Süleyman Demirel Üniversitesi Fen Edebiyat Fakültesi Fen Dergisi https://dergipark.org.tr/sdufeffd
Süleyman Demirel University Faculty of Arts and Sciences Journal of Science e-ISSN: 1306-7575
Research Article, 2024, 19(2): 117-126, DOI: 10.29233/sdufeffd.1395816

# Effectiveness of a Novel CTGF LNA GapmeR Sequence in Gastric Cancer Cells

Deniz Sezlev Bilecen[1]

[1]Department of Molecular Biology and Genetics, Faculty of Agriculture and Natural Sciences, Konya Food and Agriculture University, 42080, Konya, TURKEY
https://orcid.org/0000-0002-5708-2276

(Received: 25.11.2023, Accepted: 04.07.2024, Published: 25.11.2024)

***Abstract:*** Gastric cancer is the third most common cause of malignancy worldwide and the prognosis is poor due to drug resistance and molecular diversity of the disease. Therefore, development of novel therapies is required. Connective Tissue Growth Factor (CTGF) is involved in the extracellular matrix production, cell proliferation and migration which makes it a target for the treatment of disease. Nucleic acid-based therapies are used to reduce the expression of specific mRNA sequences. The purpose of this study was to reduce the migration, and proliferation of gastric cancer cells through the inhibition of CTGF expression. On this purpose, a novel locked nucleic acid GapmeR sequence was identified as an inhibitor of CTGF expression, and the effectiveness of the sequence was shown in the gastric cancer cells. The gastric adenocarcinoma cells were transfected with GapmeR and changes in gene expressions of CTGF and collagen type I (COL1A1) were studied by qRT-PCR. The CTGF protein levels and proliferation were studied by Western Blot analysis and Alamar Blue Assay. The sequence caused significant reductions in CTGF and COL1A1 mRNA levels and proliferation of cells. These results might lead to the development of delivery system towards gastric cancer cells by using this sequence.

***Key words:*** Gastric Cancer, LNA GapmeR, CTGF

## 1. Introduction

Gastric cancer is the fifth most common diagnosed cancer and third most common malignancy globally. The primary cause of gastric cancer development is chronic *Helicobacter pylori* infection [1,2]. Depending on the metastatic property of the developed cancer, different treatment approaches such as chemotherapy, chemoradiation, fluoropyrimidines, platinums, taxanes, irinotecan or immunotherapy are being used [3].

However, acquired drug resistance and the molecular and phenotypic diversity within tumors [4] cause the verification of prognosis among patients [5]. Therefore, identification of new targets, therapeutic agents and effective therapies are crucial towards developing alternative treatment strategies to improve the survival rate of gastric cancer patients.

Connective tissue growth factor (CTGF) which is also known as cellular communication network factor 2 (CCN2), is a member of CCN family secretory proteins including cysteine-rich protein 61 (CCN1), nephroblastoma-overexpressed gene (CCN3), Wisp-1/elm1 (CCN4), Wisp-2/rCop1 (CCN5) and Wisp-3 (CCN6) [6,7].

Under normal physiological conditions, CTGF is involved in embryonic development, wound healing and tissue repair [8]. However, cancer research has revealed that, abnormal expression of CTGF leads to development and progression of tumors in various

tissues such as lung, prostate, and breast; through the induction of cell proliferation, migration, invasion and metastasis [9]. CTGF also supports fibrotic and inflammatory tumor microenvironment which induces malignancy and metastasis [10]. In the literature it is stated that the overexpression of CTGF in gastric cancer patients, led to a higher incidence of lymph node metastasis and lower cumulative 5-year survival rate [11].

ECM proteins in tissue microenvironment play critical roles in cancer progression and invasion. The ability of cancer cells to leave the original tissue, invade and locate into the metastatic niches is known to be related to the extracellular matrix (ECM) composition and remodeling rate of the tumor microenvironment [12]. ECM remodeling at the tumor microenvironment takes place with the combined actions of increased levels of ECM remodeling enzymes (namely matrix metalloproteinases, MMPs), their tissue inhibitors and elevated collagen deposition. MMP proteins are known to be important mediators of ECM degradation and thus they contribute to the disruption of basement membrane and particularly the increased expression of MMP-9 in gastric carcinoma tissues is associated with the depth of cancer invasion [13]. In addition to MMPs, the expression of other ECM components such as collagen are elevated in gastric cancer tissues [14,15]. Abnormal cell-cell adhesion, increased collagen expression leading to ECM stiffness, increased matrix metalloproteinase (MMP) activity inducing ECM disruption and remodeling result in cancer cell invasion [16].

Studies performed by using gastric adenocarcinoma cells revealed that MMP-9 and collagen gene expressions are elevated in gastric cells and are related to CTGF expression [17]. The overexpression of COL1A1 in gastric cells is known to promote invasion and migration of the cells in addition to their increased proliferation rate. Moreover, miRNA associated suppression of these genes, inhibited their proliferation, invasion, and migration [17,18]. It is also demonstrated that, the inhibition of MMP-9 expression resulted in reduced migration, invasion and growth of gastric cancer cells [19]. Therefore, development of therapeutic approaches addressing the reduction of increased ECM proteins such as MMP-9 and COL1A1 might be promising for reducing migration, invasion and proliferation of gastric cancer cells.

In the literature it is stated that the knockdown of high CTGF expression in gastric cancer cells reduces MMP-9 expression in both mRNA and protein levels. Also, the restoring of CTGF levels through conditioned media reversed that reduction suggesting that MMP-9 expression is correlated with CTGF levels in the cells [20].

It is also known that collagen, being the main component in the ECM of tumors, is a substrate of MMPs and is also recognized as tumor-related gene [21]. Moreover, CTGF acts as a downstream mediator of TGF-β and increases the collagen type I deposition in tumor microenvironment [22]. The knockdown of COL1A1 was shown to be associated with low cell migration, invasion and proliferation of gastric cancer cells. Although not performed with gastric cancer cells, it is worth mentioning that the CTGF knockdown caused reduced COL1A1 expression in hepatocarcinoma spheroids [22]. In particular, CTGF overexpression in gastric cancer cells leads to enhanced cell migration and thus metastasis by inducing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway. In addition, the elevated collagen expression is known to be related with tumor enlargement in gastric cancer [23]. Taking all this information together, CTGF may be considered as a potential target for the treatment of gastric cancer. The purpose of the study is to reduce CTGF levels in gastric cancer cells so that the reduction might potentially lead to suppression of collagen and MMP expressions, thus ultimately reduce cell migration, invasion, and tumor enlargement.

The use of nucleic acids as therapeutics is an emerging field. Antisense oligonucleotide (AON) based therapies have been studied in several cancer states. There are also several clinical trials with these therapeutics [24]. The therapeutic action of AON relies on the ability of specific nucleic acid sequences to modulate the expression of target mRNA. AONs are single-stranded 16 to 21-mer long sequences which function either by degrading the mRNA (through RNase H activity), by modifying the splicing of mRNA or by sterically blocking the translation of mRNA. The use of AON as it is, has some limitations such as poor cellular penetration, easy degradation by nucleases and possible toxicity due to degradation products of AONs which might possess antiproliferative effects [24,25]. Therefore, numerous modifications have been implemented to overcome these limitations. These modifications have led to the emergence of three different types of AONs known as the first, second and third generation AONs.

The "Antisense locked nucleic acid (LNA) GapmeR" used in this study, falls under the category of third generation AONs. It is a 16 nucleotide long single-stranded antisense oligonucleotide that induces RNase H activity. The flanking regions (both 5' and 3') are modified with LNA and the central region is composed of LNA-free DNA nucleotide. The central region is responsible for inducing the RNase H activity and the LNA flanking regions increase the targeting efficiency. It has a fully phosphorothioate (PS) modified backbone to increase the resistance to enzymatic degradation.

In this study, the custom designed and synthesized Antisense LNA GapmeR was investigated in terms of its effectiveness in suppressing CTGF expression in gastric cancer cells. The possible outcome of CTGF inhibition was also studied by analyzing COL1A1 and MMP-9 mRNA expressions along with the changes in the proliferation of gastric cancer cells.

## 2. Material and Method

### *2.1. Cell culture conditions*

Human gastric adenocarcinoma cell line, AGS (American Type Culture Collection, ATCC CRL-1739) was a kind gift from the laboratory of Prof. Nazlı Arda (Istanbul University, Turkey). This commercially available cell line which exhibits epithelial-like morphology was isolated in 1979 from the stomach tissue of a 54-year-old, Caucasian, female patient with gastric adenocarcinoma with no prior anti-cancer treatment. The cells were cultured under normal conditions (37 °C, 5% $CO_2$) in RPMI medium, supplemented with 10% FBS, penicillin/streptomycin (1%) and 2 mM L-glutamine.

### *2.2. GapmeR sequences and transfection studies*

The CTGF mRNA specific GapmeR sequence was custom designed by Qiagen with the product number LG00789631. The scrambled negative control was also purchased from Qiagen (LG00000002). For the transfection studies, a commercially available transfection reagent, HiPerFect Transfection Reagent (HP) (Qiagen, Germany) was used with a fast forward transfection technique, as follows.

For gene expression studies, HP (3 µL) was diluted in OptiMem™ Reduced Serum Medium (100 µL) and then mixed with AON sequences (10 µM, 1.5 µL). The mixture was incubated for 10 min for complete complexation of AON sequences with HP. Meanwhile, AGS cells ($1.2x10^5$ cells) were seeded in 12 well plates. At the end of incubation, the HP-AON complexes were added onto the cell suspension within the 12 well plates and the medium was completed to 500 µL with a 1:1 (v/v) OptiMem™: RPMI

full medium. Gene silencing was monitored at 48 h, post transfection. Cells that are not treated with any type of AON were used as a control group for the experiment.

For protein expression analysis studies, HP (12 µL) was diluted in OptiMem$^{TM}$ medium, mixed with AON (10 µM, 6 µL) and incubated for 10 min. The HP-AONs complexes were added dropwise onto AGS cell suspension ($6x10^5$ cells) in 6-well plates. The final volume was completed to 2.5 mL with 1:1 (v/v) OptiMem$^{TM}$ and RPMI full mediums. The cells that were not treated with AON sequences were used as a control group for the experiment.

### *2.3. Gene expression studies*

In order to determine the efficiency of AON sequence to knockdown CTGF gene expression and the indirect effects of CTGF downregulation on the AGS cells, quantitative real time PCR (qRT-PCR) was performed (CFX96 Touch Real Time PCR System, BioRad). The primer pairs used for the study are given in Table 1.

**Table 1.** qRT-PCR primer pairs and the amplicon sizes

| | **Primers (5' - 3')** | **Sequence** | **Amplicon Size (bp)** |
|---|---|---|---|
| **CTGF** | Forward | GAAGAGAACATTAAGAAGGGCAAA | 97 |
| | Reverse | ATGTCTTCATGCTGGTGCAG | |
| **Collagen Type I** | Forward | GTTGTGCGATGACGTGATCTGTGA | 110 |
| | Reverse | TTCTTGGTCGGTGGGTGACTCTG | |
| **MMP 9** | Forward | CGTCTTCCAGTACCGAGAGA | 124 |
| | Reverse | GCAGGATGTCATAGGTCACG | |
| **GAPDH** | Forward | TGCACCACCAACTGCTTAGC | 87 |
| | Reverse | GGCATGGACTGTGGTCATGAG | |

On day 2, the RNA was isolated (NucleoSpin RNA isolation kit, Macherey Nagel) and converted to cDNA (OneScript Plus cDNA Synthesis Kit, ABM) according to manufacturer's protocols. For qRT-PCR analysis BlasTaq™ 2X qPCR MasterMix (ABM, Canada) was used with primers given (6 pmol). The qRT-PCR cycling conditions for all the primers were as follows: 95°C, 10 min initial denaturation, followed by denaturation at 95°C 15 s and annealing-extension 65°C 60 s for 35 cycles.

The reaction was terminated with melt curve analysis (65°C-95°C; 1°C/1 cycle). The fold changes of CTGF, COL1A1 and MMP9 was calculated by normalization to GAPDH as an internal control using the $2^{-\Delta\Delta Ct}$ relative expression equation:

$$\Delta C_t \text{ (treated)} = C_t \text{ (target gene)} - C_t \text{ (ref. gene)} \quad (1)$$

$$\Delta C_t \text{ (control)} = C_t \text{ (target gene)} - C_t \text{ (ref. gene)} \quad (2)$$

$$\Delta\Delta C_t = \Delta C_t \text{ (treated)} - \Delta C_t \text{ (control)} \quad (3)$$

$$\text{Target normalized to reference gene and relative to control (Fold Change)} = 2^{-\Delta\Delta Ct} \quad (4)$$

### *2.4. Western blotting*

72 h post-transfection, the cells were washed with cold PBS, scraped in RIPA buffer supplemented with PMSF (1 mM) and incubated for 30 min at +4°C with constant shaking. The resultant suspension was centrifuged 10000 rpm for 10 min at +4°C and the protein concentration was measured from the supernatant by BCA method. For western

blot analysis, equal amounts of protein sample were separated in SDS-PAGE (4% stacking-12% separating gel). The samples were run at 75V for 90 min and transferred to nitrocellulose membranes using Trans-Blot Turbo Transfer System (Bio-Rad, USA). The membranes were blocked with 5% skim milk (1h at RT) and incubated at +4°C overnight with CTGF primary antibody (Proteintech, 25474-1AP; 1/1000), washed with TBS-T. The membranes were then incubated with HRP-conjugated anti-rabbit secondary antibody (1/2000 at RT) and finally visualized with ECL reagent (SignalFire$^{TM}$, CST) using gel documentation system (GelDoc EZ System, Bio-Rad). The intensity of the protein bands was analyzed using Image Lab 6.1 Software.

### *2.5. Effect of AON on the proliferation of AGS cells*

AGS cells ($10^4$ cells/well) were seeded on 24 well plates. The cells were transfected with scrambled and CTGF AON sequences by using HP as described in section 2.2. The cells were incubated for 48 h and effect of AON sequences on the cell proliferation was observed by Alamar Blue Cell Viability Assay. For analysis, the cells were washed twice with PBS, colorless DMEM containing 10% Alamar Blue Solution was added to each well and incubated (1.5 hours at 37°C under 5% $CO_2$). The OD values (570 nm and 595 nm) of the individual wells were used to calculate percent reduction. Data is represented as percent viability calculated with respect to percent reduction of the positive control.

### *2.6. Statistical analysis*

All qualitative data presented were expressed as mean ± standard deviations. Statistical analyzes were carried out using GraphPad Prism (GraphPad Software 8.0.2). Statistical differences between the groups were determined using one-way ANOVA with Tukey post-hoc analysis. Statistical significance was expressed as $p$ <0.05 *.

## 3. Results and Discussion

### *3.1 Gene expression studies*

In this study, a novel GapmeR sequence was studied for its efficiency in inhibiting CTGF mRNA in gastric cancer cells. For this intent, the GapmeR sequence targeted specifically to *CTGF* mRNA was administered to AGS cells with a transfection reagent (HiPerFect Transfection Reagent, Qiagen). As negative control a scrambled GapmeR sequence was used to eliminate the nonspecific inhibitory effect of the GapmeR presence on gene expression. The fold changes of both scrambled and CTGF targeted GapmeR sequences were calculated with respect to TCPS (tissue culture polystyrene) control group, where none of the GapmeR sequences or HiPerFect Reagent were applied. The changes in the gene expressions of the groups were studied with qRT-PCR technique and analyzed by $2^{-\Delta\Delta Ct}$ relative quantification method (Figure 1). The primer pairs used for the study are provided in Table 1.

48 h post transfection, statistically significant reduction was observed in CTGF mRNA levels. The fold change of CTGF mRNA was 0.36 ± 0.05 leading to 64% inhibition. In addition, the scrambled GapmeR sequence did not lead to any reduction in the fold change of CTGF mRNA. This result shows that the inhibition of CTGF mRNA in CTGF GapmeR administered cells was not a nonspecific reduction. The suppression in CTGF levels is due to the GapmeR sequence used specifically against CTGF [26].

After confirming the reduction in CTGF mRNA levels in gastric cancer cells, the ultimate effect of CTGF inhibition on the expression of ECM components, such as COL1A1 and MMP9 gene expression levels were also studied by qRT-PCR technique. COL1A1

mRNA expression was also found to be significantly reduced (80%) by the treatment with the CTGF GapmeR (AON) sequence. The fold change of Col1A1 mRNA was found to be 0.20 ± 0.06. This result is very important for this study, because COL1A1 is closely related to tumor regulation, functioning as an oncogene [27,28]. Many studies confirmed that, in different kinds of cancers, COL1A1 is associated with the metastatic ability, invasion and proliferation of cancer cells and the same results were observed with gastric cancer cells. For instance, the downregulation of COL1A1 through miRNA resulted in reduction in cell invasion and migration capability in gastric cancer cells. In addition, in the same study, siRNA mediated knockdown of COL1A1 caused reduced cell viability and proliferation [29]. It is known that the increased COL1A1 levels activate the TGFβ signaling and thus ultimately leads to increased migration and invasion. Therefore, the suppression of COL1A1 might be advantageous in reducing the migration of gastric cancer cells. In addition, CTGF, being the downstream mediator of TGFβ, is known to induce the migration of gastric cancer cells, which can be reversed by RNAi induced knockdown of CTGF. Taking the information about the involvement of CTGF in cell migration and the effect of COL1A1 expression in cell invasion, migration and proliferation of gastric cancer cells, finding effective therapeutic agents acting on CTGF and COL1A1 can be considered as an advantageous treatment strategy. In this study, the GapmeR mediated suppression of CTGF also resulted in inhibition in COL1A1 expression. This result can be considered as a good starting point to further explore the response of the cells to this reduction. Therefore, further studies such as wound healing assay and Matrigel-coated invasion assays [30] are required to observe the effectiveness of the inhibition of these mRNA levels on migration and invasion.

Since cancer invasion and metastasis requires the action of matrix metalloproteinases (MMP), reduction in the MMP levels can be considered as a powerful impact of the therapeutic reagent. Alteration in MMP-9 levels was chosen as a parameter to be analyzed in the present study because of its importance in invasion in addition to the direct effect of CTGF on MMP-9 levels and activity [31]. The transfection of AGS cells with CTGF targeting AON sequence, however, did not cause any inhibition in *MMP 9* levels. This was an interesting result, as mentioned before, the direct effect of inhibition of CTGF in gastric cancer cells resulted in the reduction in MMP-9 levels as determined by qRT-PCR and western blot analysis. The unsuccessful inhibition of *MMP 9* in AGS cells might be due to AON dose used for the study. The proliferation of cells during experiments ultimately may lead to reduction in the AON concentration per cell, therefore by increasing the dosing, effective *MMP9* inhibition might be observed. Moreover, time course studies might be performed to determine whether the unsuccessful mRNA suppression is because of insufficient dose, or due to RNA turnover.

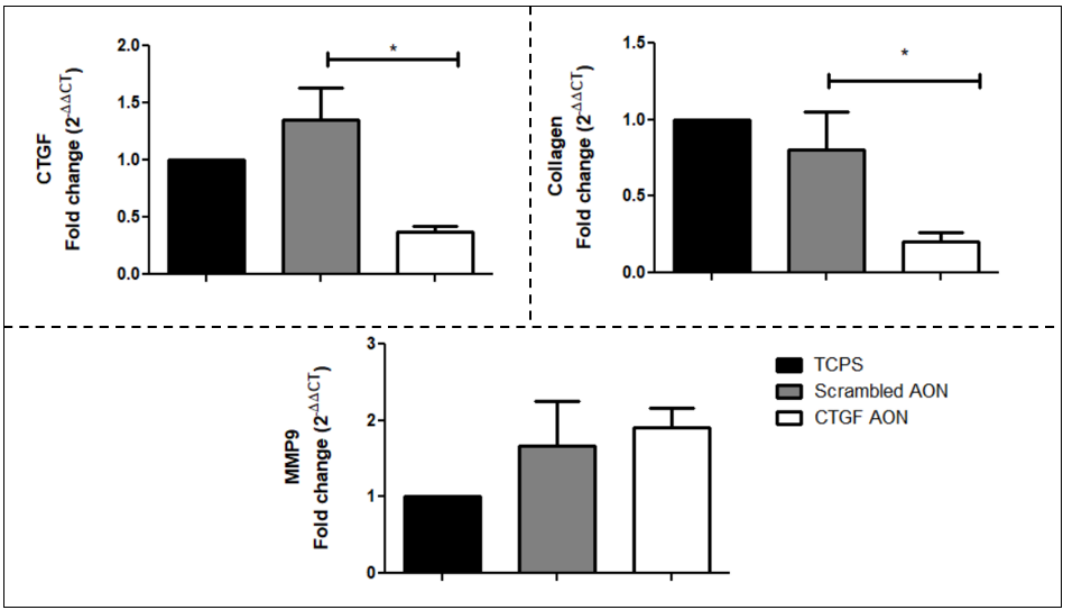


**Figure 1**. Fold changes of *CTGF*, *collagen* (*Col1A1*) and *MMP9* mRNA expression after administration of GapmeR sequence. TCPS: TCPS control; Scrambled AON: Treatment with scrambled AON sequence, CTGF AON: Treatment with CTGF AON sequence. The fold changes are given as mean ± STDEV ($p$<0.05 *)

### *3.2 Western blotting*

The effect of CTGF GapmeR AON on the CTGF protein levels were also studied by western blot technique (Figure 2). The CTGF band intensity was normalized to the corresponding band intensity of β-actin. With respect to TCPS control group, a 44% of inhibition in CTGF protein levels was observed, when the AGS cells were treated with the GapmeR AON sequence. The achievement of reduction in CTGF protein levels implies that the amount of AON administered to AGS cells was sufficient to suppress both the CTGF mRNA and protein levels.

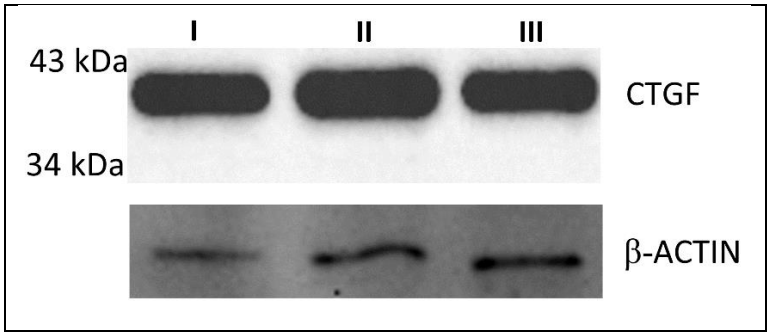


**Figure 2.** CTGF protein expression of AGS cells after treatment with CTGF GapmeR AON sequence. β-actin serves as loading control. I: TCPS Control, II: Treatment with scrambled AON sequence, III: Treatment with CTGF AON sequence.

### *3.3 Effect of AON on the proliferation of AGS cells*

It is known that abnormal expression of CTGF in different types of tumors such as prostate cancer, gliomas, breast cancer is also observed in gastric cancer, and it leads to increased cell proliferation along with increased migration and metastasis. Therefore, it is important to observe reduced proliferation of gastric cancer cells following CTGF GapmeR AON treatment. The proliferation ability of the AGS cells were studied with Alamar Blue Cell Viability Assay. It can be clearly observed from Figure 3 that, with the treatment of cells with CTGF GapmeR AON, the proliferation of the cells reduced significantly (by 50%) when compared to TCPS. More importantly, the scrambled AON

did not cause reduction in cell viability, revealing that the reduction in AGS cell proliferation with CTGF AON administered group is not through the toxicity of the GapmeR presence, but through the inhibition in abnormal CTGF expression in gastric cancer cells. There are studies in the literature suggesting the possible reason for reduced gastric cell proliferation due to CTGF inhibition. Cyclin D1 levels, one of the regulators of cell cycle, in addition to the c-Myc levels were found to be correlated with the overexpression of CTGF and thus the proliferation [32]. Therefore, suppressing CTGF levels might reduce the proliferation, however further studies are needed to understand the background of reduced cell proliferation with suppression of CTGF in AGS cells.

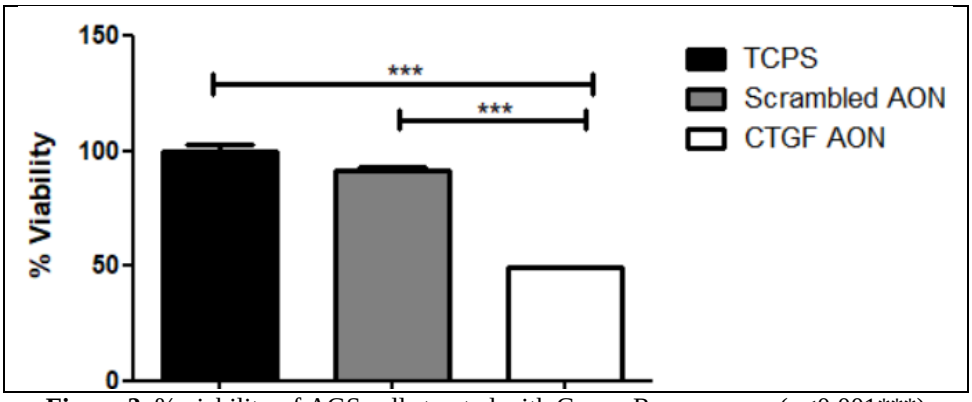


**Figure 3.** % viability of AGS cells treated with GapmeR sequences. ($p<0.001$***)

## 4. Conclusion

In summary, the custom designed CTGF GapmeR AON, used in this study is highly effective in inhibiting the CTGF mRNA expression in gastric cancer cells. The reduction is also followed by the reduced expression of collagen from the AGS cells, which might be the indicator of reduced ECM production. The CTGF reduction was also observed in the protein levels and caused significant suppression in the proliferation of AGS cells. Further studies with mentioned GapmeR sequence on gastric cancer cell proliferation, migration and invasion, may lead to the development of alternative effective ways for reversing the tumor enlargement, invasion and metastasis.

### *Authorship contribution statement*

**D. Sezlev Bilecen**: Conceptualization, Methodology, Experimental Procedures, Data Collection, Writing.

### *Declaration of competing interest*

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### *Acknowledgment*

The authors acknowledge Konya Food and Agriculture University for laboratory facilities.

### *Ethics Committee Approval and/or Informed Consent Information*

As the authors of this study, we declare that we do not have any ethics committee approval and/or informed consent statement.

## References


[1] E. C. Smyth, M. Nilsson, H. I. Grabsch, N. Ct Van Grieken, and F. Lordick, "Gastric cancer Epidemiology and risk factors", *The Lancet*, 396, 635–48, 2020.

[2] H. Sung, J. Ferlay, R. L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, and F. Bray, "Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries", *CA: A Cancer Journal for Clinicians*, 71(3), 209–249, 2021.

[3] S. S. Joshi and B. D. Badgwell, "Current treatment and recent progress in gastric cancer", *CA: A Cancer Journal for Clinicians*, 71(3), 264–279, 2021.

[4] S. W. T. Ho and P. Tan, "Dissection of gastric cancer heterogeneity for precision oncology", *Cancer Science*, 110, 3405–3414, 2019.

[5] X. F. Zhang, C. M. Huang, H. S. Lu, X. Y. Wu, C. Wang, G. X. Guang, J. Z. Zhang, and C. H. Zheng, "Surgical treatment and prognosis of gastric cancer in 2 613 patients", *World Journal of Gastroenterology*, 10(23), 3405-3408, 2004.

[6] P. Bork, "The modular architecture of a new family of growth regulators related to connective tissue growth factor", *Federation of European Biochemical Societies*, 327(2), 125-130, 1993.

[7] L. F. Lau and S. C-T Lam, "The CCN Family of Angiogenic Regulators: The Integrin Connection", *Experimental Cell Research*, 248, 44–57, 1999.

[8] L. Kular, J. Pakradouni, P. Kitabgi, M. Laurent, and C. Martinerie, "The CCN family: A new class of inflammation modulators?", *Biochimie*, 93(3), 377–388, 2011.

[9] Z. Mao, X. Ma, Y. Rong, L. Cui, X. Wang, W. Wu, J. Zhang, and D. Jin, "Connective tissue growth factor enhances the migration of gastric cancer through downregulation of E-cadherin via the NF-κB pathway", *Cancer Science*, 102(1), 104–110, 2011.

[10] J. Hutchenreuther, K. Vincent, C. Norley, M. Racanelli., S. B. Gruber, T. M. Johnson, D. R. Fullen, L. Raskin, B. Perbal, D. W. Holdsworth, L. M. Postovit, and A. Leask, "Activation of cancer-associated fibroblasts is required for tumor neovascularization in a murine model of melanoma", *Matrix Biology*, 74, 52–61, 2018.

[11] L. Y. Liu, Y. C. Han, S. H. Wu, and Z. H. Lv, "Expression of connective tissue growth factor in tumor tissues is an independent predictor of poor prognosis in patients with gastric cancer", World *Journal of Gastroenterology*, 14(13), 2110–2114, 2008.

[12] A. M. Moreira, J. Pereira, S. Melo, M. S. Fernandes, P. Carneiro, R. Seruca, and J. Figueiredo, "The Extracellular Matrix: An Accomplice in Gastric Cancer Development and Progression", *Cells*, 9(2), 394-417, 2020.

[13] S. Z. Chen, H. Q. Yao, S. Z. Zhu, Q. Y. Li, G. H. Guo, and J. Yu, "Expression levels of matrix metalloproteinase-9 in human gastric carcinoma", *Oncology Letters*, 9: 915-919, 2015.

[14] Q. N. Zhang, H.L. Zhu, M.T. Xia, J. Liao, X.T. Huang, J.W. Xiao, and C. Yuan, "A panel of collagen genes are associated with prognosis of patients with gastric cancer and regulated by microRNA-29c-3p: An integrated bioinformatics analysis and experimental validation", *Cancer Management and Research*.11, 4757–4772, 2019.

[15] J. Li, Y. Ding, and A. Li, "Identification of COL1A1 and COL1A2 as candidate prognostic factors in gastric cancer", *World Journal of Surgical Oncology*, 14, 297, 2016.

[16] Y. Zhao, T. Zhou, A. Li, H. Yao, F. He, L. Wang, and J. Si, "A potential role of collagens expression in distinguishing between premalignant and malignant lesions in stomach", *Anatomical Record*, 292(5), 692–700, 2009.

[17] Y. Guo, G. Lu, H. Mao, S. Zhou, X. Tong, J. Wu, Q. Sun, H. Xu, and F. Fang, "miR-133b Suppresses Invasion and Migration of Gastric Cancer Cells via the COL1A1/TGF-β Axis", *OncoTargets and Therapy*, 13: 7985-7995, 2020.

[18] Q. Wang and J. Yu, "MiR-129-5p suppresses gastric cancer cell invasion and proliferation by inhibiting COL1A1" *Biochemistry and Cell Biolology*, 96: 19-25, 2018.

[19] A. Baghbanzadeh, E. Baghbani, K. Hajiasgharzadeh, S. Noorolyai, V. Khaze, B. Mansoori, M. Shirmohamadi, B. Baradaran, and A. Mokhtarzadeh, "microRNA-193a-5p Suppresses the Migratory Ability of Human KATO III Gastric Cancer Cells through Inhibition of Vimentin and MMP-9", *Advanced Pharmaceutical Bulletin*, 12(1): 169-175, 2022.

[20] C. G. Jiang, L. Lv, F. R. Liu, Z. N. Wang, F. N. Liu, Y. S. Li, C. Y. Wang, H. Y. Zhang, Z. Sun, and H. M. Xu, "Downregulation of connective tissue growth factor inhibits the growth and invasion of gastric cancer cells and attenuates peritoneal dissemination", *Molecular Cancer*. 28 (10), 122, 2011.

[21] I. Radziejewska, K. Supruniuk, J. Nazaruk, E. Karna, B. Popławska, A. Bielawska, and A. Galicka, "Rosmarinic acid influences collagen, MMPs, TIMPs, glycosylation and MUC1 in CRL-1739 gastric cancer cell line", *Biomedicine & Pharmacotherapy* 107: 397–407, 2018.

[22] Y. Song, J. S. Kim, E. K. Choi, J. Kim, K. M. Kim, and H. R. Seo, "TGF-β–independent CTGF induction regulates cell adhesion mediated drug resistance by increasing collagen I in HCC", *Oncotarget*, 8(13): 21650-21662, 2017.

[23] H. Sun, Y. Wang, S. Wang, Y. Xie, K. Sun, S. Li, W. Cui, and K. Wang, "The involvement of collagen family genes in tumor enlargement of gastric cancer", *Scientific Reports*, 13(1), 100-111, 2023.

[24] S. Karaki, C. Paris, and P. Rocchi, "Antisense Oligonucleotides, A Novel Developing Targeting Therapy", in Antisense Therapy, Editors, S. Sharad, S. Kapur, doi: 10.5772/intechopen.73736, 2019.

[25] C. F. Bennett and E. E. Swayze, "RNA targeting therapeutics: Molecular mechanisms of antisense oligonucleotides as a therapeutic platform", *Annual Review of Pharmacology and Toxicology*, 50, 259–293, 2010.

[26] T. Tabaglio, D. H. P. Low, W. K. L. Teo, P. A. Goy, P. Cywoniuk, H. Wollmann, J. Ho, D. Tan, J. Aw, A. Pavesi, K. Sobczak, D. K. B. Wee, and E. Guccione, "MBNL1 alternative splicing isoforms play opposing roles in cancer", *Life Science Alliance*, 1(5): e201800157, 2018.

[27] P. Sengupta, Y. Xu, L. Wang, R. Widom, and B. D. Smith, "Collagen alpha1(I) gene (COL1A1) is repressed by RFX family", *Journal of Biological Chemistry*, 280(22): 21004–21014, 2005.

[28] M. Hayashi, S. Nomoto, M. Hishida, Y. Inokawa, M. Kanda, and Y. Okamura, "Identification of the collagen type 1 alpha 1 gene (COL1A1) as a candidate survival-related factor associated with hepatocellular carcinoma", *BMC Cancer*, 14: 108, 2014.

[29] Y. Shi, Z. Duan, X. Zhang, X. Zhang, G. Wang, and F. Li, "Down-regulation of the let-7i facilitates gastric cancer invasion and metastasis by targeting COL1A1" *Protein & Cell*. Feb;10(2):143-148. 2019.

[30] K. I. Hulkower and R. L. Herber, "Cell Migration and Invasion Assays as Tools for Drug Discovery" *Pharmaceutics*, 3, 107-124, 2011.

[31] H. S. Kim, M. H. Kim, M. Jeong, Y. S. Hwang, S. H. Lim, B. A. Shin, B. W. Ahn, and Y. D. Jung, "EGCG blocks tumor promoter-induced MMP-9 expression via suppression of MAPK and AP-1 activation in human gastric AGS cells", *Anticancer Research*, 24(2B):747-53, 2004.

[32] Y. Z. Deng, P.P. Chen, Y. Wang, D. Yin, H. P. Koeffler, B. Li, X. J. Tong, and D. Xie, "Connective tissue growth factor is overexpressed in esophageal squamous cell carcinoma and promotes tumorigenicity through beta-catenin-T-cell factor/Lef signaling", *Journal of Biological Chemistry*, 14;282(50):36571-81, 2007.