

Platelet Factor 4: A targetable chemokine induces antineoplastic effects in colorectal cancer cells

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Abstract

Background: Platelet Factor 4 plays an important role in tumor cell proliferation, migration and invasion. Platelet Factor 4 is an angio-static chemokine that acts as an immunosuppressant in tumor microenvironment by inhibiting T cell mediated anti-tumor immune response. Due to chemoattractant properties, it appears to be an important marker in cancer progression. In this study, Platelet Factor 4 was inhibited in the colorectal cancer cells followed by concomitant effects on functional properties of the colorectal cancer cells.

Methods: Platelet Factor 4 gene was knocked down by siRNA in human (Caco2) and rat (CC531) colorectal cancer cells. MTT assay was used to observe colorectal cancer cell survival following the Platelet Factor 4 knockdown, while trans-well migration assay, scratch assay and colony forming assays were used to investigate the ability of colorectal cancer cells to migrate and form colonies respectively.

Results: Knockdown of Platelet Factor 4 causes decrease in proliferation of the colorectal cancer cells. Inhibition of the gene also reduced the migration, colony formation and wound healing properties of colorectal cancer cells. The effects were visible and comparable in the human and rat colorectal cancer cell lines.

Conclusion: Down-regulation of Platelet Factor 4 gene may serve as a therapeutic target in colorectal cancer.

Key Words: Colorectal cancer, Chemokine, Platelet Factor 4, Therapeutics

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INTRODUCTION

Chemokine are small molecular weight proteins belonging to cytokine superfamily, playing role in inducing the recruitment and migration of specific cells [1]. Four families of chemokine (CC, CXC, CX3C and XC) are based on the arrangement of two cysteine residues near the N-terminal region. Chemokine along with the seven-transmembrane-spanning G protein-receptors (GPCR) are found in a variety of cells including leukocytes. These proteins are involved in intercellular signaling and plays role in biological processes such as migration, adhesion, differentiation and chemotaxis [2]. Based on their role in immune system and inflammatory responses, chemokine can also be divided in different classes such as inflammatory, homeostatic or dual action chemokine [3]. Tumor cells may secrete chemokine that act as chemoattractant and play an important role in tumor growth, progression, metastasis and angiogenesis. The C-X-C chemokine have been recognized as important prognostic markers that can play an important role in therapeutic applications [4].

CXC chemokine are divided into two subtypes: ELR+ or ELR- based on the presence or absence of an ELR (Glu-Leu-Arg) motif on its amino terminus. The chemokines without ELR motif are anti-angiogenic and include Platelet Factor 4 whereas chemokine with ELR motif shows pro-angiogenic properties. Inhibiting CXC chemokine leads to reduced motility of tumor cells as they are involved in various hallmarks of cancer such as angiogenesis, inflammation and metastasis [5].

Platelets and megakaryocytes produce Platelet Factor 4 (also known as CXCL4) that regulates leucocyte recruitment, inhibit angiogenesis and pro-tumor effects [6]. It is an angio-static chemokine that inhibits endothelial cell chemotaxis and angiogenic effects of VEGF. In a recent study, Platelet Factor 4 was found as an immunosuppressant in tumor microenvironment while decreasing cytotoxic T lymphocytes (CTLs) proliferation and interferon gamma (IFN- γ) production and enhancing CTL apoptosis and PD-1 expression promoting colorectal cancer cell growth [7]. In circulation, Platelet Factor 4 exists in two forms as Platelet Factor 4 and Platelet

Factor 4L1. These two isoforms are recognized based on three amino acids that are different in the variant isoform (Platelet Factor 4L1). Platelet Factor 4L1 is also secreted by platelets but in smaller amounts, possess angio-static properties but is a weak chemoattractant [8].

In an experimental *in vivo* study, done by Imke Atreya in Germany, increased expression of Platelet Factor 4 acting as CXCR3 ligand increased the proliferation and activation of T regulator cells while inhibiting CD8⁺ T-cell mediated anti-tumor immune response leading to a decreased survival time in colorectal cancer patients [9]. Platelet Factor 4 also leads to an increased risk of metastasis by inhibiting CD8⁺ T cells that affect immune response. An increased Platelet Factor 4 expression leads to increased rate of metastasis and poor overall survival in patients [10].

Due to their role in angiogenesis, inflammation, immunity and migration of tumor cells, chemokines are being investigated extensively. In this study, we explored the effects of Platelet Factor 4 knockdown on functional aspects of the colorectal cancer cells.

METHODS

Colorectal Cancer Cell Lines

Culturing of 2 colorectal adenocarcinoma cell lines (CC531, rat origin) and (Caco2, human origin) were done in RPMI-1640 medium, that is supplemented with L-glutamine (2mM), fetal bovine serum (10%), streptomycin (100µg/ml) and penicillin (100IU/ml). The cultures were maintained in a humidified atmosphere, 5% CO₂ and 37°C. Splitting of these cell lines was done 2-3 times every week to maintain the proliferation of cells exponentially. For this purpose, the cells were washed with PBS, dissociation reagent (Trypsin 0.25%/EDTA) was added and centrifuged at 1500rpm for 5 minutes to collect the cell pellet for sub-culturing. Neubauer chamber was used to count the desired cell density for further experimental purposes.

RNA Extraction and cDNA Synthesis

The cells (2×10⁵/well) were seeded in 6 well plates, knocked down for 48 h (gene specific or mock siRNA). Afterwards, total RNA was extracted by Qiagen RNeasy Mini kit and the concentration was measured by GeneQuant pro spectrophotometer. A total of 1000ng of RNA per sample was used to synthesize cDNA. 20µl reaction mix was prepared by adding 1X RT buffer, RNase inhibitor (10 units), 1µl dNTPs (10mM), 1µl oligo-dT-primer (10mM) and Maxima reverse transcriptase (200 units).

Quantitative Real-Time PCR

Universal probe library kit was used along with LC480 2X master mix (Roche) for expressional profiling that

was done in triplicate on LightCycler 480 Real-Time system. 384-well plate was utilized in which 2µl cDNA (25-100ng) was pipetted into each well, making a total of 10µl volume containing optimized primer for amplification. A reference gene (γ-Tubulin) was used for data normalization. The inhibition of Platelet Factor 4 transcript in cell lines was assessed. The primers used for this purpose are shown in Table 2.

Transfection of Colorectal Cancer Cells

To knock down Platelet Factor 4, small interfering RNA duplexes (siRNA) were obtained from Invitrogen, and non-specific siRNA was purchased from Ambion. The cells were transfected using Lipofectamine 2000 agent with 100-300nM siRNAs for 24-72 h in 6, 12, 24 and 96-well culture plates according to the requirement of experiment. Sequence of siRNA used is shown in Table 1.

Cell Proliferation Assay

Platelet Factor 4 gene was knocked down by siRNA for 24-72 h. Following this treatment, MTT solution (10mg/ml) was added and incubated for 3 h. After discarding the old media, an acidified solvent (0.04N HCl in 2-propanol) (100µl/well) was added to dissolve the formazan crystals. ELISA plate reader was used to measure the optical density with absorbance wavelength at 540nm and reference filters at 690nm. Inhibitory effects were calculated as percentages whereas percentages of controls transfected with mock siRNAs were set to 100% survival.

Clonogenic Assay

The assay was used to evaluate the effects of targeting Platelet Factor 4 on colony formation ability of colorectal cancer cells. A total of 5×10² cells/1.5ml semiliquid medium (RPMI-1640 containing 0.4 % methylcellulose and 30 % FBS) were transferred to 6-well plates following siRNA mediated knockdown of Platelet Factor 4 (48 h). The cluster of cells was counted under an inverted microscope (Leitz fluovert FU Microscope) after an incubation period of 6-8 days. Colony forming units were categorized as small (<30 cells) or large (≥30 cells) and were presented as clusters with more than 10 cells. Presentation of data sets were shown as percentage of controls (mock transfected siRNA).

Trans-well Migration Assay

A two-chamber model divided by an 8µm polycarbonate membrane (Millicell, Millipore) with a chemoattractant (FBS) in the lower compartment was used to study the effects of targeting Platelet Factor 4 (knocked down by siRNA) on migration of cells. For building a chemotaxis gradient, a chemoattractant (250µl FBS) was layered at the bottom of 24-well plate covered by 650µl semi-liquid medium and incubated under standard conditions for 24 h. The

knocked down cells (siRNA transfected) were counted (5×10⁴ cells/200ul Optimem media) and seeded into suspended Millicell slots with polycarbonate membrane. The non-moving cells were placed each day into new wells with fresh chemoattractant and the migrating cells were counted by the help of an inverted microscope for 24, 48 and 72 h.

Scratch Assay

The cells (1×10⁵/well) allowed to proliferate in 12-well plates under standard conditions, were knocked down on the next day by siRNA (gene specific/mock) for 48 h and a straight scratch was made on the monolayer of cells with the help of a 200µl sterile

pipette tip. Detached cells were removed and fresh RPMI-1640 media (500µl/well) with reduced FBS (0.5%) was added. The images were captured by Axio Observer Z1 microscope to observe the scratch healing process taking place by the cells (knocked down by siRNA) after zero and 48 h of seeding.

Data Analysis Data was entered and analyzed by using SPSS 29.0. Mean values were used and given for quantitative data, while qualitative variables were expressed as frequencies/percentage. Independent *t-test* was used to compare numerical variables between the groups. A p-value of ≤0.05 was considered as statistically significant.

Table 1: Sequence of siRNA for Human (Caco2) and rat (CC531) cell lines

Specie	siRNA Sequence (5`- 3`sequence)	siRNA	Transfection Agent
Human	AGCAAAUGCACACACGUAGGCAGCU	100 nM	Lipofectamine 2000
Rat	UUCACACACACAGCUAAGAUCUC	100 nM	Lipofectamine 2000

Table 2: Sequence of primers for expressional profiling in human (Caco-2) and rat (CC531) cell lines

Gene	Forward Primer (5`- 3`sequence)	Reverse Primer (3`- 5`sequence)
Human	AGCCTGGAGGTGATCAAGG	CCATTCTTCAGCGTGGCTA
Rat	CCAGGATCCATCTCAAACG	CCATTCTTCAGCGTGGCTA

RESULTS

Knockdown of Platelet Factor 4

Platelet Factor 4 was knocked down by using 100nM of gene specific siRNA in human (Caco-2) and rat (CC531) cell lines and observed for 24-72 h. Significant expression knockdown (≥70%) was seen at mRNA level after 24 h. For later intervals (48 and 72 h), the knockdown efficiency was reduced in both cell lines.

Effect of Platelet Factor 4 Knockdown on Cell Proliferation and Migration

After Platelet Factor 4 knockdown, both cell lines were observed for 24-72 h and displayed significant inhibition of cell proliferation and migration. However, human cells (Caco-2) showed substantial inhibition of proliferation (≥60%) after Platelet Factor 4 knockdown (72 h) as compared to rat (CC531) cell line proliferation inhibition (~20%). As far as trans-well migration of the cells is concerned, a maximum

migratory inhibition was seen in rat CC531 cells after 72 h (76%), while human cell line showed significant migration inhibition peek (64%) after 48 h. Overall, anti-proliferative and anti-migratory effects were clearly visible in both CRC cell lines following the Platelet Factor 4 knockdown (Figure 2).

Platelet Factor 4 Knockdown Inhibits Colony Formation and Wound Healing

Platelet Factor 4 knockdown led to the inhibition of colony formation and wound healing in colorectal cancer cells as shown in Figure 3. Large colonies (>30 cells) showed marked inhibition in both human (43% in Caco-2) and rat (45% in CC531) cell lines as compared to small colonies, where percentage of inhibition was 21 and 10% respectively. Similar results were observed during the scratch assay that showed wound healing inhibition over a period of 24 h in human Caco-2 (44%) and rat CC531 (46%) cell lines after Platelet Factor 4 knockdown.

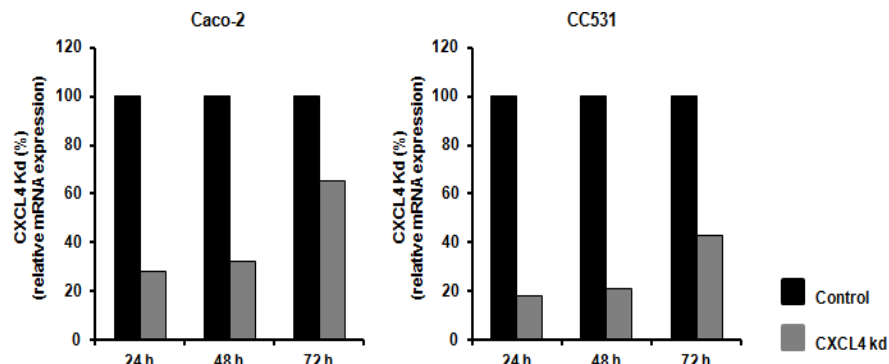


Figure 1: Downregulation of Platelet Factor 4 in human (Caco2) and rat (CC531) cell lines.

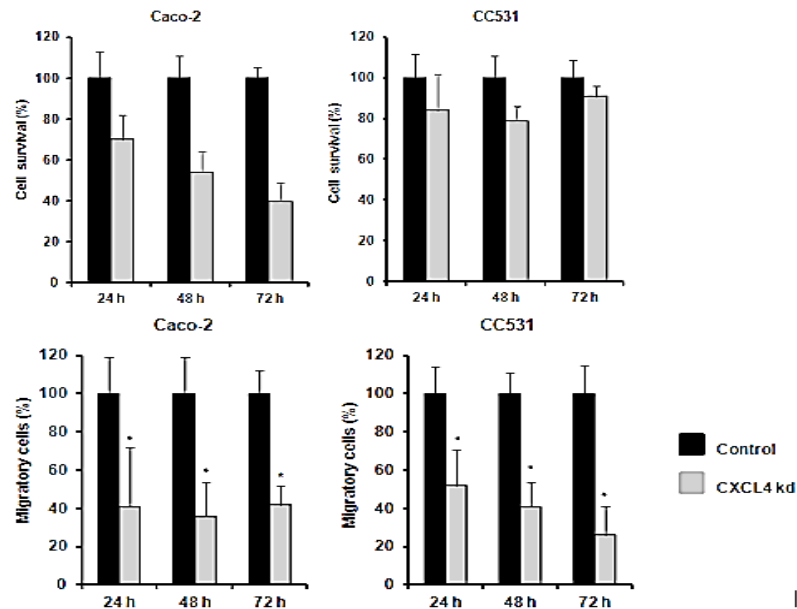


Figure 2: Proliferation and migratory (trans-well chamber) of colorectal cancer cells after Platelet Factor 4 Knockdown in human (Caco-2) and rat (CC531) cell lines.

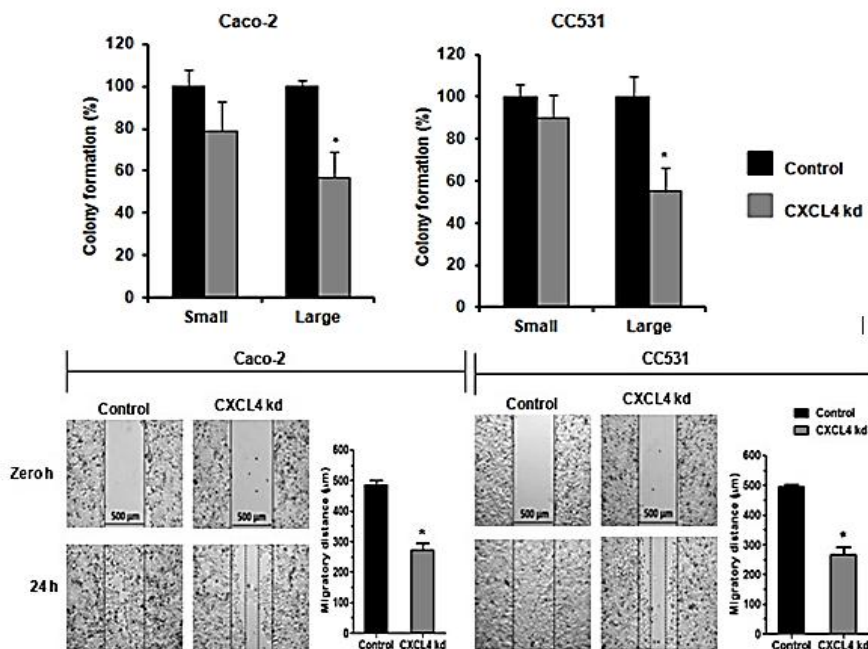


Figure 3: Inhibition in colony formation and wound healing observed after Platelet Factor 4 knockdown in human (Caco-2) and rat (CC531) cell lines.

DISCUSSION

One of the most common malignant tumor of digestive system worldwide is colorectal cancer with more than 1.6 million new cases and a high mortality rate of $\geq 900,000$ deaths each year [11]. Due to its late diagnosis, the overall survival rate for colorectal cancer patients is less than 50% that is further reduced in patients with metastasis (10-20%) [12]. Chemokines and their respective receptors play important role in tumor growth, migration of tumor cells and metastasis serving as prognostic markers in colorectal cancer [13]. In this study, the effects of one of the chemokine, Platelet Factor 4, was studied in human and rat colorectal cancer cell lines.

Platelet Factor 4 is secreted by platelets in tumor microenvironment and possess immunosuppressive effects, thus displaying a pro-cancer role. In the study, human (Caco2) and rat colorectal cancer cell line (CC531) were used to investigate the role of Platelet Factor 4 on colorectal cancer cells. Firstly, Platelet Factor 4 gene was knocked down using gene specific siRNA where maximum knockdown ($<70\%$) was seen during early time interval (24 h) with gradual decrease (minimal knockdown) for later time (48 and 72 h). This quick knockdown may be due to high efficiency of designed siRNA and/or maybe easy access to target gene. Afterwards, knockdown effects were investigated on cell survival in the colorectal cancer cell lines where with a linear inhibition of proliferation (up to 60%) was observed with increased period (72 h) in human cell line. In rat cell line, inhibition of proliferation was mild (21%). This differential inhibition of proliferation reflects that the knockdown of Platelet Factor 4 imposes variable effects based on the cell origin and associated molecular features.

The knockdown of Platelet Factor 4 gene resulted in anti-colonizing effects during the colony formation assay in human as well as rat cell lines. After the knockdown of Platelet Factor 4, clonogenic assay was performed and showed decreased number of colonies in selected cell lines. Substantial inhibition of colony formation was seen in rat cells (45%, CC531) and human cell lines (41%, Caco2). This, in turn, reflects that inhibition of Platelet Factor 4 can be instrumental while reducing the colonization of cancer cells.

Trans-well migration assay and scratch assays showed significant inhibition of migration in human (64% after 48 h during migration assay, 44% during scratch assay) and rat cell line (74% after 72 h during migration assay, 46% during scratch assay) following Platelet Factor 4 knockdown. This fact is of particular importance, especially with reference to metastatic procedures, where cancer cells have to migrate towards the distant organs/sites and can be reduced by inhibiting the Platelet Factor 4.

In another study done by Robiya Joseph in 2021, involvement of Platelet Factor 4 in metastasis of cancer was studied using two mouse mammary cancer cell lines (67Nr and 4T1) with the help of tissue microarray, trans-well migration assay and immunohistochemistry. It was demonstrated that Platelet Factor 4 secreted by platelets in TME and CD8+ cells have a strong inverse correlation as the Platelet Factor 4 leads to the differentiation of monocytes into Myeloid differentiating stem cells (MDSCs) which in turn inhibits CD8+ cells, leading to increased metastasis of cancer. Poor overall survival was seen in the patients with high levels of platelets and Platelet Factor 4 than patients with low level of platelets and Platelet Factor 4 [10]. These findings are consistent with our research observation that the knockdown of Platelet Factor 4 in colorectal cancer cell lines leads to inhibition of proliferation in tumor cells.

To conclude, Platelet Factor 4 can be targeted in colorectal cancer as it leads to anti-cancer effects. However, the specific role and mechanism of action of Platelet Factor 4 in tumor microenvironment is still unclear. Further research on this topic may improve its importance regarding future therapeutic approach in colorectal cancer.

REFERENCES

1. Zou Q, Lei X, Xu A, Li Z, He Q, Huang X, et al. Chemokines in progression, chemoresistance, diagnosis, and prognosis of colorectal cancer. *Frontiers in Immunology*. 2022;13:724139.
2. Khare T, Bissonnette M, Khare S. CXCL12-CXCR4/CXCR7 axis in colorectal cancer: therapeutic target in preclinical and clinical studies. *International journal of molecular sciences*. 2021;22(14):7371.
3. Łukaszewicz-Zajac M, Pączek S, Mroczko P, Kulczyńska-Przybik A. The significance of CXCL1 and CXCL8 as well as their specific receptors in colorectal cancer. *Cancer Management and Research*. 2020:8435-43.
4. Zeng Q, Sun S, Li Y, Li X, Li Z, Liang H. Identification of therapeutic targets and prognostic biomarkers among CXC chemokines in the renal cell carcinoma microenvironment. *Frontiers in oncology*. 2020;9:1555.
5. Wu T, Yang W, Sun A, Wei Z, Lin Q. The role of CXC chemokines in cancer progression. *Cancers*. 2022;15(1):167.
6. Xie J, Xing S, Jiang H, Zhang J, Li D, Niu S, et al. Extracellular vesicles-derived CXCL4 is a candidate serum tumor biomarker for colorectal cancer. *Iscience*. 2024;27(4).
7. Zhang Y, Rajput A, Jin N, Wang J. Mechanisms of immunosuppression in colorectal cancer. *Cancers*. 2020;12(12):3850.
8. George E, Andrew M, Maria T, Argyro V, Elias K. Angiogenic Chemokines Production by Colonic Cancer Cell Lines. *Onco*. 2022;2(2):69-84.
9. Atreya I, Neurath MF. How the Tumor Microenvironment Modulates the Recruitment and Activation of Colorectal Cancer-Infiltrating Lymphocytes. *Biomedicines*. 2022;10(11):2940.
10. Joseph R, Soundararajan R, Vasaikar S, Yang F, Allton KL, Tian L, et al. CD8+ T cells inhibit metastasis and CXCL4 regulates its function. *British journal of cancer*. 2021;125(2):176-89.
11. Yin H, Xie J, Xing S, Lu X, Yu Y, Ren Y, et al. Machine learning-based analysis identifies and validates serum exosomal proteomic signatures for the diagnosis of colorectal cancer. *Cell Reports Medicine*. 2024;5(8).
12. Xie Y-H, Chen Y-X, Fang J-Y. Comprehensive review of targeted therapy for colorectal cancer. *Signal transduction and targeted therapy*. 2020;5(1):22.
13. Song Y, Chen M, Wei Y, Ma X, Shi H. Signaling pathways in colorectal cancer implications for the target therapies. *Molecular Biomedicine*. 2024;5(1):1-24.

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