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Research Article

Inhibition of pro-platelet basic protein (CXCL7) induces antineoplastic effects in colorectal cancer cells

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Abstract
Background: Colorectal cancer (CRC), a 3rd leading cause of cancer-related deaths worldwide, is characterized by high metastatic potential and poor survival rates in advanced stages. Pro-platelet basic protein (CXCL7) is a chemokine and plays crucial roles in promoting tumor progression, angiogenesis, immune modulation and metastasis. Elevated CXCL7 expression correlates with increased tumor vascularization, immune cell recruitment, and poor clinical outcomes. This study investigates the potential of targeting CXCL7 to inhibit CRC progression and metastasis related properties.
Methods: The role of CXCL7 in CRC was evaluated by using SW480 (human) and CC531 (rat) cell lines. CXCL7 expression was knocked down using siRNA, and its effects on cell proliferation, colony formation, migration, wound healing and apoptosis were assessed through MTT assays, colony formation assays, migration assays, scratch-healing assays and nuclear staining, respectively. RNA expression levels were analyzed through real-time PCR.
Results: CXCL7 knockdown was achieved significantly at mRNA levels, with a maximum reduction of 76% and 81% in SW480 and CC531 cells, respectively, within 24 hours. This led to reduced cell proliferation, where SW480 cells showed a maximum inhibition of 56% compared to 39% in CC531 cells. Colony formation declined significantly in both cell lines, with a more pronounced effect on large colonies. Migration and scratch healing capacities were reduced by up to 26% in SW480 cells and 67% in CC531 cells, demonstrating impaired motility. Apoptotic markers, including nuclear condensation and fragmentation, were observed after CXCL7 inhibition.
Conclusion: Targeting CXCL7 in CRC reduces tumor cell proliferation, colony formation, migration, and wound healing while inducing apoptosis. These findings highlight CXCL7 as a promising therapeutic target for CRC management and warrant further exploration in clinical settings.
Key Words: Colorectal cancer, CXCL7, Chemokine, siRNA, Gene knockdown, Anticancer

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INTRODUCTION

Colorectal Cancer (CRC) is referred to as cancerous manifestations in the colon and rectum, and it is responsible for approximately 10% of all global cancer cases and 9.4% of cancer related deaths worldwide. It stands as the 3rd leading cause of cancer worldwide as of GLOBOCON 2020's data [1]. CRC is a highly malignant cancer and about fifty percent of the CRC patients acquire metastases during the disease's progression, primarily to the liver because it is the primary vascular bed for circulating tumor cells followed by the lungs, bones, and brain [2]. A vast majority of individuals with metastatic forms of CRC are incurable. For individuals diagnosed with advanced stages of CRC (stage III, IV), 5-year survival rate is reported to be under 10%. [3]. CRC is heterogeneous in nature especially at advanced stages. Despite the efforts to identify biomarkers for early detection, accurate prognosis, and effective management of CRC, progress remains limited, and mortality rates continue to be alarmingly high. CRC progression is a multistep process that depends on multiple factors like genetic mutations, environmental factors, tumor microenvironment, which includes stromal cells, immune cells, and complicated signaling networks.

Chemokines, a class of small-secreted cytokines (8-14kDa), have gained attention for their role in pathophysiology of different types of cancers, including CRC. They play a key role in regulating cell migration, inflammation, angiogenesis and immune

response. Dysregulation of chemokine expression contributes to tumor promoting processes. Consequently, chemokines and their receptors have become an attractive target for cancer therapy. New methodologies are being developed to target the chemokine network with antibodies, antagonists, and other molecules under preclinical and clinical investigations [4].

CXCL7 activity is mediated by binding with CXCR1 and CXCR2 receptors and is a potent chemokine implicated in the progression of CRC [5]. It is released by the cells such as megakaryocytes, neutrophils, and monocytes. CXCL7 plays roles in cell proliferation, angiogenesis, and immune cell recruitment. Studies indicate that CXCL7 is expressed by several types of tumors, particularly CRC [6]. Elevated expression of CXCL7 in CRC has been linked to increased neutrophil infiltration, tumor vascularization, and poor patient outcomes including shorter disease-free survival [7]. High expression levels of CXCL7 were reported and were positively correlated with lymph node metastasis in gastric cancer [8]. Its critical role in promoting angiogenesis and maintaining a pro-inflammatory tumor microenvironment highlights CXCL7 as a prospective therapeutic target for cancer immunotherapy [9]. Current treatment options for CRC include surgical resection, chemotherapy, and targeted therapies yet, these means of treatment are frequently inadequate to contain the metastatic disease [10] highlighting the need for innovative approaches of therapy. In this context, targeting chemokine network like CXCL7 and its downstream channels offers a promising way to disrupt the inflammatory and angiogenic processes that contribute to CRC advancement and metastasis to improve the prognosis as well as quality of life for CRC patients.

The present study was designed to assess the role of CXCL7 in CRC progression related properties using *in vitro* models. After targeting the CXCL7 expression through knock-down strategies, its concomitant effects on proliferation, migration, colony formation and apoptosis related properties were explored in CRC cells.

METHODS

Cell Cultures

Two colorectal adenocarcinoma cell lines of rat (CC531) and human (SW480) origin, free of pathogenic contaminations, were cultured routinely in RPMI-1640 medium which was supplemented with FBS (10%), L-glutamine (2mM), antibiotics like streptomycin (100 µg/ml) and penicillin (100 IU/ml). All cell culture work was maintained under optimal cell culture conditions which included a humidified atmosphere, 5% CO_2 supply, and 37°C temperature. Cells were sub-cultured twice or thrice a week to sustain a logarithmically growing population. For propagation, the cells were washed using 1X PBS, then trypsinized via using 0.25% trypsin, following centrifugation for 5 minutes at 1500–2000 rpm to form the pellets. Using Neubauer chamber, the cells were counted and sub-cultured at appropriate cell concentrations as per the experimental demands.

Knockdown of CXCL7

For knockdown experiments, small interfering RNA duplexes (siRNAs) targeting the CXCL7 gene in human and rat were designed. Specified siRNAs and nonspecific siRNAs (mock) were purchased from Invitrogen and Ambion (cat#AM4615) respectively. As per the kit manufacturer's guidelines of the transfection reagents, cells were transfected with a range of 100-300nM siRNAs for 24-72 h in cell culture plates. In brief, the cells were grown for overnight up to 50% of confluency and transfected with gentle handling procedures while using the transfecting agent and the siRNAs. Details about siRNAs and transfection agents are given in Table 1.

RNA Extraction and cDNA Synthesis

For RNA isolation, the cells from all cell lines were seeded in respective culture plates (6-well) ensuring a desired cell concentration of $1\text{-}1.5\times10^5$ cells per well, after that knock-down were done accordingly for a duration of 24, 48, and 72 h. The cell pellets were collected afterwards, and RNA was extracted using a commercially available Qiagen RNeasy Mini kit. A spectrophotometer (Gene Quant pro) was used to measure the purity and concentration of extracted RNA. A total of 250-1000 ng of fine-quality total RNA (having a 260/280 ratio between 1.8-2.0) per sample was used as a template to synthesize complementary DNA (cDNA). For cDNA synthesis, a mixture of RT buffer (1X), RNAse inhibitor (10 units), 1 µl dNTPs (10 mM), 1 µl oligo-dT-primer (10 µM), and reverse transcriptase (200 units) were used making a total 20 µl volume. Maxima reverse transcriptase enzyme was used in this procedure.

Real-Time PCRs

The expression profile of the CXCL7 gene after knock-down was studied by Real-Time PCR (Light Cycler 480). For amplification, the Universal Probe Library kit along with Roche's 2X Master Mix LC480 was used. Two µl of prepared cDNA (25-100 ng) per sample was added into each well of 384-well PCR plates in triplicate manners with a final reaction volume of 10 µl/sample. To normalize the data expression, levels of the GAPDH gene were used. Corresponding changes in expression levels of the CXCL7 gene were analyzed using the Livak ($2^{-\Delta\Delta CT}$) method. Primer sequences used in these amplification procedures were purchased from Roche.

Cell Proliferation Assay

The MTT dye reduction method was utilized to assess cell proliferation; after counting cells via Neubauer's chamber, the desired cell suspension in RPMI-1640 medium was made and the cells were seeded into 96-well culture plates to ensure the final desired density of 5×10^3 cells/100 µl medium/well. Following 24 hours of incubation, the CXCL7 gene was knocked down by specific siRNA and after the treatment intervals (24, 48, or 72 h), 10 µl of MTT solution (10 mg/ml in PBS) was added per well and incubated for an additional 3 hours. Following removal of the medium, 100µl per well of acidified solvent (0.04N HCl in 2-propanol) was added to each well to dissolve the formazan crystals. An ELISA plate reader was used to measure the optical density at 540 nm wavelength via a 690 nm filter. Cell survival percentages were calculated relative to mock siRNA. In this and all following functional assay, the experiments were carried out at least in triplicates.

Colony Formation Assay

The clonogenic potential of CRC cells in response to CXCL7 targeting was evaluated using a colony formation assay. Cells were either with siRNA for CXCL7 knockdown for 48 h. Following treatment, 5×10^2 cells were suspended in a 1.5 ml semiliquid media (RPMI-1640 having 0.4% methylcellulose and 30% FBS) and transferred to 6-well plates. After 6–8 days of incubation under optimal culture conditions, cell colonies were counted using an inverted microscope. Colonies containing more than 10 cells were designated as colony-forming units. The colonies were divided into two categories small and large containing less than 30 cells or containing more than 30 cells respectively, and results were reported as a percentage of control samples.

Migration Assay

To assess how cell migration is affected by CXCL7 targeting (siRNA), a two-compartment model with an 8µm polycarbonate membrane was used with an FBS (which is a chemo-attractant) in the lower chamber. To establish a chemotaxis gradient, 24-well culture plates were prepared but pouring 250µl of FBS at the base, followed by a 650µl overlay of semi-liquid media containing 20% FBS, and 0.4% methylcellulose in RPMI-1640 and incubation under optimum conditions was given for 24 h. Following 48 h of transfection with CXCL7 or mock siRNA, equal number of cells (5×10^4/well) were seeded in 200µl of Optimum medium into Millicell inserts with 8µm membranes. Using an inverted microscope, migrating cells were counted, and non-migrating cells were relocated to new plates containing a fresh chemotaxis gradient each day.

Wound Healing Assay

To assess CRC cell motility, a wound-healing assay was used to determine the effect of CXCL7 knockdown. Cells were seeded in 12-well plates to achieve a monolayer with a density of 1×10^5 cells per well, On the subsequent day the cells were subjected to the treatment with siRNAs (CXCL7-specific or mock) for a period of 48 hours. A scratch was introduced using a 200 µl pipette tip. Residue or floating cells were washed away, and 500 µl of 0.5% FBS medium was added to each well. To evaluate the scratch healing process, images were taken at the beginning (0 h) and after 24 hours using an Axio Observer Z1 microscope.

Table 1: siRNA sequence of CXCL7

Gene	Specie	siRNA Sequence (5`- 3`sequence)	siRNA Concentration	Transfection Agent
CXCL7	Human	CCACCAAAGGACAAACUAA	200 nM	X-tremeGENE
	Rat	AGGUUACGAAGAGAGCUGG	200 nM	X-tremeGENE

Table 2: Primer sequences

Gene	Specie	Sequence (5`- 3`sequence)	Sequence (3`- 5`sequence)
CXCL7	Human	TCATTGCTGCTGACTGCTCT	CGGAGTTCAGCATACAAGTCAC
GAPDH		AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
CXCL7	Rat	GCTTCAGACTCAGACCTACATCC	AAATGGCTCGTTGTTATCAGG
γ- Tubulin		TCTACAACCCAGAGAACATCTACCT	TGATGTCAAAAATGTCCTCGTG

RESULTS

CXCL7 Knockdown by siRNA

A significant knockdown of the CXCL7 gene was achieved in SW480 (human) and CC531 (rat) cell lines using 200nM specific siRNA. Following a period of 24-72 h, maximum knockdown of the target transcript was achieved after 24 h with a maximum inhibition of 76% in SW480 and 81 % in CC531 cells. The knockdown effect became less pronounced over time, as maximum inhibition was 38% and 57 % after 72 h intervals in SW480 and CC531 cells, respectively.

Inhibition of Cell Proliferation

The knockdown of the CXCL7 gene resulted in the inhibition of the proliferation of CRC cells. Human CRC cells were more sensitive in this regard as the maximum inhibition of proliferation was 56% in SW480 cells as compared to 39% inhibitory effects in CC531 cells. Moreover, the effects were more profound at later time intervals (48 and 72 h) in SW480 cells, while in CC531 cells, the effects were minimal after 72 h of the transfection procedure.

Colony Formation Inhibition

A substantial decline in colony formation, particularly large colonies, was observed following CXCL7 knockdown in CRC cells. In SW480 cells, the maximum inhibition of colony formation was 46% and 31% for small and large colonies, respectively. In CC531 cells, a slight stimulation was observed for small colony formation (maximally by 12%) followed by a striking decrease in large colony formation (maximally by 66%).

CXCL7 and Migratory Behavior of Cells

Inhibition of CXCL7 expression reduced the ability of both cell lines to migrate toward a chemoattractant (FBS). The maximum inhibition of migration was 26% in SW480 and 67% in CC531 cells. The effects were relatively moderate and steady in human CRC cells (SW480). On the contrary, in rat CRC cells (CC531), the effects were more significant initially (24 h) but reduced over the later time intervals (48 and 72 h).

Scratch Healing Inhibition

Following inhibition of the CXCL7 expression, the capacity of CRC cells to cover the scratched space between the two created boundaries was monitored.
A statistically significant reduction was found in both cell lines to cover the empty area as compared to the control cells. The effects were identical in both cell lines, where almost a 50% reduction in scratch healing properties was observed after CXCL7 knockdown.

Nuclear Staining and CXCL7 Inhibition

Inhibition of the CXCL7 imposed apoptotic effects in selected CRC cells. These effects, including condensation of nuclear content and fragmentation of DNA/nuclei, were visible in both CRC cell lines.

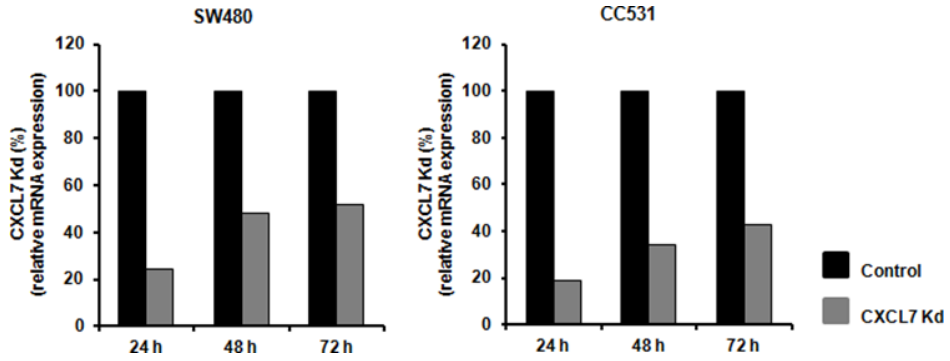

Figure 1: CXCL7 knockdown in the cells by using siRNA methodology and confirmation by real-time PCR.

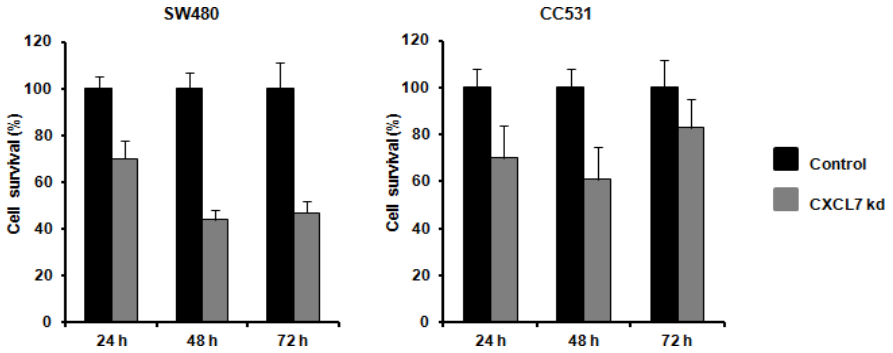

Figure 2: Antiproliferative effects of CXCL7 knockdown in the cells identified by MTT assay.

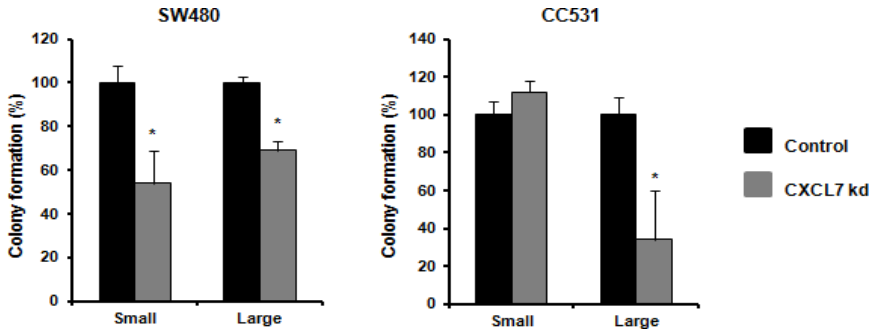

Figure 3: Colony formation inhibition after CXCL7 Knockdown in the cells.

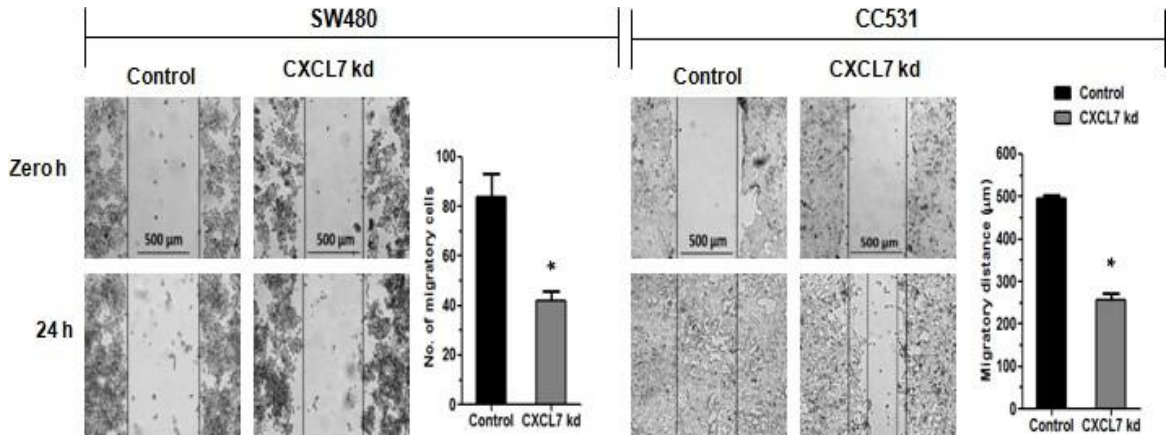

Figure 4: Inhibition of scratch healing process in the cells after CXCL7 knockdown.

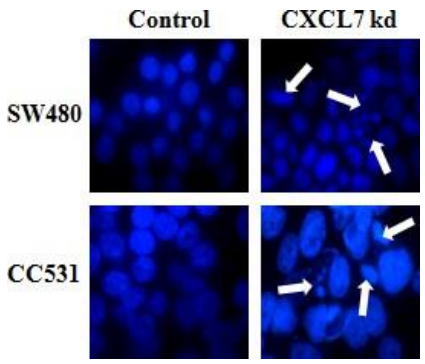

Figure 5: Induction of nuclear condensation/fragmentation in the cells after CXCL7 knockdown.

DISCUSSION

The selected chemokine ligand (CXCL7) is a key platelet factor. Platelets closely associated with carcinogenesis but how the platelet factors of cancer cell origin assist in this whole process, is poorly understood. The role of CXCL7 was studied in human CRC (SW480) and rat CRC (CC531) cell lines. Expression of the gene was confirmed by RT-PCR as the first step in their respective selected cell lines (results not shown). Knockdown of CXCL7 was achieved by corresponding gene-specific siRNA. Interestingly, maximum siRNA-mediated knockdown of the gene was achieved at early periods (24 h) in the selected cell lines, whereafter the effects gradually decreased with time (minimum knockdown at 72 h). This shows the possibility of quick targeting and efficient knockdown of the gene at least at the mRNA level. Possible reasons for this quick knockdown could be easy access of the target transcript by the siRNA species, highly effective RNAi machinery, and/or high efficiency of designed siRNAs. Knockdown of the CXCL7 gene resulted in significant anti-proliferative effects in respective CRC cell lines. The effects were more profound in human CRC cells (56%), which, in turn, indicate that human CRC cells are more sensitive to the expression of this chemokine and their corresponding induction of proliferation. The studies have reported a link between CXCL expression profile and proliferation of the CRC cells [11]. Following knockdown of the gene, the colony formation assay showed inhibition of colony-forming abilities in the selected CRC cells. Interestingly, the effects were more profound in rat CRC cells as compared to the inhibitory effects observed in human CRC cells. In literature, mechanistic aspects of the colony formation by cancer cells and their association with the platelet factors are not well defined, but in this study, rat CRC cells were found and confirmed to be more sensitive to these inhibitory effects. Regarding migration, under the influence of a mixture of growth factors (FBS), the inhibition of migration was observed in human and rat CRC cells after the CXCL7 knockdown. Many study reports have highlighted the promoting effects of CXCL7 on cell motility either in normal leukocytes or cancer cells [12-14]. Our results align with a study by Guo et al., that reported the role of CXCL7 in promoting chemotaxis and metastasis in cancer [15]. A second assay about the migratory behavior of the cells (scratch assay) also showed profound inhibitory effects on cellular migration following the CXCL7 knockdown. Although the stimuli for migration in these assays were dissimilar (cell-based growth factors/chemokine receptors vs FBS factors), the outcomes from the two assays supported each other. This, in turn, shows that CXCL7 chemokine could be of greater importance regarding the metastatic phase of the disease, where cancer cells must leave their primary site of origin, enter the circulatory or lymphatic systems, and invade the specific target organs. Knockdown of the CXCL7 also induces signs of apoptosis at morphological levels, as indicated by the nuclear staining procedures. These signs included the shrinkage and fragmentation of nuclei/DNA in CRC cells. In conclusion, this platelet factor can be an important therapeutic target in CRC, as the inhibition of this factor (at least *in vitro*) illustrated anti-neoplastic effects on CRC cells. Higher levels of this factor (CXCL7) have also been identified in different cancers, so they can also serve as initial biomarkers for cancer presence and progression.

In summary, results from current study highlight the importance of CXCL7 chemokine, and its role in CRC progression. CXCL7 is a pivotal chemokine involved in the proliferation, migration, and survival of CRC cells. Its knockdown exerts substantial antiproliferative, anti-migratory and pro-apoptotic effects. Thus, CXCL7 is a promising therapeutic target in CRC and its inhibition can be used as a novel strategy in CRC management.

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Ethics Approval: Not Applicable
Competing Interests: None
Data Availability: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.