

Site specific expression of CXCL-12 β and its regulation by miR-200a in ulcerative colitis

Abstract

Purpose: CXCL-12 β is a chemokine playing an important role in inflammation and cancer. Altered cytokine and chemokine expression is a hallmark of ulcerative colitis. Emerging evidence suggest that microRNAs are involved in inflammatory process and cancer development during chronic inflammation. This study was done to find the regulation of CXCL-12 expression via different miRNAs in UC.

Methods: Biopsy samples were collected from UC patients and non-IBD controls. CXCL-12 β expression was analyzed at mRNA as well as protein level in UC and compared to controls. Expression of miRNAs targeting CXCL-12 β was analyzed by microarray and real time PCR. In vitro studies were done to validate CXCL-12 β as a target of miR-200a-3p.

Results: Expression of CXCL-12 β was increased in UC compared to controls. MiR-141-3p and miR-200a-3p were predicted to target CXCL-12 β . Expression of miR-141-3p was upregulated and expression of miR-200a was downregulated in UC compared to controls. In vitro studies using reporter assay proved CXCL-12 β as a target miR-200a. Expression of CXCL-12 β increased while expression of miR-200a decreased in the cancer prone, rectosigmoid area of colon.

Conclusion: Our study reveals that miR-200a-3p regulates the expression of CXCL-12 β in UC. Both CXCL-12 β and miR-200a also show site specific altered expression in the colon of UC patients. Our data shows that regulation of CXCL-12 β expression using miRNA may be a promising therapeutic approach in UC pathogenesis.

Keywords: Ulcerative Colitis, Cancer, MiRNA, CXCL12

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Introduction

IBD is a chronic inflammatory disease of gastrointestinal tract which involve complex interactions between immune system, genetic makeup, gut microbiota and environmental factors.¹ Ulcerative Colitis (UC) is one of the two main subtypes of Inflammatory Bowel Disease (IBD).² Along with other pathogenic factors, altered cytokine and chemokine expression play important role in the pathogenesis of UC.^{3,4} Patients with prolonged UC have increased risk of developing colorectal cancer (CRC).⁵⁻⁸

Expression of pro-inflammatory cytokines and chemokines are known to increase in UC.^{3,9} The degree of inflammation and tissue damage is dependent on the concentration of local chemokines. Thus over expression of chemokines leads to enhanced recruitment of immune cells to the inflammatory site.⁹ Experimental evidences show that CXCR4/CXCR7/CXCL12 chemokine axis play crucial role in inflammation as well as cancer. CXCL12/CXCR4 chemokine axis is known to be involved in pathogenesis of IBD specially UC.¹⁰ CXCL12 is expressed by normal intestinal epithelial cells (IECs) and upregulated in inflamed IECs of IBD patients.¹¹ Interestingly, enhanced expression of CXCL-12 was observed in UC compared to CD and more specifically in inflamed compared to non-inflamed mucosa.¹² Upregulation of CXCL12 in the inflamed colonic mucosa is specifically due to the variant CXCL12 β upregulation.¹³ Polymorphisms in CXCL12 are also reported as risk factor for IBD development.^{14,15} Blockade of CXCL12/CXCR4 axis has been shown to ameliorate colitis in both DSS induced as well as in IL-10 KO

mice model of colitis. Antagonist of CXCR4 also showed potent anti-inflammatory effect.¹⁰

Micro RNAs (miRNA) are small non-coding RNA molecules that are emerging key players in pathology of many diseases including IBD and cancer.^{16,17} MiRNA are differentially expressed in colonic mucosa and serum of IBD patients and regulate important genes involved in the disease pathogenesis.^{11,18,19} It has been shown that miRNA plays important role in fine-tuning of TLR signaling and maintaining homeostasis of gut microbiota, two very important players in the pathology of IBD.^{20,21}

There is increased risk of cancer development due to chronic inflammation.^{22,23} There is also an enhanced risk of CRC development in IBD patients specially UC.³ In UC, cancer development predominantly occurs in the rectosigmoid part of colon.^{6,25} MiR-141 regulate the expression of CXCL-12 β , which further mediate leukocyte migration in murine model of colitis and CD.¹³ MiRNAs are reported as connecting link between inflammation and cancer.^{22,23,26} CXCL12 is a pro-angiogenic chemokine that act as important player in chronic inflammation as well as cancer development.^{27,28} Different miRNAs are reported to regulate the expression of CXCL12 in various cancers.^{13, 27,29-39} Earlier studies have reported that tumor promoting miRNAs are overexpressed and tumor suppressing miRNAs are downregulated in cancer prone rectosigmoid part of colon in UC.⁴

In the present study we investigated the regulation of CXCL12 β through miRNA in UC. CXCL12 β expression was determined in colon biopsy samples as well as serum samples of UC patients and controls.

The impact of miR-200a over-expression on level of CXCL12 β was determined.

Materials and Methods

Patient Samples and cell lines: 30 UC patients and 25 non-IBD controls were included in this study. 20 samples from UC patients were from rectosigmoid region of colon and 10 were from ascending region of colon. Control samples were non IBD individuals without any IBD symptoms and without any colon inflammation. All control and patient samples were age and sex matched. Diagnosis of UC patients were based on the clinical findings based on ECCO guidelines [5]. UC was confirmed by histopathological analysis of colon biopsy samples. This study was approved by Institutional Ethics Review Board, JNU (REF no. 2012/student/28) and the ethical committee of All India Institute of Medical Sciences, New Delhi, India (Ref no. IEC/NP-320/2012). A written consent was taken from all the participants included in this study. Human colorectal adenocarcinoma cell line, HT-29 (National Center for Cell Sciences, Pune, India) were cultured in Dulbecco's Modified Eagle's medium (Gibco, Thermo Fisher Scientific, USA) for *in vitro* studies.

RNA isolation and RNA quality check: mirVana miRNA isolation kit (Ambion INC, TX 78744, USA) was used for total RNA isolation. RNA was stored at -80°C until further analysis. RNA quality was assessed by running it on 1.5% agarose gel for evaluating the three bands 28S, 18S and 5S rRNA. RIN value for the total RNA was

calculated using bioanalyser (Agilent Technologies, California, USA). Samples with RIN value more than 7 were included for microarray analysis.

Microarray Analysis: MiRNA microarray was performed by Exiqon A/S, Denmark via miRCURY LNA™ microRNA Array (7th gen). Five RNA samples were pooled in each category in equal concentration.⁶ The array data has been submitted in NCBI Gene Expression Omnibus (GEO) under the entry series GSE66932.

MiRNA target prediction and qRT-PCR: Target prediction for miRNAs targeting 3'UTR of CXCL12 β was done using online target prediction tools (Targetscan, Diana-microT and PicTar). RNA was reverse transcribed to cDNA using miRNA specific stem loop primers for miRNAs⁷ and Random Hexamer primers were used for cDNA synthesis for other genes by revert aid cDNA synthesis kit (Fermentas St. Leon Rot, Germany). Table 1 shows the primers sequences used for reverse transcription and real time PCR analysis. Real time PCR was performed using ABI PRISM 7500 Real time PCR system (Applied Biosystems). U6 snRNA and GAPDH were used as internal controls. Parameters for real time were- initial denaturation- 94°C for 2 min, denaturation- 94°C for 30 sec, annealing- 60°C for 1 min for 40 cycles. 20 μ l reaction containing 7 μ l MQ water, 10 μ l SYBER Green universal PCR Master Mix (Applied Biosystems, California, USA), 1 μ l of each forward and reverse primer (4 picomole/ μ l), 1 μ l cDNA. The relative expression was calculated using the equation $2^{-\Delta\Delta CT}$ where $\Delta\Delta CT = \Delta CT_{\text{target}} - \Delta CT_{\text{control}}$.

Table 1 Sequence of primers used in Reverse Transcription and qRT-PCR.*

Name		Primer (5'-3')
Reverse Universal Primer for miRNA		GTGCAGGGTCCGAGGT
hsa-miR-141-3p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGACTGGATACGCCATCTTT
	Forward	TAACACTGTCTGGTAAAGAT
hsa-miR-200a-3p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGACTGGATACGACATCGTT
	Forward	TAACACTGTCTGGTAAACGAT
CXCL-12β	Forward	CCTGCCACAGCCTCCCTT
	Reverse	TAAGCTGCTACGTGTCGCC

* RT primers were used for cDNA synthesis in each case; Reverse universal and forward primers were used for real time analysis for each miRNA

Cloning and transfection: miR-200a was cloned in pBABE-puro vector. DNA fragment containing sequence of miR-200a and its flanking region was used for cloning. Primers used for miR-200a cloning were Forward miR-200a 5'-CGCGGATCCACAGCCATCTTCCCTCCTG- 3' and Reverse miR-200a 5'-CCGGAATTCGAGCTGACACAGGCCCTC- 3'.⁸ 3'UTR of CXCL12 β was cloned in pMIR-Report vector downstream of luciferase gene. Primers used for cloning were Forward CXCL12 β 5'-CGAGCTCGGGTCAGACGCCTGAGGAAC-3' and Reverse CXCL12 β 5'-CGACGCGTACATTGAGTACAGAACTTTATT-3'. Transfection of plasmids was done using Lipofectamine 3000 (ThermoFisher Scientific, USA) following manufacturer's protocol.

Enzyme Linked Immunosorbent Assay (ELISA): CXCL-12 β concentration in the patient serum samples and cell culture supernatant was measured using ELISA kit (Sunred Biological, Shanghai). The assay was performed according to manufacturer's protocol and data was analysed using curve expert 1.4 software.

Luciferase assay: Luciferase assay was performed using Dual luciferase assay kit (Promega, USA). PRL-TK vector was used for normalization. CXCL12 β 3'UTR containing luciferase vector was

transfected in HT-29 cells with or without miR-200a overexpression.

Statistical analysis: Statistical analysis was done using two-way student's t test. Data analysis was done using Graphpad Prism 5.0. A probability level of $p < 0.05$ was considered statistically significant.

Results

CXCL12 β expression in UC vs Control individuals: CXCL12 β expression was analyzed both at mRNA as well as protein level. There was significant increase in CXCL12 β expression in biopsy samples from UC patients compared to controls (Figure 1a). Level of CXCL12 β was increased in the serum samples of UC patients compared to non IBD controls (Figure1b).

MiRNAs targeting 3'UTR of CXCL12 β : Target prediction analysis showed miR-141-3p and miR-200a, members of miR-200 family, targeting 3'UTR of CXCL12 β . Both target same two sites, position 51-58 and 2940-2948 in the 3'UTR of CXCL12 β (http://www.targetscan.org/cgi-bin/targetscan/vert_71/view_gene.cgi?rs=ENST00000374429.2&taxid=9606&member_s=&showcnc=0&shownc=0&showncf1=&showncf2=&subset=1).

Figure 1a

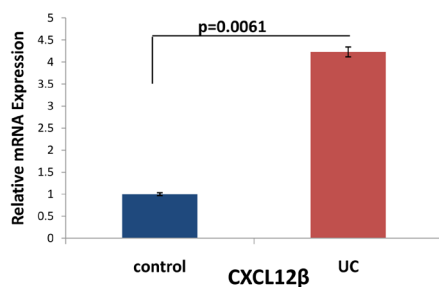


Figure 1b

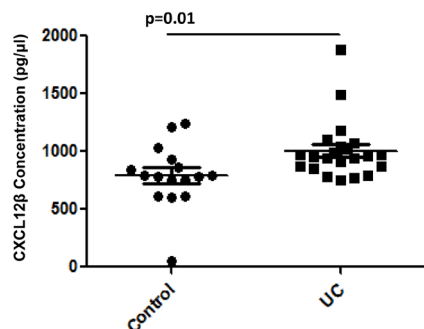


Figure 1 Increased expression of CXCL12 β in UC: **a)** Total RNA was isolated from colon biopsy samples from healthy individuals and UC patients and reverse transcribed to cDNA using random hexamer primers. Data derived from quantitative real-time PCR. Normalization was performed with the GAPDH. Sample size was n=25 for UC and n=25 for controls. There was significant increase in expression of CXCL12 β in UC patients compared to controls. Bars represent Standard Error of mean (SEM). **b)** Concentration of CXCL12 β measured in human serum samples by ELISA, n=25 in each category. There was significant increase in serum concentration of CXCL12 β in UC patients compared to controls.

Expression of miR-141-3p and miR-200a: miRNAs expression was analysed in UC compared to non-IBD controls using microarray

analysis. Figure 2 represents the heatmap for the microarray data describing the expression profile of miRNAs in UC and non-IBD controls. Expression of both the miRNAs was decreased in UC compared to control. Microarray data was validated using qRT-PCR. MiR-141-3p showed upregulation in our qRT-PCR analysis in contrary to microarray data (Figure 3a). Expression of miR-200a however decreased significantly in UC patients compared to controls in qRT-PCR results similar to microarray data (Figure 3b).

In vitro validation of CXCL12 β as a target of miR-200a: *In vitro* validation was carried out by miR-200a overexpression and reporter assay.

Effect of miR-200a overexpression on CXCL12 β secretion: We looked at the effect of miR-200a overexpression on CXCL12 β secretion in HT-29 cell line. Secretion of CXCL12 β decreased in the supernatant of HT-29 cells overexpressing miR-200a compared to control transfected and non-transfected cells (Figure 4).

Dual Luciferase assay: Further validation of CXCL12 β as miR-200a target was done using 3'UTR reporter gene assay. Luciferase was cloned upstream of 3'UTR of CXCL12 β and was used as a reporter. So the luciferase activity was controlled by miR-200a. The level of luciferase activity was decreased by overexpression of miR-200a (Figure 5).

Site specific expression of miR-200a and CXCL12 β in colon of UC patients: We looked at the site specific expression of CXCL12 β and miR-200a in rectosigmoid vs ascending colon of UC patients. Expression of miR-200a and CXCL12 β showed contrasting pattern of expression. Expression of CXCL12 β was increased in rectosigmoid colon in UC patients compared to controls. Expression of the chemokine in ascending colon was comparable to non-IBD controls (Figure 6a). Expression of miR-200a was decreased in rectosigmoid colon compared to ascending colon of UC patients and to non-IBD controls (Figure 6b).

Figure 2

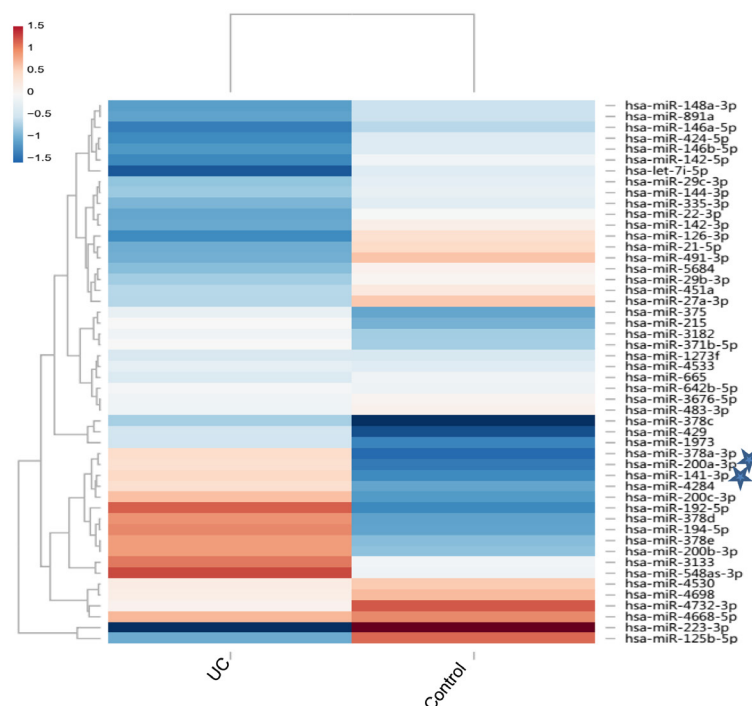


Figure 2 Heatmap and unsupervised hierarchical clustering of miRNAs. Heatmap showing differential miRNA expression in UC patients vs Controls.

Figure 3a

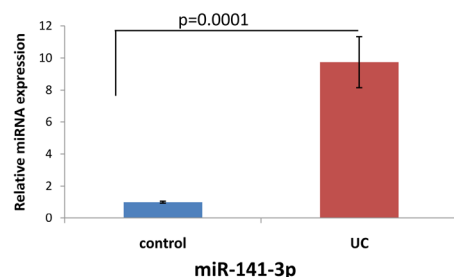


Figure 3b

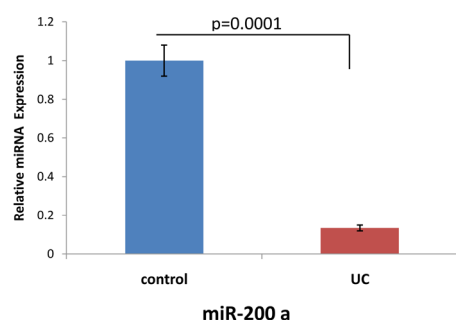


Figure 3 Relative Expression of CXCL-12 β targeting miRNAs. Total RNA was isolated from colon biopsy samples from healthy subjects and UC patients and reverse transcribed to cDNA using specific primers. Data derived from quantitative real-time PCR. Normalization of samples was performed with the small nuclear U6 snRNA. **a)** Relative expression of miR-141-3p in UC vs controls. **b)** Relative Expression of miR-200a-3p in UC vs controls. n=25 for each category. Bars represent SEM.

Figure 4

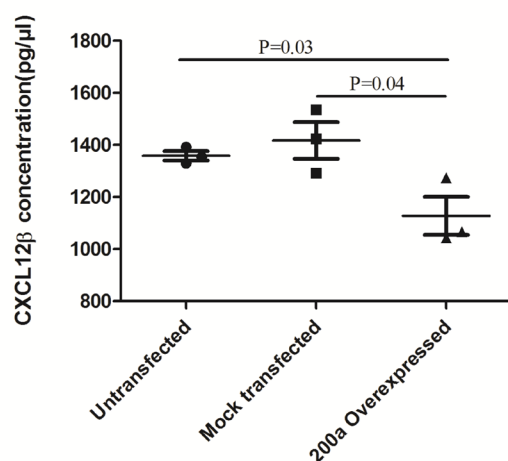


Figure 4 Effect of miR-200a-3p overexpression on CXCL-12 β secretion. miR-200a gene was cloned in pBABE-puro vector and transfected in HT-29 cell line. Untransfected cells and mock transfected cell were taken as controls. Cell supernatant was collected after 48hrs of transfection and CXCL-12 β concentration was measured using ELISA. Bars represent SEM.

Discussion

CXCL12 β is reported to play important role in chronic inflammation and various type of cancers.^{12,27,28} Different miRNAs were reported to regulate CXCL12 expression in different diseases.^{13,27,29–39} In this study we investigated how the expression of CXCL12 β is regulated by miRNA during UC. We also looked at site specific expression of

CXCL12 β and its targeting miRNA in context to CRC susceptibility of various parts of colon.

CXCL12 is a constitutive cytokine reported to play important role in various inflammatory diseases including lung, brain, joint and intestinal inflammation. CXCL12 is constitutively expressed by colon epithelial cells. Its expression was reported to be increased in IBD. Its expression is more pronounced in UC compared to CD and in inflamed mucosa compared to non-inflamed mucosa. CXCL12-CXCR4 axis contributes to intestinal epithelial cells migration, barrier function, restitution and cAMP mediated cellular functions in normal intestinal mucosa.^{44,45} Increased CXCL12 expression attracts immune cells which infiltrate and secrete proinflammatory cytokines in inflamed mucosa.¹³ CXCL-12 has different splice variants. Due to four additional residues at its C-terminus, CXCL-12 β variant is found more resistant to proteolysis. It can maintain a more potent concentration in the blood in order to recruit more leukocytes.^{46,47} Increased expression of CXCL12 in colonic mucosa is mainly contributed by increased expression of CXCL12 β .¹³ We looked at the expression of CXCL12 β in UC vs non-IBD controls. We also found increased expression of CXCL12 β at mRNA level in colonic mucosa (Figure 1a) and at protein level in the serum (Figure 1b) of UC patients compared to non-IBD controls.

We looked for the miRNAs targeting 3'UTR of CXCL12 β . It was predicted to be target of miR-141-3p and miR-200a. Both are member of miR-200 family and target same site in the 3'UTR of CXCL12 β . Expression of both miR-141-3p and miR-200a was decreased in our microarray analysis (Figure 2). We looked at the miR-141-3p expression in the colon of UC patients and controls. MiR-141-3p was reported to regulate CXCL12 β expression in the colon of CD patients.¹³ Expression of miR-141-3p was increased in the colon of UC patients compared to non-IBD controls in our real time analysis (Figure 3a). As miR-141-3p showed same expression as that of target gene, it revealed that probably miR-141-3p was not regulating the expression of CXCL-12 β in case of UC. Our real time expression data was in disagreement with our microarray data. Microarray data was often reported to show disagreement with real time analysis.⁹ We next did real time PCR to validate the expression of miR-200a-3p. Real time expression analysis showed that there was a significant decrease in the expression of miR-200a-3p (Figure 3b), as found in microarray analysis. This indicated that miR-200a-3p might be regulating the expression of CXCL-12 β in UC.

MiR-200a-3p was transfected and overexpressed in HT-29 cell line. Increase in expression of miR-200a-3p resulted in significant decrease in secretion of CXCL-12 β from HT-29 cell line (Figure 4). We performed dual luciferase assay in order to confirm if the regulation of CXCL-12 β is mediated by miR-200a-3p. Increase in expression of miR-200a-3p leads to significant decrease in luciferase activity (Figure 5).

During the process of intestinal inflammation, increased level of CXCL-12 β attract different immune cells, which further secrete a large volume of pro-inflammatory cytokines. CXCL12 is a constitutive and inflammatory chemokine in the intestinal immune system. Selective manipulation of the CXCL12/CXCR4 axis had been suggested as potential therapeutic mechanism in IBD.¹³ Blockade of CXCL12/CXCR4 Axis ameliorates murine colitis in experimental mice model of colitis.¹⁰ It has been hypothesized that the CXCL12 β targeted by miR-141 in colonic epithelial cells serves as an important mechanism regulating colonic CXCL12 β /total-CXCL12 expression and control immune cell trafficking during both experimental colitis models

and the CD dysfunction process in humans. Our expression data in patients and validation by in vitro studies proves our hypothesis that the expression of miR-200a-3p regulates the level of CXCL-12 β chemokine in the colonic cells during ulcerative colitis.

Figure 5

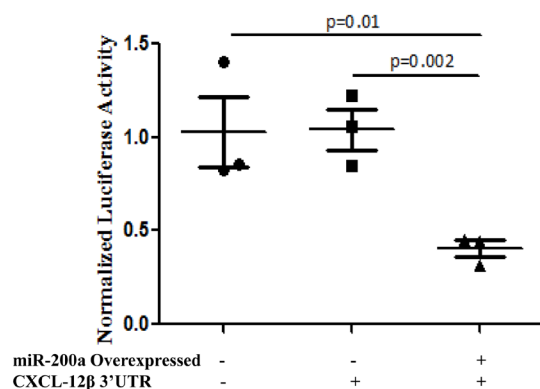


Figure 5 Dual Luciferase assay. 3'UTR reporter gene assay was performed to confirm CXCL-12 β as a target of miR-200a-3p. Luciferase was cloned upstream of 3'UTR of CXCL12 β and used as a reporter. Effect of miR-200a-3p overexpression was observed by measuring the luciferase activity. Bars represent SEM.

Figure 6a

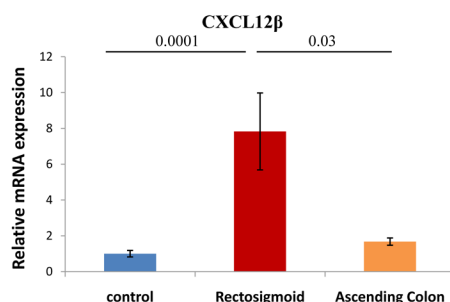


Figure 6b

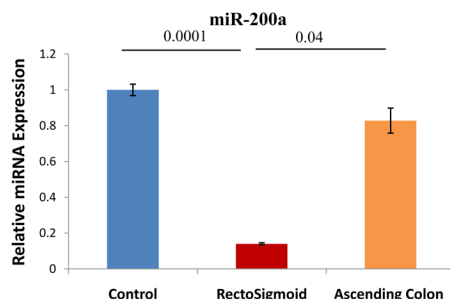


Figure 6 Site specific expression of CXCL-12 β and its targeting miRNA in colon. Expression of (a) CXCL-12 β (b) miR-200a was analyzed in Ascending colon and Rectosigmoid part of colon of UC patients. n=25 for controls, n=20 for Rectosigmoid and n=10 for Ascending Colon. Bars represent SEM.

Rectosigmoid part of the colon of UC patients was reported to be cancer prone in inflammation associated carcinoma development. MiRNA expression was also altered in the rectosigmoid part of colon compared to ascending colon in UC.⁴ Proinflammatory CXCL12-CXCR4 signaling axis has been reported to play important role in cancer development.¹⁰ We looked at the site specific expression of CXCL12 β and its targeting miRNA. There was significant increase in expression of CXCL12 β and the expression of miR-200a decreased significantly in the rectosigmoid part of colon compared to ascending colon and non-IBD controls (Figure 6a and 6b). This further strengthen

our analysis that miR-200a regulate the expression of CXCL12 β in UC and do so in a site specific manner. It also showed that CXCL12 may be an important factor in UC associated CRC development as it exhibited increased expression in the cancer prone rectosigmoid part of colon.

Conclusion

In conclusion our study show regulation of CXCL-12 β in UC is mediated by miR-200a, not by miR-141-3p as in CD. Our results further revealed that miR-200a was predominantly decreased in the colonic epithelial cells of UC patients leading to increase in expression of CXCL-12 β in the colon. Both CXCL12 β and its targeting miRNA showed site specific expression in UC. Inhibiting CXCL-12 β expression using miRNA may prove to be a valuable approach for treatment of IBD and to prevent IBD associated cancer development.

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Conflicts of interest

The authors declare that no conflicts of interest.

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