens and the interaction between the tumors and their surrounding microenvironment have not been established.

The aim of our pilot study was to understand the expression pattern of *CCN1 (CYR61)* gene in human PDAC tumor tissues and evaluate the correlation between CCN1 staining in stromal cells composed mainly of fibroblasts surrounding the tumor, pancreatic intraepithelial neoplasia (PanIN) and pancreatic ductal adenocarcinoma cells (PDAC) on one hand, and CCN1 staining in normal ductal cells (acinar cells) and normal stromal cells away from the tumor on the other hand, in resected PDAC specimens. Results from our study refer to the role of CCN1 as a potential diagnostic marker and/or therapeutic target in PDAC.

## 2. Materials and methods

### 2.1. Patient selection

Forty-two deceased patients who had undergone pancreaticoduodenectomy at the American University of Beirut Medical Center (AUB-MC), Beirut, Lebanon, between January 2009 and December 2015 were included in this study. All experimental protocols described herein were carried out in accordance with relevant guidelines and regulations, and in agreement with The Code of Ethics of the World Medical Association (Declaration of Helsinki). After Institutional Review Board (IRB) determination of non-human subject research, paraffin-embedded tumor specimens were retrieved and data from medical charts were reviewed. No informed consent was obtained since all patients included are deceased.

### 2.2. Patient tissue specimens

Archival paraffin blocks were retrieved from the Department of Pathology and Laboratory Medicine of the American University of Beirut Medical Center and paraffin-embedded tissue sections were prepared. These sections were stained with hematoxylin and eosin (H&E) and examined by a specialized pathologist using light microscopy to identify the area of highest histologic tumor grade (Figures 1A, B, C and D). Immunohistochemical staining (IHC) was then performed to estimate levels of CCN1 expression.

### 2.3. Immunohistochemical (IHC) staining

Unstained paraffin-embedded tissue sections were deparaffinized, and antigen retrieval was performed in a citrate buffer in a steamer at 100 °C for 40 min followed by 30 min incubation at room temperature. Slides were stained with the rabbit polyclonal anti-CCN1 (1/200 dilution) (ab127988; Abcam), according to the Novolink Polymer Kit protocol (Leica biosystems, UK). All slides were counterstained with hematoxylin.

### 2.4. IHC Evaluation and scoring

CCN1 expression was assessed by specialized pathologist in normal ductal cells (acinar cells), normal cells away from the tumor cells, stromal

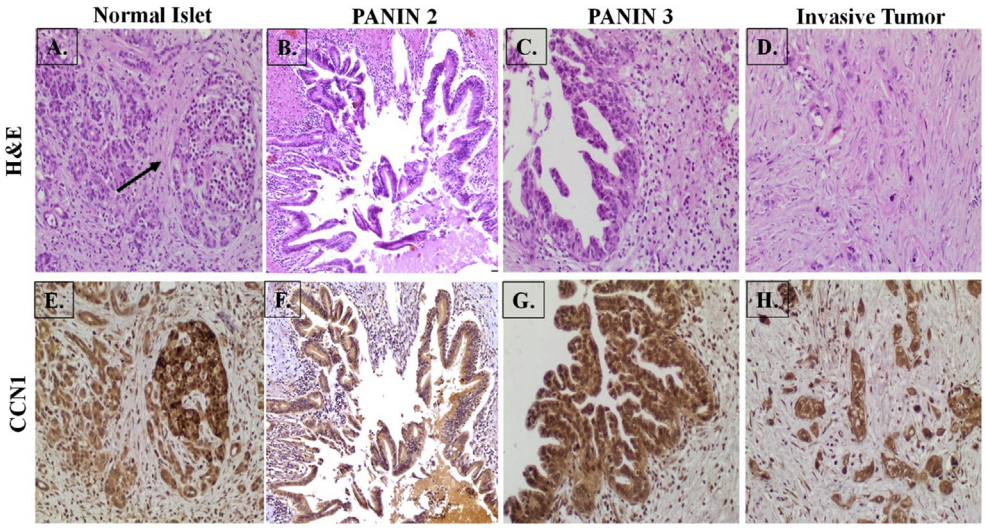


**Figure 1. Immunohistological analysis of a PDAC patient**. Serial sections of pancreatic adenocarcinoma tissues were stained with H&E (A–D) and with CCN1 antibody (E–H). Representative images of normal islet, PanIN2, PanIN3 and invasive tumor (x20) were shown. Normal stromal cells are indicated by an arrow.

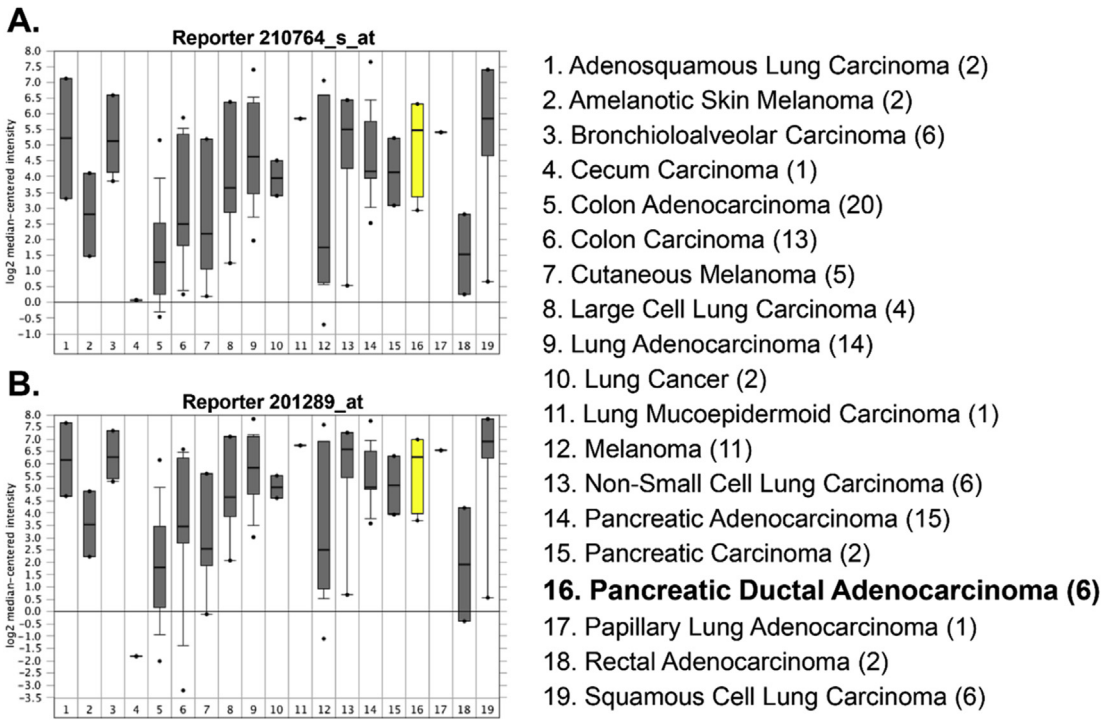


**Figure 2. Expression levels of *CCN1* (*CYR61*) mRNA were assessed in the two probes (Reporters 201289_at in (A) and 210764_s_at in (B)) of an array set comprised of human pan-tumor samples (Wagner CellLine Statistics).** Expression within tumor tissues was presented by log (base 2) median-centered expression of *CCN1* (*CYR61*). Box and whiskers plots indicate median and interquartile range. *p* values were obtained using *t*-tests (Wagner CellLine Statistics, 119 samples; data retrieved from Oncomine.org).

cells adjacent to the tumor, PanIN and ductal cancer cells. CCN1 expression was scored using a semi-quantitative system as follows: 0 (no staining), 1+ (Weak granular cytoplasmic staining), 2+ (Moderate granular cytoplasmic staining) and 3+ (Intense granular cytoplasmic staining) based on the intensity of cells stained. Islet cells were used as an internal control because of their strong CCN1 staining with intensity 3+/3 in all cases.

### 2.5. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences statistical package 21.0 software (SPSS, Inc.). Chi- Square test and Fisher's exact test were used to study the association of the dependent variable categorized into 4 groups depending on the score of the stain with the specimens clinicopathological characteristics such as PanIN type, cancer stage and grade, in addition to the association between the CCN1 score in PanIN, ductal cancer cells, and adjacent stromal cells. A Mantel–Haenszel test of trend was run to determine whether a linear association existed between PanIN grade and PanIN CCN1 score. A predictive model was built for time-to-death data using Cox regression. P-values less than or equal to 0.05 were considered significant.

## 3. Results

### 3.1. CCN1 (CYR61) mRNA expression patterns in human PDAC tissues

In the present study, we aimed at better understanding the expression pattern of *CCN1* (*CYR61*) gene in human PDAC tumor tissues compared to other body tumor tissues. We surveyed a publicly available dataset (Wagner CellLine, 119 samples; data retrieved from Oncomine.org) comprised of human tumor tissues from different organs. Interestingly, analysis revealed that *CCN1* (*CYR61*) gene was highly expressed in PDAC tissues relative to other organ specific tumor tissues in the two probes of the datasets (**Fold change = 2.969; *p* = *3.96E-5***) (Figure 2).

### 3.2. Clinicopathological characteristics of PDAC patients

A total of 42 PDAC tissues were analyzed by IHC and examined for CCN1 immunoreactivity. Summary of the clinicopathological characteristics, including cancer stage, grade and IHC staining results of the 42 patients are shown in Table 1. CCN1 expression in islet cells, normal ductal cells (acinar cells) and normal stromal cells was then analyzed and shown in Table 2.

### 3.3. CCN1 expression

CCN1 expression was seen with an intensity of none or 1+ in normal stroma cells and normal ductal cells (acinar cells) of 41/42 of patients, whereas it was identified with an intensity of 3+ in islets (control) of 41/42 of patients (Figure 1E) (Table 2). CCN1 expression was seen in the PanIN of 31/42 of patients with an intensity of 1+ in 5/42, 2+ in 19/42 (Figure 1F) and 3+ in 7/42 (Figure 1G) (Table 3A). High CCN1 expression (score 3+) was identified in ductal cancer cells in 18/42 of tumors (Figure 1H and Table 3B).

Thirty-one of the total 42 specimens were scored for PanIN CCN1 stain. Of the total specimens, 11/42 did not have PanIN. This group was

**Table 1.** Characteristics of the patients.

| | Total N | Categories | n |
|---|---|---|---|
| Age (in years) | 42 | Mean (±SD) | 62.2 (±9) |
| Cancer Stage | 41 | 1A | 3 |
| | | 1B | 4 |
| | | 2A | 10 |
| | | 2B | 24 |
| Cancer Grade | 40 | 1 | 7 |
| | | 2 | 20 |
| | | 3 | 13 |

**Table 2.** CCN1 expression in islet cells, normal ductal cells (acinar cells) and normal stromal cells.

| Clinicopathological Parameters | Total number of patients | Categories | Number of patients |
|---|---|---|---|
| CCN1 expression intensity in islets cells | 42 | Neg | 0 |
| | | + | 0 |
| | | ++ | 0 |
| | | +++ | 41 |
| | | N/A | 1 |
| CCN1 expression intensity in normal duct cells (acinar cells) | 42 | Neg | 1 |
| | | + | 40 |
| | | ++ | 0 |
| | | +++ | 0 |
| | | N/A | 1 |
| CCN1 expression in normal stromal cells | 42 | Neg | 1 |
| | | + | 40 |
| | | ++ | 0 |
| | | +++ | 0 |
| | | N/A | 1 |

**Abbreviations:** Neg: negative; N/A: not applicable.

excluded from the following associations as they lack PanIN, and consequently absent CCN1 stain. Higher PanIN grade was associated with statistically significant increase in CCN1 expression. A Mantel-Haenszel test of trend was run to determine whether a linear association exists between the PanIN grade and the PanIN CCN1 score. The Mantel-Haenszel test of trend showed a statistically significant linear association between them, $\chi 2(1) = 19.778$, $p < .0001$, r = 695. Number of patients in each PanIN grade versus the intensity of CCN1 expression in PanIN is shown in Table 3A.

A stratification of the PanIN CCN1 score has been made according to PanIN type, cancer stage, cancer grade, ductal cancer cells and stromal cancer cells as shown in Table 4. In studying the association between PanIN CCN1 score and cancer stage, 2/5 of those who had 1 + CCN1 score had a 1B cancer stage, 16/19 from the 2 + CCN1 scores had 2B cancer stage, and finally, 3/6 of those with 3+++ CCN1 score had 2A cancer stage (Table 4). There was a statistically significant association between PanIN CCN1 scores and cancer stage as assessed by Fisher's exact test ($p$-value = 0.006), where advanced cancer stage 2B was associated with higher CCN1 score in PanIN ++. Another significant association was observed between PanIN CNN1 score and cancer grade as shown in Table 4 ($p$-value = 0.031), where high cancer grade of 2 was associated with higher CCN1 score in PanIN of ++.

When investigating the relationship between CCN1 score in PanIN to that of ductal and adjacent stromal CCN1 scores, a highly significant association was observed. As shown in Table 4, all tumor specimens in the 1 + PanIN CCN1 score had a similar ductal and adjacent stromal CCN1 score (1+/2+), and all specimens of 3 + scores had a parallel 3 + score in the ductal and adjacent stromal CCN1 scores ($P$-values $\leq$ 0.0001).

A Mantel-Haenszel test of trend was run to determine whether a linear association exists between the CCN1 score of the ductal cancer and CCN1 score of the adjacent stroma. The Mantel-Haenszel test of trend showed a statistically significant linear association between them, $\chi^2(1) = 39.311$, $p < .0001$, $r = 979$. Higher ductal cancer CCN1 score is associated with a higher adjacent stromal CCN1 score vice-versa (Figure 3 and Table 3B). We also assessed the association between CCN1 expression in PanIN and that of ductal cancer (Figure 4 and Table 5A), and between CCN1 expression in PanIN and that of stromal cancer (Figure 5 and Table 5B). The Mantel-Haenszel test of trend showed no statistically significant linear association between them, $\chi^2(1) = 2.095$, $p = 0.148$, r = 0.226 (same results with the ductal and stromal as they have similar counts).

### 3.4. Survival analysis

We found no significant difference in median overall survival between cases with high CCN1 expression (+++) in ductal and stromal cancer cells compared to cases with low to moderate CCN1 staining (+ or ++) (Figure 6). In multivariable model adjusting for age, cancer stage and cancer grade, CCN1 staining was not an independent factor associated with survival in this cohort (data not shown).

## 4. Discussion

To the best of our knowledge, this is the first pilot study to investigate the relationship comparing CCN1 expression in PanIN to CCN1 in ductal tumor cells and the adjacent stroma in resected PDAC specimens. Although this pilot study is limited by a small sample size, we have demonstrated that CCN1 moderate (2+) and overexpression (3+) is higher in PanIN grade 3 in this cohort of patients. Almost two-thirds of PanIN CCN1 2+ and 3+ were found in PanIN grade 3 (Table 4). A statistically significant linear correlation was seen between CCN1 score of the ductal cancer and CCN1 score of the adjacent stroma. Also, significant association was found between CCN1 expression in PanIN on one hand, and CCN1 expression in ductal tumor cells and adjacent stromal cells on the other hand, where all tumor specimens in the 1 + PanIN CCN1 score had a similar ductal and adjacent stromal CCN1 score (1+/2+), and all specimens of 3 + scores had a parallel 3 + score in the ductal and

**Table 3.** Descriptive table showing the number of patients in each PanIN grade versus the intensity of CCN1 expression in PanIN (A) and number of patients in the different stromal cancer CCN1 scores versus that of ductal CCN1 scores (B).

| A | | PanIN Grade | | | | Total |
|---|---|---|---|---|---|---|
| | | **1** | **2** | 3 | No PanIN Observed | |
| CCN1 expression in PanIN | + | 0 | 2 | 3 | 0 | 5 |
| | ++ | 1 | 6 | 12 | 0 | 19 |
| | +++ | 0 | 2 | 5 | 0 | 7 |
| | **N/A** | 0 | 0 | 0 | 11 | 11 |
| Total | | 1 | 10 | 20 | 11 | 42 |

| B | | Stromal Cancer CCN1 Score | | Total |
|---|---|---|---|---|
| | | +/++ | +++ | |
| CCN1 expression in ductal cancer cells | +/++ | 24 | 0 | 24 |
| | +++ | 0 | 18 | 18 |
| Total | | 24 | 18 | 42 |

**Abbreviations:** PanIN: Pancreatic intraepithelial neoplasia; N/A: not applicable.

**Table 4.** Association between several clinicopathological factors and expression levels of CCN1 in PanIN.

| Clinicopathological Parameters | Categories | CCN1 Score in PanIN | | | | *P*-value |
|---|---|---|---|---|---|---|
| | | + | ++ | +++ | **Total** | |
| | | **N** | **N** | **N** | **N** | |
| PanIN Type | **1** | 0 | 1 | 0 | 1 | 1 |
| | **2** | 2 | 6 | 2 | 10 | |
| | **3** | 3 | 12 | 5 | 20 | |
| | **Total** | 5 | 19 | 7 | 31 | |
| Cancer Stage | **1A** | 1 | 1 | 0 | 2 | 0.006* |
| | **1B** | 2 | 0 | 1 | 3 | |
| | **2A** | 1 | 2 | 3 | 6 | |
| | **2B** | 1 | 16 | 2 | 19 | |
| | **Total** | 5 | 19 | 6 | 30 | |
| Cancer Grade | **1** | 1 | 1 | 3 | 5 | 0.031* |
| | **2** | 4 | 10 | 1 | 15 | |
| | **3** | 0 | 8 | 2 | 10 | |
| | **Total** | 5 | 19 | 6 | 30 | |
| CCN1 expression in ductal cancer cells | +/++ | 5 | 12 | 0 | 17 | <0.0001* |
| | +++ | 0 | 7 | 7 | 14 | |
| CCN1 expression in stromal cancer cells | +/++ | 5 | 12 | 0 | 17 | <0.0001* |
| | +++ | 0 | 7 | 7 | 14 | |

**Abbreviations:** PanIN: Pancreatic intraepithelial neoplasia.
Significant *P*-values are referred to with an **asterisk (*).**

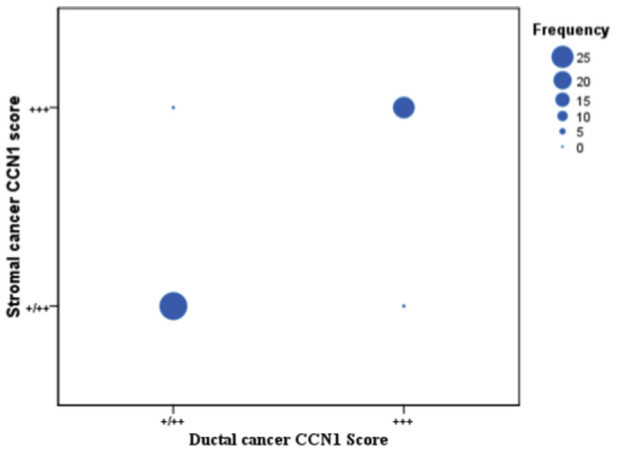


**Figure 3.** Scatterplot showing the linear trend between stromal cancer CCN1 score and ductal cancer CCN1 score.

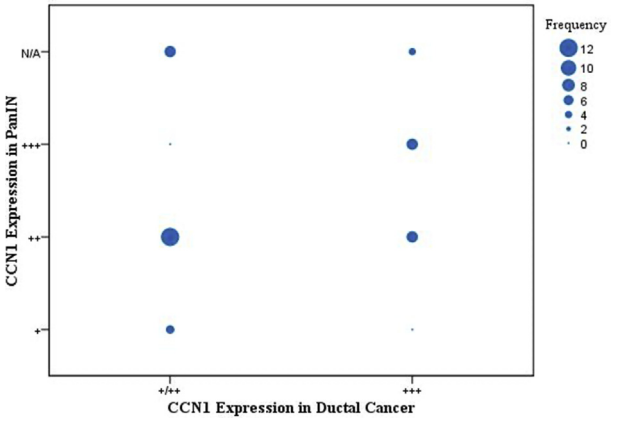


**Figure 4.** Scatterplot showing the relation between CCN1 expression in PanIN and that of ductal cancer.

adjacent stromal CCN1 scores (*P*-values $\leq$ 0.0001). Yet, in this small cohort, CCN1 expression in ductal and stromal tumor cells was not found to be associated with median overall survival of patients.

Review of literature on the role of CCN1 in different other cancers, it was also found to be expressed at elevated levels in advanced breast cancers where it is thought to promote invasiveness [18, 22]. In laryngeal squamous cell cancer, overexpression of CCN1 was shown to induce epithelial-mesenchymal transition (EMT), therefore leading to invasion, metastasis and poor prognosis [23]. Furthermore, CCN members (including CCN1) were highly dysregulated in colorectal cancer, and appear to be involved in initiation, development, and progression of the disease [22]. CCN1 silencing was also found to potentially blunt tumor vasculature and slow growth of osteosarcoma cells [24], thereby limiting the number of corresponding lung metastases.

In a study by Sano *et al.*, authors employed gene expression profiling to identify target molecules of the WNT/β-catenin signaling pathway in pancreatic cancer cells *in vitro* [25]. Results from this study revealed that WNT/β-catenin signaling pathway enhances pancreatic cancer development and malignancy via up-regulation of CYR61. Another study by Haque *et al.* demonstrated the involvement of Cyr61/CCN1 in pancreatic carcinogenesis via promoting EMT and stemness [26]. Besides, the Sonic Hedgehog (SHh) and Notch 1 pathways have been shown to serve as critical regulators in pancreatic carcinogenesis through CCN1-dependent cell migration and tumorigenicity [27], and through promoting endothelial cell migration and aberrant neovascularization [28].

The associations we have demonstrated between CCN1 expression in PanIN and tumor-adjacent stroma suggest that CCN1 might play an important role in the interaction between the pancreatic tumors and their surrounding stromal microenvironment in additional to their microscopic precursors, i.e. the PanINs. Studies have shown that components of the PDAC microenvironment, including the ECM, matrix metalloproteinase (MMP), growth factors, and TGFβ, contribute to desmoplasia and are associated with poor patient prognosis [8]. Reports from basic research studies are consistent with this where Haque *et al.* [26] showed that CCN1 mRNA and protein expression was elevated in pancreatic cell lines and that silencing CCN1 blocked cell migration, EMT and tumor formation in mice. CCN1 was also found to activate the sonic hedgehog (SHh) and the notch pathways through autocrine-paracrine

**Table 5.** Descriptive table showing the number of patients with different intensities of CCN1 expression in PanIN versus that in ductal cancer cells **(A)** and that of stromal cancer cells **(B)**.

| | | CCN1 Expression in PanIN | | | | Total |
|---|---|---|---|---|---|---|
| | | + | ++ | +++ | N/A | |
| A | | | | | | |
| CCN1 expression in ductal cancer cells | +/++ | 5 | 12 | 0 | 7 | 24 |
| | +++ | 0 | 7 | 7 | 4 | 18 |
| **Total** | | 5 | 19 | 7 | 11 | 42 |
| B | | | | | | |
| CCN1 expression in stromal cancer cells | +/++ | 5 | 12 | 0 | 7 | 24 |
| | +++ | 0 | 7 | 7 | 4 | 18 |
| **Total** | | 5 | 19 | 7 | 11 | 42 |

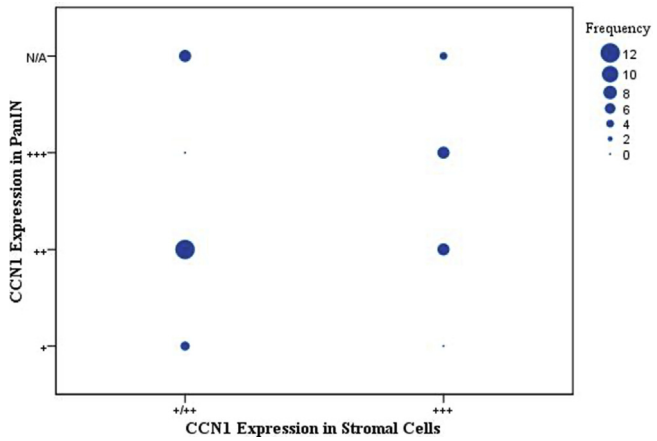


**Figure 5.** Scatterplot showing the relation between CCN1 expression in PanIN and that of stromal cancer.

circuits to promote endothelial cell migration and tumor angiogenesis [27]. SHh is known to affect tumor growth by promoting desmoplasia, a network of fibrotic tissue facilitating tumor invasion [28, 29]. Additional studies on cell lines have suggested that CCN1 overexpression promotes resistance to gemcitabine chemotherapy through the regulation of cancer cell-secreted connective tissue growth factor (CTGF), a regulator of desmoplasia, and deoxycytidine kinase (dCK), an enzyme that enhances gemcitabine sensitivity and efficacy in cancer cells [30, 31].

Further mechanistic studies are on-going to elucidate the role of CCN1 in the development and progression of pancreatic adenocarcinoma with small-molecule inhibitors of the hedgehog pathway, a potential therapeutic strategy in clinical development [32].

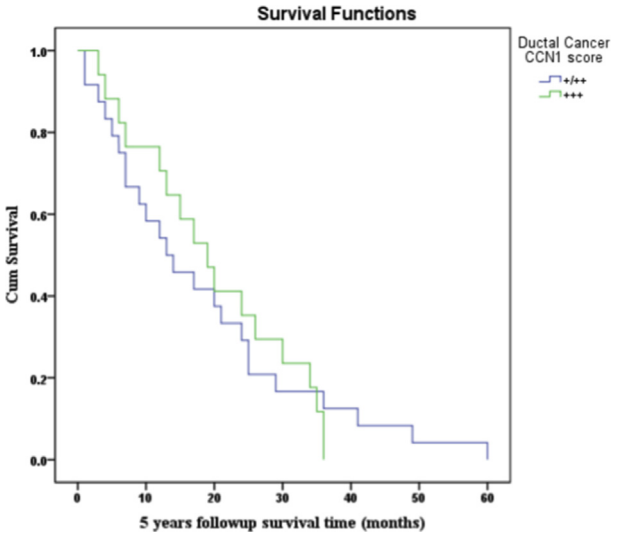


**Figure 6.** Kaplan-Meier survival curves for patients with CCN1 staining in ductal cancer cells (+++) compared to low to moderate staining (+/++).

## 5. Limitations

We believe that our pilot study has some limitations. First, although this is the first translational research paper to evaluate the expression patterns of *CCN1* (*CYR61*) in human PDAC tumor tissues and surrounding microenvironment, the sample size is relatively small and we acknowledge that we have some missing data in the medical records of patients, which might have contributed to the absence of significant prognostic correlation between CCN1 expression and patients' survival. Henceforth, the results obtained require conducting subsequent studies on a larger cohort and avoiding missing data. In accordance, we believe that more data and follow up is required to assess the correlation of CCN1 expression with clinical outcomes. Second, our study focuses on deciphering the correlation between CCN1 expression in tumor cells and their microenvironment, rather than on the prognostic value of CCN1. In this milieu, it has been postulated that the pancreatic tumor microenvironment plays a pivotal role in expediting the initiation and progression of pancreatic cancer. Therefore, our aim was to assess the patterns of CCN1/CYR61 expression and significance in human PDAC tissue specimens and the interaction between the tumors and their surrounding microenvironment. Results from our study reveal that CCN1 might constitute a viable diagnostic marker and/or a clinical therapeutic target for PDAC. Third, samples were collected retrospectively over the period of 6 years between January 2009 and December 2015, which attests to the restricted sample size obtained. Fourth, our small sample size and retrospective collection of data with some missing information might explain the possible lack of significant association between increased ductal tumor CCN1 expression and poor outcome. Lastly, we acknowledge that islets of Langerhan's immunoreaction might exhibit false-positive staining.

## 6. Conclusions

In a sample of resected adenocarcinoma specimens, we have demonstrated that CCN1 moderate and overexpression in PanIN is higher in PanIN grade 3. Significant association was seen between PanIN CCN1 expression, ductal tumor cell CCN1 expression and CCN1 expression in adjacent stromal cells. These results reinforce the potential role of CCN1 as a diagnostic marker and/or therapeutic target in PDAC. Although increased ductal tumor CCN1 expression was not associated with poor outcome in our small cohort, studies in our lab targeting the CCN1-mediated tumor-stromal interaction in pancreatic cancer are still

ongoing. Further studies could be also employed to correlate between CCN1 expression and other important molecular factors in pancreatic tumor like Wnt signaling or Ki67.

## Declarations

### *Author contribution statement*

W. Abou-Kheir, D. Mukherji, I. Khalifeh and W. Faraj: Conceived and designed the experiments; Wrote the paper.

O. Hadadeh, E. Saleh, and H. Bahmad: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Kanso, M.Khalifeh, A. Shamseddine, S. Tamraz, C. Dagher, and R. Jaafar: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### *Competing interest statement*

The authors declare no conflict of interest.

### *Additional information*

No additional information is available for this paper.

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## References

[1] S. Gillen, T. Schuster, C. Meyer Zum Buschenfelde, H. Friess, J. Kleeff, Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages, PLoS Med. 7 (2010), e1000267.

[2] A.L. Cubilla, P.J. Fitzgerald, Morphological lesions associated with human primary invasive nonendocrine pancreas cancer, Cancer Res. 36 (1976) 2690–2698.

[3] C. Shi, A.P. Klein, M. Goggins, A. Maitra, M. Canto, S. Ali, R. Schulick, E. Palmisano, R.H. Hruban, Increased prevalence of precursor lesions in familial pancreatic cancer patients, Clin. Cancer. Res. Offic J. Am. Assoc. Cancer Res. 15 (2009) 7737–7743.

[4] A. Andea, F. Sarkar, V.N. Adsay, Clinicopathological correlates of pancreatic intraepithelial neoplasia: a comparative analysis of 82 cases with and 152 cases without pancreatic ductal adenocarcinoma, Mod. Pathol. Offic. J. United States Can. Acad. Pathol. Inc 16 (2003) 996–1006.

[5] K. Brune, T. Abe, M. Canto, L. O'Malley, A.P. Klein, A. Maitra, N. Volkan Adsay, E.K. Fishman, J.L. Cameron, C.J. Yeo, S.E. Kern, M. Goggins, R.H. Hruban, Multifocal neoplastic precursor lesions associated with lobular atrophy of the pancreas in patients having a strong family history of pancreatic cancer, Am. J. Surg. Pathol. 30 (2006) 1067–1076.

[6] C. Feig, A. Gopinathan, A. Neesse, D.S. Chan, N. Cook, D.A. Tuveson, The pancreas cancer microenvironment, Clin. Cancer. Res. – Offic. J. Am. Assoc. Cancer Res. 18 (2012) 4266–4276.

[7] M. Jewer, S.D. Findlay, L.M. Postovit, Post-transcriptional regulation in cancer progression : microenvironmental control of alternative splicing and translation, J. Cell Commun. Signal 6 (2012) 233–248.

[8] B. Ren, M. Cui, G. Yang, H. Wang, M. Feng, L. You, Y. Zhao, Tumor microenvironment participates in metastasis of pancreatic cancer, Mol. Cancer 17 (2018), 108-108.

[9] C.J. Whatcott, C.H. Diep, P. Jiang, A. Watanabe, J. LoBello, C. Sima, G. Hostetter, H.M. Shepard, D.D. Von Hoff, H. Han, Desmoplasia in primary tumors and metastatic lesions of pancreatic cancer, Clin. Cancer. Res. – Offic. J. Am. Assoc. Cancer Res. 21 (2015) 3561–3568.

[10] C. Roma-Rodrigues, R. Mendes, P.V. Baptista, A.R. Fernandes, Targeting tumor microenvironment for cancer therapy, Int. J. Mol. Sci. 20 (2019) 840.

[11] P. Bork, The modular architecture of a new family of growth regulators related to connective tissue growth factor, FEBS Lett. 327 (1993) 125–130.

[12] N. Chen, C.C. Chen, L.F. Lau, Adhesion of human skin fibroblasts to Cyr61 is mediated through integrin alpha 6beta 1 and cell surface heparan sulfate proteoglycans, J. Biol. Chem. 275 (2000) 24953–24961.

[13] D.R. Brigstock, R. Goldschmeding, K.I. Katsube, S.C. Lam, L.F. Lau, K. Lyons, C. Naus, B. Perbal, B. Riser, M. Takigawa, H. Yeger, Proposal for a unified CCN nomenclature, Mol. Pathol. 56 (2003) 127–128.

[14] B. Perbal, CCN proteins: multifunctional signalling regulators, Lancet 363 (2004) 62–64.

[15] G.R. Grotendorst, Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts, Cytokine Growth Factor Rev. 8 (1997) 171–179.

[16] Y. Mori, M. Hinchcliff, M. Wu, M. Warner-Blankenship, K.M. Lyons, J. Varga, Connective tissue growth factor/CCN2-null mouse embryonic fibroblasts retain intact transforming growth factor-beta responsiveness, Exp. Cell Res. 314 (2008) 1094–1104.

[17] H. Chintala, I. Krupska, L. Yan, L. Lau, M. Grant, B. Chaqour, The matricellular protein CCN1 controls retinal angiogenesis by targeting VEGF, Src homology 2 domain phosphatase-1 and Notch signaling, Development (Cambridge, England) 142 (2015) 2364–2374.

[18] L. Ruth, D. Clara, D. Manjari, E. Ingrid, CCN1: a new target for chemotherapeutical drugs in breast cancer treatment, in: A. Perbal, M. Takigawa, B. Perbal (Eds.), CCN Proteins in Health and Disease, Springer, NY, USA, 2010, p. 314.

[19] C.R. Goodwin, B. Lal, X. Zhou, S. Ho, S. Xia, A. Taeger, J. Murray, J. Laterra, Cyr61 mediates hepatocyte growth factor-dependent tumor cell growth, migration, and Akt activation, Cancer Res. 70 (2010) 2932–2941.

[20] M. Brown, R. Robinson, C. Hart, CYR61/CCN1 Mediated HGF Linked Metastatic Behavior and Expression Is Associated with a Poor Prognosis in Muscle Invasive Bladder Cancer NCRI 2014 Poster.

[21] P.P. Chen, W.J. Li, Y. Wang, S. Zhao, D.Y. Li, L.Y. Feng, X.L. Shi, H.P. Koeffler, X.J. Tong, D. Xie, Expression of Cyr61, CTGF, and WISP-1 correlates with clinical features of lung cancer, PloS One 2 (2007) e534.

[22] C.C. Chang, B.R. Lin, T.S. Wu, Y.M. Jeng, M.L. Kuo, Input of microenvironmental regulation on colorectal cancer: role of the CCN family, World J. Gastroenterol. 20 (2014) 6826–6831.

[23] Y. Liu, Y.D. Zhou, Y.L. Xiao, M.H. Li, Y. Wang, X. Kan, Q.Y. Li, J.G. Lu, D.J. Jin, Cyr61/CCN1 overexpression induces epithelial-mesenchymal transition leading to laryngeal tumor invasion and metastasis and poor prognosis, Asian Pac. J. Cancer Prev. 16 (2015) 2659–2664.

[24] N. Habel, M. Vilalta, O. Bawa, P. Opolon, J. Blanco, O. Fromigue, Cyr61 silencing reduces vascularization and dissemination of osteosarcoma tumors, Oncogene 34 (2015) 3207–3213.

[25] M. Sano, D.R. Driscoll, W.E. DeJesus-Monge, B. Quattrochi, V.A. Appleman, J. Ou, L.J. Zhu, N. Yoshida, S. Yamazaki, T. Takayama, M. Sugitani, N. Nemoto, D.S. Klimstra, B.C. Lewis, Activation of WNT/beta-Catenin signaling enhances pancreatic cancer development and the malignant potential via up-regulation of Cyr61, Neoplasia (New York, N.Y.) 18 (2016) 785–794.

[26] I. Haque, S. Mehta, M. Majumder, K. Dhar, A. De, D. McGregor, P.J. Van Veldhuizen, S.K. Banerjee, S. Banerjee, Cyr61/CCN1 signaling is critical for epithelial-mesenchymal transition and stemness and promotes pancreatic carcinogenesis, Mol. Cancer 10 (2011) 8.

[27] I. Haque, A. De, M. Majumder, S. Mehta, D. McGregor, S.K. Banerjee, P. Van Veldhuizen, S. Banerjee, The matricellular protein CCN1/Cyr61 is a critical regulator of Sonic Hedgehog in pancreatic carcinogenesis, J. Biol. Chem. 287 (2012) 38569–38579.

[28] G. Maity, S. Mehta, I. Haque, K. Dhar, S. Sarkar, S.K. Banerjee, S. Banerjee, Pancreatic tumor cell secreted CCN1/Cyr61 promotes endothelial cell migration and aberrant neovascularization, Sci. Rep. 4 (2014) 4995.

[29] J.M. Bailey, B.J. Swanson, T. Hamada, J.P. Eggers, P.K. Singh, T. Caffery, M.M. Ouellette, M.A. Hollingsworth, Sonic hedgehog promotes desmoplasia in pancreatic cancer, Clin. Cancer. Res. – Offic. J. Am. Assoc. Cancer Res. 14 (2008) 5995–6004.

[30] V.G. Gupta, G. Maity, I. Haque, S.K. Banerjee, S. Banerjee, CCN1/Cyr61regulation of gemcitabine-resistant phenotype in pancreatic cancer: involvement of CTGF and dCK, in: AACR annual meeting 2017, Washington, DC, 2017.

[31] G. Maity, A. Ghosh, V. Gupta, I. Haque, S. Sarkar, A. Das, K. Dhar, S. Bhavanasi, S.S. Gunewardena, D.D. Von Hoff, S. Mallik, S. Kambhampati, S.K. Banerjee, S. Banerjee, CYR61/CCN1 regulates dCK and CTGF and causes gemcitabine-resistant phenotype in pancreatic ductal adenocarcinoma, Mol. Cancer Therapeut. 18 (2019) 788–800.

[32] V. Kumar, A.K. Chaudhary, Y. Dong, H.A. Zhong, G. Mondal, F. Lin, V. Kumar, R.I. Mahato, Design, synthesis and biological evaluation of novel hedgehog inhibitors for treating pancreatic cancer, Sci. Rep. 7 (2017) 1665.