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Cross Sectional Study

Correlation between CXCL12 and MMP-7 Expression with Lymph Node Metastasis Status in Colorectal Adenocarcinoma

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ABSTRACT

Colorectal cancer is the third most frequent cancer worldwide, whereas histopathologically, adenocarcinoma is the most common type. High expression of CXCL12 in tumor cells influences tumor cell metastasis. CXCL12 will activate CXCR4, which progressively upregulates MMP-7 expression and degrades the extracellular matrix, causing the migration of cancer cells. The status of regional lymph node metastases influences prognosis. Therefore, accurate biomarkers are required to diagnose lymph node metastases. The research analyzed the expression of CXCL12 and MMP-7 to reveal their role in the process of colorectal cancer regional lymph node metastasis. This research applies an analytical observational method with a cross-sectional approach. The number of samples was 47, divided into 3 groups based on regional metastasis stage (N0, N1, and N2). Immunohistochemistry was performed with CXCL12 and MMP-7 antibodies. The relationship between CXCL12 and MMP-7 expression was analyzed using statistical tests. There were significant differences in CXCL12 and MMP-7 expression at various N stages ($p = 0.005$ and $p = 0.001$). There was a positive correlation between CXCL12 expression and MMP-7 expression in various N stage of colorectal adenocarcinoma ($p=0.004$). According to this study, CXCL12 and MMP-7 expression are associated with regional lymph node metastases in colorectal adenocarcinoma. Results suggest CXCL12 and MMP-7 represent an essential role in lymph node metastasis in colorectal adenocarcinoma.

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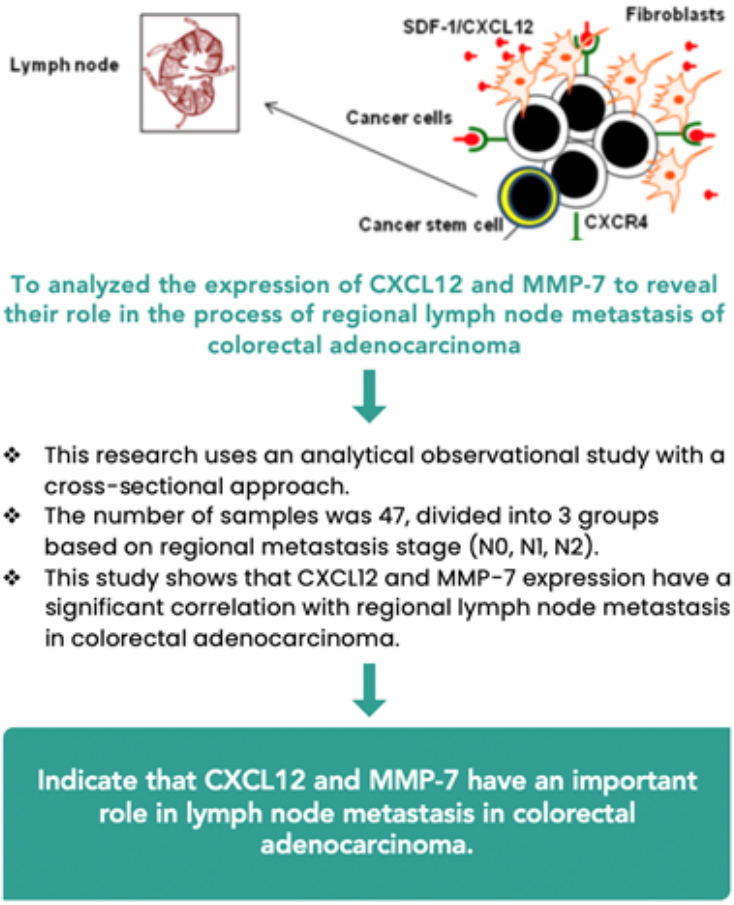
GRAPHICAL ABSTRACT



Introduction

The most prevalent type of gastrointestinal malignancy is colorectal cancer. There are approximately 1.8 million new cases, with 0.86 million deaths world wide, it is currently the third greatest factor in death in men and the second greatest cause of death in women [1-4]. According to 2020 GLOBOCAN data, the number of colorectal cancer cases in Indonesia is relatively high, ranking fourth with 34,189 new cases, accounting for around 8.8% of overall cancer cases. Adenocarcinoma is the most common histopathological type of colorectal cancer [5]. TNM staging was used to assess tumor progression. The survival of colorectal cancer patients is determined by stage [6, 7]. The more nodal metastases there are, the higher the stage, and the worse the prognosis. According to one study, if more than six nodal metastases are discovered, the patient's survival rate drops by 10% every five years, and if more than sixteen nodal metastases are discovered, the patient dies within five years [8, 9]. Colorectal cancer has a favourable prognosis if caught early before metastases develop. Within 5 years of surgery, 40% of cases had recurrence or metastasis, which is the main reason the prognosis worsens [10]. The causal factors are non-specific initial symptoms, a lack of patient knowledge, and delays in diagnosis by medical personnel, so more accurate biomarkers to determine the status of colorectal cancer lymph node metastases are hoped to be discovered [1, 11]. Chemokines are chemoattractant chemicals that can attract specific cells to a site, aid in cancer metastasis, and disseminate cancer from the main tumor to distant regions in the body. CXC chemokines, such as CXCL12, are involved in cancer metastasis [12].

In several cancers, the chemokine CXCL12 and its receptor CXCR4 play a role in metastasis [10, 13]. CXCL12 expression has been observed in tumor cell membranes. This is related to the cell's potential to spread or metastasize to regions far from the original tumor location, whereas expression in the cytoplasm is related to cell growth and development regulation. CXCL12 regulates cell proliferation and development in this way. CXCL12 expression in colorectal cancer cells is an important component in colorectal cancer prognosis [14, 15]. According to one study, CXCL12 is the most potent chemokine that stimulates proliferation, angiogenesis, invasion, and migration, as well as facilitating tumor spread. However, several researchers disagree: there is a decrease in CXCL12 expression levels in colorectal cancer [4, 16]. Proteolytic enzymes (matrix metalloproteinase) have a role in controlling extracellular matrix (ECM) degradation, which occurs in numerous biological processes, which occurs in many biological processes; including angiogenesis, cell proliferation, differentiation, and tumor cell migration [13-17]. Humans have over 21 MMPs. Based on their affinity for extracellular matrix (ECM) components; MMPs are classified as collagenase, gelatinase, stromelysin, and matrilysin [18]. The smallest member of the MMP family is MMP-7 (Matrilysin, Pump 1) [19]. Matrix metalloproteinase-7 expression is normally found in the cell membrane. However, in colorectal cancer cells, it can be found in the cytoplasm as well. Matrix metalloproteinase-7 expression in cancer cells' cytoplasm might accelerate the process of extracellular matrix disintegration, allowing cancer cells to migrate to neighboring tissues and organs, worsening the prognosis and increasing the potential of metastasis [20]. MMP-7 expression was not connected with colorectal cancer prognosis, according to Schwandner *et al.*, however, this contradicts multiple previous research [21, 22]. Using immunohistochemical methodologies, this study examined the association between CXCL12 and MMP-7 expression in relation to regional lymph node metastatic status. This study was carried out because it is unclear how the link between CXCL12 and MMP-7 expression can be employed as an appropriate prognostic indicator in determining the existence of metastases in regional lymph nodes in patients with colorectal adenocarcinoma.

Materials and Methods

Study design

A cross-sectional, retrospective, and analytical observational design was used in this research. A simple random sampling strategy was used to gather 47 samples. The samples were paraffin blocks from surgical tissue from colorectal adenocarcinoma patients with various N stages (N0, N1, and N2), as determined by histopathological pictures at RSUD. Dr. Soetomo Surabaya from January 2016 to December 2020. The inclusion criteria were paraffin blocks derived from colorectal adenocarcinoma tumor tissue with a minimum number of regional lymph nodes of 12 nodules according to the recommendations of the CAP [7, 23]. The paraffin blocks were of acceptable quality and contained tumor cells that were fairly representative for immunohistochemical evaluation. The qualifications for exclusion are that there is an additional cancer diagnosis in the preparation.

Immunohistochemistry

Paraffin blocks of HE preparations are cut to a thickness of 3-5 microns and mounted on special slides. Deparaffinization using xylol solution was followed by rehydration with 96%, 90%, and 80% alcohol for 2 minutes each, then washing with running water and distilled water for 5 minutes each. The slide is then immersed in a 3% H_2O_2 methanol solution for fifteen minutes at the ambient temperature. After that, the slides will be cleaned using purified water for 5 minutes before being heated in a decloaking chamber for 20 minutes at 95 degrees Celsius using Target Retrieval Solution (TRS) or Buffer Citrace at pH 6. The next step is to drop a background snipper for 15 minutes, then the primary antibody is dropped, namely a monoclonal antibody against CXCL12, namely rabbit polyclonal antibody IgG (A18225) (dilution 1:100, ABclonal Biotechnology), and a monoclonal antibody against MMP-7, namely rabbit monoclonal

antibody IgG (A20701) (dilution 1:1,500, ABclonal Biotechnology). The slides were then incubated for 60 minutes at the ambient temperature before being washed with PBS for 5 minutes. Secondary antibodies (Trekkie Link) were dropped and incubated for 20 minutes. PBS was washed for 5 minutes. Next, the second secondary antibody (HRP label) was dropped for 10 minutes. PBS was washed for 5 minutes. Diaminobenzidine (DAB) was dropped and incubated at the ambient temperature for 5 minutes, and then it was washed with running water for 5 minutes and placed in Mayer Hematoxylin at room temperature for 10 minutes. Running water was used to clean and dehydrated with 80%, 90%, and 96% alcohol for 2 minutes each. Finally, put it in Xylol and cover the slide with a cover glass. The assessment will be conducted by two experts in anatomical pathology. Olympus CX31 binocular light microscope A double-headed microscope was used to assess differences in observation results.

Evaluation of immunohistochemistry expression levels

The histochemical scoring evaluation (H-score) scoring method is used to interpret immunoreactivity, with CXCL12 expression seen in the membrane and cytoplasm of tumor cells and MMP-7 expression seen in the cytoplasm [24]. This evaluation entails adding together all of the intensity scores seen in tumor cells (negative intensity = 0, weak intensity = 1, moderate intensity = 2, to high intensity = 3), the percentage of stained tumor cells in each category was recorded, and the H score value was calculated by the following method: (3x% strong staining) + (2x% moderate staining) + (1x% weak staining)+ (0x% weak staining). This algorithm yields an H-score ranging from 0 to 300. The H-score value range is explained as follows: 0 represents a negative outcome, 1-100 represents a weak result, 101-200 represents a moderate result, and 201-300 represents a strong result. The assessment will be conducted by two experts in anatomic pathology. If the score between researchers has a difference of more than 30, a joint assessment will be carried out.

Statistical analysis

The EZR program was used for analysis of statistics. Using the Spearman correlation test, we examined the correlation across CXCL12 with MMP-7 expression, as well as their associated with various metastatic statuses in regional lymph nodes. If the p value of a statistical test is less than 0.05, the result is considered as significant.

Ethics approval and consent to participate

The authors employed colorectal cancer paraffin blocks as research subjects instead of humans or animals. On Juny 18th, 2023, the Dr. Soetomo General Academic Hospital Research Ethical Committee approved this study in accordance with the regulations of the Office for Human Research Protection (OHRP), with Reference No.1341/LOE/301.4.2/VI/2023.

Results and Discussion

The results of this research include CXCL12 and MMP-7 expression, as well as gender, age, N stage (N0, N1, and N2), tumor location, and tumor grading (Table 1).

Patient and tumor characteristics

The average age of patients with colorectal adenocarcinoma in this study was ± 56.36 years. The youngest age upon diagnosis was 29 years, while the oldest was 79 years. The sample distribution was then sorted into five groups with 10-year intervals depending on age. The 50-59-year age group had the most participants in this research sample, with 15 instances (31.92%), while the 40-year age group had the fewest, with 3 cases (6.38%).

The average age of sufferers was found to be 56.36 years, with the biggest age group of sufferers being in the 50-59 year age range with 15 cases (31.92%), and the lowest number being in the 40-year age group with 3 instances (6.38%).

Table 1: Characteristics of colorectal adenocarcinoma patients

Characteristics	Total
Population	47
Gender	
Male	24 (51.06%)
Female	23 (48.94%)
Age	
<40	3 (6.38%)
40- 49	11 (23.40%)
50-59	15 (31.92%)
60- 69	9 (19.15%)
70-79	8 (17.02%)
N stage	
N0	20 (42.55%)
N1	15 (31.92%)
N2	12 (25.53%)
Location	
Colon ascendens	11 (23.40%)
Colon transversum	2 (4.26%)
Colon descendens	6 (12.77%)
Rectum	28 (59.58%)
Tumor grading	
Low grade (well and moderately differentiated)	42 (89.36%)
High grade (poorly differentiated)	5 (10.64%)

This study is consistent with previous research, who found that the highest incidence of colorectal adenocarcinoma occurred in patients over 50 years old and the lowest in those under 40 years old, with only four cases (1%) [25, 26]. There are various reasons why adults beyond the age of 50 years become at greater risk to be diagnosed with colorectal cancer.: Chronic Inflammatory Bowel Disease (IBD), which often occurs in individuals over the age of 50, is believed to have been correlated with an increased probability for developing colorectal cancer. As age increases, the cells in the body experience the possibility of genetic mutations that can lead to the development of colorectal cancer. A family history of colorectal cancer or genetic mutations such as Familial Adenomatous Polyposis (FAP) syndrome or Lynch syndrome all are some examples of risk factors [27]. The sample for this study included 47 cases of colorectal adenocarcinoma, with male patients having a greater incidence of cases than female patients, with 24 (51.06%) male patients and 23 (48.94%) female patients. According to the literature, the risk of colorectal adenocarcinoma occurs more frequently in men rather than women, and it is related to hormonal factors, obesity, alcohol usage, red meat consumption, and smoking [5]. The tumor site is essential because it affects the type of therapy advised, increases the risk of metastasis, and reflects changes in molecular characteristics [28]. The rectum was the most common tumor location in this study, accounting for 28 cases (59.58%), followed by the ascending colon, which had 11 instances (23.40%), the descending colon, which had 6 cases (12.77%), and the transverse colon, which had 2 cases (4.26%). The results of this research are consistent with previous research, who found that the rectum had the highest rate of colorectal adenocarcinoma at 320 (46.18%), followed by the ascending colon at 174 (25.11%), and the transverse colon at 30 (4.33%) and the anus at 11 (1.59%) [29]. Different risk factors can contribute to the increased prevalence of rectal cancer compared to colon cancer. Rectal cancer risk factors include a family history of the disease and a history of rectal polyps. Aside from that, the distinct structure of the colon and rectum can influence cancer prevalence. Because of its closer

position to the anus, the rectum tends to have more easily detectable symptoms, such as the presence of blood in the stool or changes in bowel patterns. These symptoms make rectal cancer more frequently detected compared to colon cancer which may not show obvious symptoms in its early stages [30]. Colorectal cancer (CRC) is graded based on the glandular formation within the tumor, which can be classified as low-grade (formerly known as well as moderately differentiated) or high-grade (poorly differentiated). This rating is based on the component with the lowest level of differentiation [28]. The degree of differentiation is an important prognostic factor in colorectal cancer, with a higher grade indicating a poorer life expectancy [31]. According to this study, the low-grade group has 42 instances (89.36%) and the high-grade group has 5 cases (10.64%). The results of this research are consistent with previous research, that examined 19 adenocarcinoma colorectal samples, 11 of which were low-grade (57.89%) and 5 of which were high-grade (26.32%) [32].

The TNM system published by the 8th edition of the American Joint Committee on Cancer (AJCC) was utilized for colorectal adenocarcinoma staging [7, 28]. Based on histological findings, this investigation determines stage N status (regional lymph node metastases). This analysis used samples from colorectal adenocarcinoma cases with a minimum of 12 lymph node [7, 28]. According to this study, the most N0 metastases were found in 20 instances (42.55%), 15 cases (31.91%), and 12 cases (25.53%). This study is similar to that of Jia *et al.*, who employed 28,014 colorectal cancer samples. Stage N lymph node metastasis was assigned to 15,989 cases (57.08%), 7,643 (27.28%) cases, and 4,382 (15.64%) cases [33]. The incidence of early stage colorectal cancer is more than the advanced stage due to several factors, one of which is the presence of symptoms that are more obvious and easily recognized. Public awareness and understanding of the symptoms and risks of colorectal cancer are factors that play a role in early diagnosis. Colorectal cancer awareness efforts and good screening programs can help improve colorectal cancer detection rates [34].

CXCL12 expression

In this study, CXCL12 expression was observed in every single group of N stage colorectal adenocarcinoma patients. Assessment of CXCL12 expression was carried out using a semiquantitative method in the form of a histochemical scoring assessment (H-score), this is an evaluation based on the intensity of staining and the percentage of cells stained at each intensity level [24]. Positive expression of CXCL12 is seen in the membrane and cytoplasm of tumor cells in colorectal adenocarcinoma (Figure 1).

The distribution of CXCL12 expression in all samples was mostly moderately positive in 25 (53.19%) samples. The highest CXCL12 expression in the N0 group was moderate positive expression in 12 (25.53%) samples, followed by strong positive expression in 6 (12.77%) samples, and weak positive expression in 2 (4.26%) samples. The highest CXCL12 expression in the N1 stage group was a moderate positive expression in 11 (23.4%) samples, followed by a strong expression in 4 (8.51%) samples. In the N1 stage group, there was no weak CXCL12 expression. The highest CXCL12 expression in group N2 was strong positive expression in 10 (21.28%) samples, followed by moderate expression in 2 (4.26%) samples. In group N2, there was no weak CXCL12 expression (Table 2).

Because the data were not normally distributed, the non-parametric Kruskal-Wallis test was used to analyze variations in CXCL12 expression at various N stages. The investigation revealed a significant difference in CXCL12 expression with stage N0, N1, and N2 colorectal adenocarcinoma, with a p-value of 0.005 (p 0.05) (Table 3).

The CXCL12 ligand, known as SDF-1, is involved in tumor growth and spread. CXCL12 expression is linked to an increased risk of metastasis.

CXCL12 expression can also influence tumor cells' ability to proliferate, migrate, and attack target organs. Studies conducted by Goita et al., the expression of CXCL12 is linked to lymph node metastasis, venous invasion, and metastases to other distant organs, as well as lower survival [9, 35].

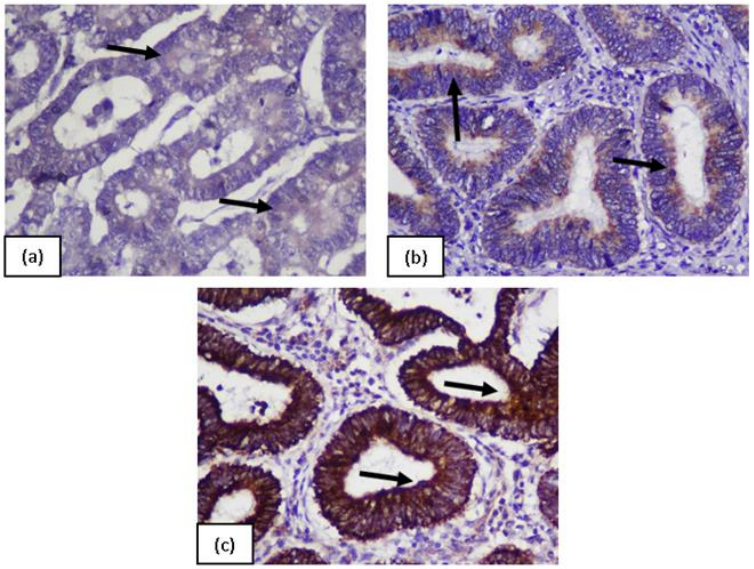


Figure 1: CXCL12 expression in colorectal adenocarcinoma tumor cells. Tumor cells are stained in the cytoplasm (black arrow). (a) weak expression in the cytoplasm and membrane of tumor cells; (b) moderate expression in the cytoplasm and membrane of tumor cells; and (c) strong expression in the cytoplasm and membrane of tumor cells. 400x magnification

Table 2: CXCL12 expression at stages N0, N1, and N2

CXCL12 expression		N Stage						Total N	*P*-value
Score H-Score	Interpretation	N0		N1		N2			
		N	%	N	%	N	%		
Score 0	Negative	0	0	0	0	0	0	0	0.005
Score 1	Weak Positive	2	4.26	0	0	0	0	2	
Score 2	Medium Positive	12	25.53	11	23.4	2	4.26	25	
Score 3	Strong positive	6	12.77	4	8.51	10	21.28	20	
Total		20	42.55	15	31.91	12	25.54	47	

Table 3: Spearman correlation test results between CXCL12 expression and MMP-7

Parameter	*P*-value
Correlation of CXCL12 expression with N stage	0.005
Correlation of MMP-7 expression with N stage	0.001
Correlation of CXCL12 and MMP-7 with N stage	0.004

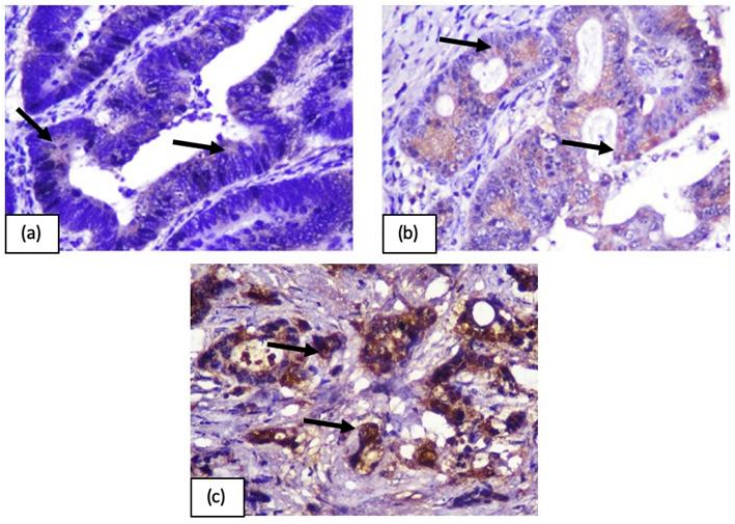


Figure 2: MMP-7 expression in colorectal adenocarcinoma tumor cells. Tumor cells are stained in the cell cytoplasm (black arrow). (a) The weak expression in the cytoplasm of tumor cells; (b) moderate expression in the cytoplasm of tumor cells; and (c) strong expression in the cytoplasm of tumor cells. 400x magnification

CXCL12 expression is a possible biomarker and therapeutic target in treatment [27]. One meta-analysis review concluded that increased CXCL12 expression is associated with an increased risk of spread to other parts of the body and a generally worse prognosis in a number of cancers, including esophageal, gastric, pancreatic, and lung cancers [37]. Chemokines are prevalent in the tumor microenvironment and serve a crucial function in modulating blood vessel development and tumor infiltration [17, 38]. CXCL12 will interact with its unique receptor, CXCR4. The CXCR4 activation pathway subsequently connects to a heterotrimeric G-protein complex on the plasma membrane's inner surface. This complex is made up of three subunits: Gα, Gβ, and Gγ [39]. When the CXCL12/CXCR4 connection is activated, the Gα subunit dissociates into the Gα subunit coupled to GTP, resulting in the formation of the GβGγ dimer. This activates phosphatidylinositol-3-kinase (PI3K) [39, 40]. Activated inositol (1, 4, 5)-trisphosphate (PI3K) will rapidly activate the AKT pathway, this is essential for cancer cell metastasis [16, 31, 41]. The Ras/ERK signaling pathway regulates gene expression. G monomers stimulate ERK via the RAS pathway, a kinase involved in the regulation of extracellular signals [31, 40].

The ERK pathway is involved in MMP-7 activation. MMP-7 activity increases separation between cells, which is directly related to the high potential for invasion and spread [10, 42].

Expression MMP-7

In this study, MMP-7 expression was observed in every single group of N stage colorectal adenocarcinoma patients. The assessment of MMP-7 expression was carried out using a semiquantitative method in the form of a histochemical scoring assessment (H-score), this is an evaluation based on the intensity of staining and the percentage of cells stained at each intensity level [24]. Positive expression of CXCL12 is seen in the cytoplasm of tumor cells in colorectal adenocarcinoma (Figure 2).

The distribution of MMP-7 expression in all samples was mostly weak positive expression in 29 (61.7%) samples. MMP-7 expression was highest in the N0 stage, with as many as 18 (38.3%) samples, followed by moderate positive expression in 2 (4.26%) samples, and in the N0 stage group there was no strong positive expression. The highest MMP-7 expression in the N1 stage group is weak expression in 8 (17.02%) samples, followed by moderate expression in 6 (12.77%) samples, and strong expression in 1 (2.13%). The highest MMP-7 expression in the N2 group was moderate positive expression in 8 (17.02%) samples, followed by weak expression in 3 (6.38%) samples, and strong positive expression in 1 (2.13%) sample (Table 4).

Numerous studies demonstrate that MMP-7 is important in tumor formation pathways that involve proliferation of tumor cells, invasion, metastasis, and angiogenesis. MMP-7 can divide ECM substrates such types IV the protein collagen, fibronectin, proteoglycans, vitronectin, laminin, gelatin, elastin, and entactin [44]. The findings of this study are consistent with previous studies demonstrating MMP-7 expression is higher in more aggressive cancer cells and is linked to tumor development, lymph node metastasis, and distant metastasis [16].

Our findings are in accordance with a previous study by Wattanawongdon and Bartpho that showed a correlation between MMP-7 expression with invasion of blood and lymphatic vessels, confirming the role of MMP-7 in the process of gastric cancer invasion and metastasis. MMP-7 expression was also significantly associated with an increased TNM grade, according to statistical analysis [43].

Previous research identified a significant correlation between higher MMP-7 expressions with N stage gastric cancer [44].

Because the data were not normally distributed, the non-parametric Kruskal-Wallis test was used to analyze variations in MMP-7 expression at various N stages. The investigation revealed a significant difference in MMP-7expression with stage N0, N1, and N2 colorectal adenocarcinoma, with a p-value of 0.005 (p 0.05) (Table 4).

Correlation between CXCL12 and MMP-7 Expression

Table 4: MMP-7 expression at stages N0, N1, and N2

MMP-7 expression		N Stage						Total N	*P*-value
Score H-score	Interpretation	N0		N1		N2			
		N	%	N	%	N	%		
Score 0	Negative	0	0	0	0	0	0	0	0.001
Score 1	Weak Positive	18	38.3	8	17.02	3	6.38	29	
Score 2	Medium Positive	2	4.26	6	12.77	8	17.02	16	
Skor 3	Strong positive	0	0	1	2.13	1	2.13	2	
	Total	20	42.56	15	31.92	12	25.53	47	

The Spearman correlation test was employed in evaluating the correlation between CXCL12 and MMP-7 expression in colorectal adenocarcinoma with various regional lymph node metastatic statuses. Spearman's non-parametric correlation test was used to examine the correlation between CXCL12 expression and MMP-7 expression with adenocarcinoma colorectal N stage. The statistical analysis indicated a significant correlation with coefficient of 41.3% and a p-value of 0.004 (p 0.05) (Table 4).

The CXCL12/CXCR4 binding activates the G subunit, which then dissociates into the GTP-bound G subunit, resulting in the formation of the GG dimer. This activates phosphatidylinositol-3-kinase (PI3K) [39, 40]. When PI3K is activated, it produces inositol triphosphate (3,4,5)-triphosphate and activates the AKT pathway. The stimulation of the PI3K/AKT pathway promotes cell survival. The PI3K/AKT pathway is important in cancer because it supports the cellular transition of EMT and cell metastasis [19]. MMP-7 expression can be altered later in the process when the PI3K/Akt pathway is activated [45]. The Ras/ERK pathway is important in gene expression regulation. G monomers activate an externally signal-controlled kinase termed ERK via the RAS pathway, which begins with the interaction of CXCL12/CXCR4. ERK subsequently enters the nucleus of the cell, where it works with other regulatory proteins to activate transcription factors. This mechanism promotes gene expression and regulates the cell cycle. Cooperation of ERK with other cellular transcription factors, particularly NF-B, contributes to CXCL12 overexpression and cell cycle progression [45]. In cancer cells, the ERK pathway is implicated in the activation of MMP-7 [46, 47]. MMP-7 induces cell dissociation and cancer cell invasion by activating the ERK pathway [48, 49]. Furthermore, the ERK pathway contributes to the maintenance of MMP-7 activity and the strengthening of the cell dissociation process, which is directly associated with cancer's high propensity for invasion and metastasis [43, 50].

Conclusion

This study analyzes the correlation between CXCL12 and MMP-7 with various regional lymph node metastatic statuses of colorectal adenocarcinoma, where the incidence colorectal adenocarcinoma cases, tends to increase in Indonesia. CXCL12 and MMP-7 were utilized as metastatic indicators in this research. These two proteins have not previously been studied combined in colorectal cancer cases. The expression of CXCL12 and MMP-7 has a significant correlation with the various regional lymph node metastatic statuses of colorectal adenocarcinoma, according to this study. According to the findings of this study, CXCL12 and MMP-7 play a crucial role in lymph node metastasis in colorectal adenocarcinoma. This research can be used to drive future research into developing early diagnoses for colorectal cancer regional lymph node metastases. The positive relationship between CXCL12 and MMP-7 expression and tumor progression can be used to guide the selection of targeted therapy for colorectal adenocarcinoma.

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the article and agreed to be responsible for all aspects of this work.

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