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Research Article

Chemokine expression modulations in primary and metastatic colorectal cancer

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Abstract

Background: Chemokine are small proteins primarily involved in cell movement, immune response, inflammation and tissue repair. Chemokine also plays an important role in disease conditions including cancer growth. The objective of this study was to analyze expressional modifications in the chemokine in primary and metastatic colorectal cancer by using clinical isolates and *in vivo* model respectively.

Methods: Serum samples from a total of 40 colorectal cancer patients (stage II and III) and healthy controls were collected following the due ethical codes. Circulatory levels of the chemokine (CCL family) were quantified using enzyme-linked immunosorbent assay (ELISA). To investigate metastasis associated expression changes of the chemokine, colorectal cancer animal model and microarray methodologies were exploited.

Results: Increased circulatory levels of CCL2, CCL17 and CCL22 were detected in stage II and III colorectal cancer patients when compared with healthy controls. In contrast, CCL11 levels were substantially low in colorectal cancer patients. As far as rat animal model for colorectal cancer liver metastasis is concerned, only CCL2 levels were considerably high during the early phase of colorectal cancer cells' implantation (Day 03) into the liver. Other chemokines did not demonstrate noticeable de-regulation during the whole period of experimental procedures (up to Day 21).

Conclusion: Differential circulatory levels of chemokine were detected in colorectal cancer patients and healthy controls. Liver metastasis of colorectal cancer cells was accompanied by a marked increase of CCL2 during early phase of settlement. The findings reflected a discrete change in the circulatory and metastasis associated with chemokine profile in colorectal cancer.

Key Words: Colorectal cancer, Chemokine, Liver metastasis, Circulatory levels, Expression changes

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INTRODUCTION

Cancer is known as the leading cause of mortality across the globe in 21st century. Colorectal cancer (CRC) is the 2nd most prevalent type of cancer in terms of mortality and ranks 3rd in terms of incidence, accounting for 9.6% of all new cancer cases globally in 2022, as based on estimations provided by the International Agency for Research on Cancer (IARC)[1-2]. Expressional changes in genes are important events during cancer onset and progression. Among these, chemokine plays a significant role. They are small sized chemo-attractive cytokines (8-14 kDa) responsible for the migration of epithelial cells, endothelial cells and leukocytes [3]. Chemokine influence tumor cell proliferation, stemness, invasiveness, and stromal processes like angiogenesis and fibrogenesis. They also modulate immune cell localization, activation, and interactions in the tumor microenvironment (TME), thus shaping immune responses [4]. Till now, over 50 chemokine have been identified and classified into four major subgroups known as the CC chemokine, CXC chemokine, CX3C chemokine, and C chemokine depending on the cysteine residues [5]. In this big family, 28 are CC chemokine [6] and among these CCL2, CCL11, CCL17, and CCL22 are under considerations in this study to explore their levels in clinical isolates and a liver metastasis model in rats. CCL2, also called Monocyte Chemoattractant Protein-1 (MCP-1), attracts macrophages and monocytes, thus participating primarily in tumor associated

inflammation. It supports the preservation of an immunosuppressive atmosphere, boosts proliferation and motility of cancer cells [7]. CCL11 (Eotaxin) principally recruits eosinophils, playing a role in inflammatory responses. CCL17 is referred as Thymus and activation-regulated chemokine (TARC) and CCL22, macrophage-derived chemokine (MDC), which controls migration of Th2 and Tregs., regulates the recruitment of these cells, which are often associated with immune suppression in tumor environment [8, 9]. The pro- or anti-cancer role of chemokine is a big debate and based on different types of chemokine, and cells/environment involved, differential response can be expected. Despite their renowned functions in different cancers, circulating levels of the above-mentioned chemokine in CRC patients and metastatic phase require further attention. Studying the variations of these chemokine levels could offer a glimpse at their potential as diagnostic/prognostic biomarkers and/or therapeutic targeting in CRC.

METHODS

Sample Collection

A total of 3-5 mL of blood from naïve CRC patients (22 stage II and 18 stage III) and gender/age matched healthy controls (40) were collected. Blood samples were transferred to BD serum separator vials. After a 30-minute coagulation period at room temperature, specimens were centrifuged at 1000 x g for 15 minutes to separate the serum. The serum was kept in nuclease-free vials at ≤ -80°C for further processing. Details about the participants are provided in Table 1. Informed consent was obtained from all patients and healthy controls. The procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

ELISA

Multi-Analyte ELISArray (Qiagen) kit was used to detect the concentrations of chemokine within the serum samples. Briefly, the healthy controls were randomly divided into two groups and pooled separately (20 samples/pool). In similar fashion, stage II and III CRC samples were divided into two groups and pooled (11samples/pool for stage II, 9samples/pool for stage III). 50µl aliquots from every pool were poured into distinct wells of the plate and the concentrations were measured as per manufacturer's guidelines. Absorbance readings were noted at 450nm wavelength utilizing a microplate reader.

Microarray

Microarray analysis was performed to highlight expressional modifications in CCL family of chemokine during the process of CRC liver metastasis. For this purpose, RFP-labelled rat CRC cells (CC531) were transplanted into the rat liver via hepatic portal vein mimicking a liver metastasis model. Afterwards, the growing cells were re-isolated at discrete time intervals (3, 6, 9, 14 and 21 days) followed by RNA extraction and cDNA microarray. A fraction of re-isolated cells was also cultured *in vitro* for 14 and 22 days to compare the results with *in vivo* grown tumor cells.

Data Analysis

Data was entered and analyzed by using SPSS 26.0 (statistical package for social science), where mean values were given for quantitative data. The qualitative variables (gender, age) were expressed as frequencies and percentage. An independent T-test was used to compare numerical variables (chemokine levels) between healthy individuals and CRC patients. A p-value of ≤0.05 was considered as statistically significant.

Table 1: Demographic data of the participants.

	Controls	Stage II CRC	Stage III CRC
Numbers	40	22	18
Gender	Male 26 (65%) Female 14 (35%)	Male 12 (55%) Female 10 (45%)	Male 11 (61%) Female 7 (39%)
Age (years)	Range 26-59 Average 48	Range 28-64 Average 49	Range 29-66 Average 51

RESULTS

Circulatory levels of CCL family of chemokine in colorectal cancer patients

The present work highlighted the circulatory levels of various chemokine ligands present in the serum of CRC patients and were compared with those of healthy controls. In our continuous sampling, most of the samples (>90%) were from stages II and III of CRC, so we restricted ourselves to the patients with these stages. The mean CCL2 level of healthy controls (56pg/ml), was slightly but not significantly lower than those of patients with stage II (62pg/ml) or stage III (74pg/ml) CRC. In contrast to this, the

average concentration of CCL11 in healthy controls was higher (122pg/ml) than that of stage II (76pg/ml) and stage III (66pg/ml) CRC patients. The average circulatory level of CCL17 was highest in stage II CRC patients (45pg/ml), whereas that of stage III patients (34pg/ml) was significantly lower ($p < 0.03$), as was that of healthy controls (29pg/ml). CCL22 followed a similar pattern as observed for CCL2, where a gradual, but non-significant increase in circulatory level was found in stage II (75pg/ml) and III (89pg/ml) CRC patients as compared to healthy controls (68pg/ml). Observed changes in levels of the CCL family are shown in Figure 1.

Expression modulations in chemokine during colorectal cancer liver metastasis

Metastasis is a complex mechanism, where cancer cells leave their primary and move to distant secondary sites for further growth. This process is accompanied by numerous expressional changes, which enable the cells for metastasis. To evaluate the potential changes in the CCL chemokine during CRC liver metastasis, CC531 cells (rat CRC) were implanted into rat livers and following re-isolation of the cells after 3, 6, 9, 14 and 21 days, expressional profiling was done via cDNA microarray. The results were compared with the microarray data of the cells re-isolated on day 21 and then cultured *in vitro* for 14 and 22 days. Overall changes in the expression levels of selected chemokine during this time period are shown in Figure 2. The majority of CCL chemokine (CCL11, CCL17, CCL22) showed marginal change (≤2fold) during the complete period of experiment. In contrast, CCL2 was the only CCL chemokine with substantial expressional modifications. More specifically, these changes were obvious during the early implantation period (Day 03) and *in vitro* culture (Day 14). To summarize, substantial de-regulation of expression was observed in CCL2 chemokine only during CRC liver metastasis (Figure 2).

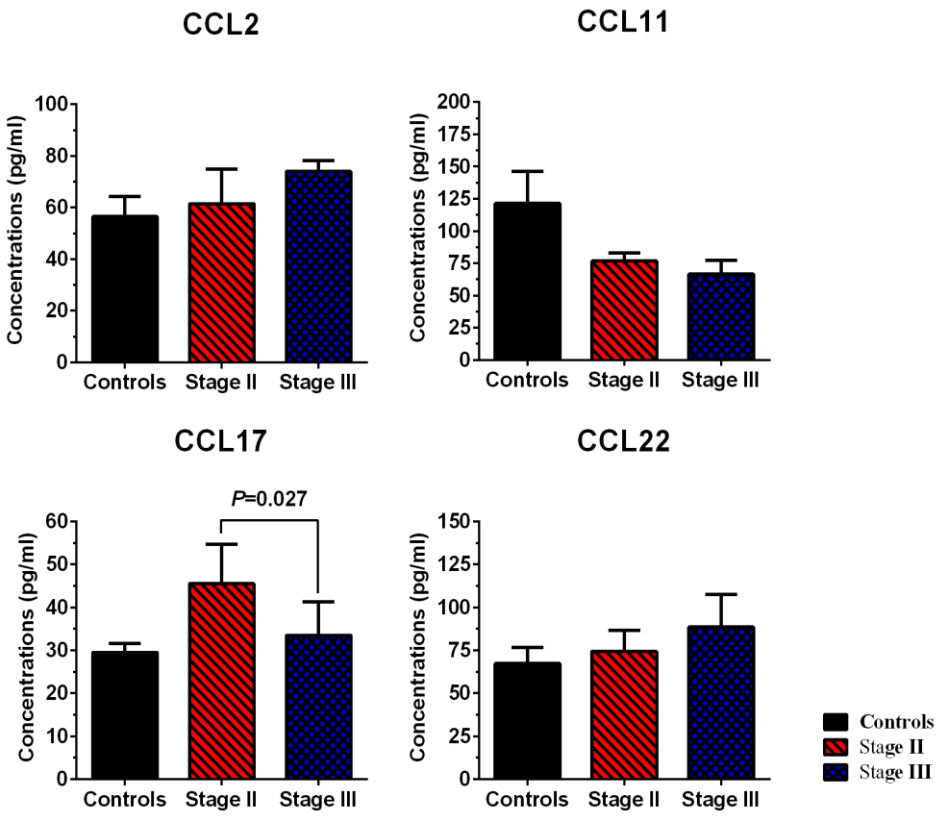

Figure 1: Chemokine concentration among healthy controls, stage II and III CRC patients. Serum samples of the patients were used to determine the levels of selected chemokine while using ELISA methodology.

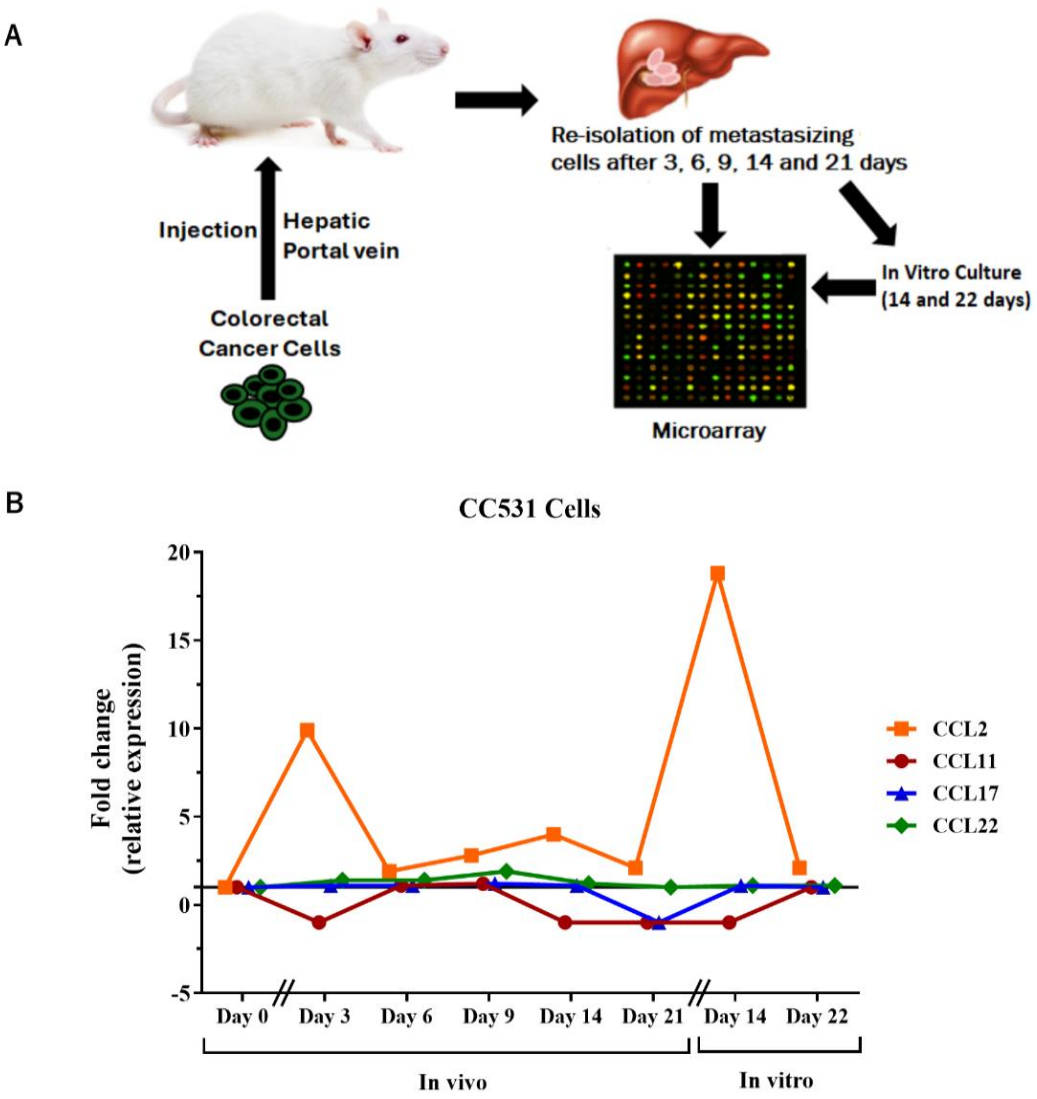

Figure 2: Evaluation of CCL chemokine expressions during colorectal cancer liver metastasis. **A.** Rat colorectal cancer cells were implanted in the liver, re-isolated after selected time intervals and used for expression analysis via cDNA microarray. **B.** Expression modulations in the chemokine' RNA are shown over a period of colorectal cancer liver metastasis, while using *in vivo* model.

DISCUSSION

Differential expressions of the chemokine pathways in disease states is a well-recognized phenomenon. Depending on their kind, concentration, and tissue involvement, chemokine might assist or hinder disease progression. In cancer, this complicated network governs immune cell infiltration, tumor development, survival, and migration. Numerous cells, including immunological, stromal, and tumor cells, express and respond to chemokine variations. Despite substantial research, specific chemokine profiles for certain cancer types remain unknown, particularly in terms of circulatory levels against tissue expression. Given chemokine network's functional involvement in cancer, more investigation is needed regarding its circulating levels. In this study, the focus was to identify the circulatory of CCL chemokine in clinical isolates of colorectal cancer patients. Over 90% of patients in this study had advanced CRC (stage II and III), likely due to late diagnosis, lack of early screening, asymptomatic disease, and/or weak local healthcare structure. To ensure statistically significant results at the end of study, we focused on stage II and III samples. Chemokine levels were measured using an ELISArray plate coated with antibodies for each chemokine, and results were compared with healthy controls. Distinct but mainly non-significant changes in chemokine concentrations were observed in CRC patients compared to healthy controls. CCL2 ligand has been reported to promote carcinogenesis and metastasis of CRC [11-12]. In this study, non-significantly elevated levels of CCL2 were observed in both stage II and III CRC patients. In fact, a gradual increase in CCL2 was observed with the increasing stage of CRC, which shows that higher circulatory

levels of CCL2 are related to more advanced stages of the disease and may play a direct role in further progression. CCL11 circulatory levels were distinctly lower in CRC patients in comparison to healthy individuals in this study. CCL11 has been mainly associated with immune cell migration to tumor lesions and plays a pivotal role in metastasis process. A significant decline in CCL11 levels has been associated with decreased immune cell infiltration to tumor area in CRC, which ultimately helps tumor cells to escape from the anti-tumor immunity [13]. This study showed higher expression of CCL17 and CCL22 in CRC patients when compared to the normal healthy controls. In our results, only CCL17 chemokine showed statistically significant differences ($P \leq 0.03$). Other studies showed elevated expressions of CCL17 and CCL22 mRNA expressions in CRC tumors which strengthen the findings of this study. Moreover Bioinformatics analysis showed that high CCL22 expression is linked to increased immune-suppressive cells and decreased antitumor immune cells [14-15]. Metastasis is a complex mechanism that imposes the major challenge while treating cancer. Considering this, precise knowledge about the molecular mechanisms involved in metastasis is crucial. In this study, while using a rat model for CRC liver metastasis, we explored the expression changes in CCL chemokine. CCL2 was the only chemokine among the selected members, which showed a substantial change in expression. These modifications were especially witnessed during early period of implantation in rat liver (Day 03) or *in vitro* culturing (Day 14). This trend shows that higher levels of CCL2 are required by the CRC cells during early settlement period in the new environment. Thus, from therapeutic perspective, targeting the CCL2 during the initial phase of metastasis can be instrumental. As we know, changes in RNA levels ultimately lead to protein level alterations and observed variations in the liver metastasis rat model can be extrapolated to human diseased conditions. In summary, chemokine showed differential level of expression in CRC patients as compared to healthy individuals. As the chemokine levels were detectable in picogram levels, they can be used as prognostic markers especially when their levels are higher in the cancerous conditions. Furthermore, discrete changes observed during *in vivo* part of this study show a potential of targeting of CCL chemokine (CCL2 for instance) during CRC liver metastasis.

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Ethics Approval: Animal experiments were approved by the relevant governmental animal ethics committee (Regierungspräsidium Karlsruhe, Germany) and all institutional guidelines for the care and use of animals were followed. For clinical investigations, the study was approved by the Ethical Review Committee for Medical and Biomedical Research, University of Health Sciences, Lahore, Pakistan.

Author Contributions: Syeda Faiza collected the clinical samples and performed the ELISA experiments. Afraz Numan analyzed the data and helped with manuscript writing. Martin R. Berger helped *in vivo* experiments. Asim Pervaiz conceived the study, supervised the experiments and wrote the manuscript draft.

Competing Interests: The authors declare that they have no conflict of interest.

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.