Research Article

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The Expression of CXCR4 and MMP13 in Colorectal Adenocarcinoma Dukes Stage A, B, C and D

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ABSTRACT

Background: Colorectal adenocarcinoma is the third most common cancer in the world with increasing incidence in Indonesia. Most presented ones were in late stage with more unfavorable prognosis. It is necessary to evaluate new markers for prognosis, identify staging and new possibilities for targeted therapy. Over-proliferating tumor cells will enhance the expression of CXCR4, a chemokine receptor. Activating CXCR4 will further activate various downstream signaling pathways, including one which will increase MMP13 secretion through MAPK/ERK signaling pathway. MMP13 then will degrade extracellular matrix, thus facilitate the migration or metastasis of tumor cells.

Methods: A cross sectional study, conducted on 32 samples of colorectal adenocarcinoma. The samples were divided into four groups based on the Dukes staging system (A, B, C and D) and stained immunohistochemically with antibody against CXCR4 and MMP13. The expressions were assessed using immunoreactive score (IRS) and were statistically analyzed.

Results: There were positive correlation between the expression of CXCR4 and MMP13 with Dukes staging, with r_s = 0,628 and r_s = 0,597, respectively. The expression of CXCR4 positively correlated with the expression of MMP13 with r_s = 0,670 (p = 0,05).

Conclusions: CXCR4 and MMP13 expressions were proven to correlate with the depth of invasion and migration of tumor cells in colorectal adenocarcinoma, hence could be considered as prognostic markers, however both could not be used as predictive marker for staging. Tailored therapies targeting these two proteins could be an interest for further investigation.

ABSTRAK

Latar Belakang: Adenokarsinoma kolorektal adalah keganasan terbanyak ketiga di dunia, dengan insidensi semakin meningkat di Indonesia. Sebagian besar kasus terdeteksi pada stadium lanjut dengan prognosis buruk. Diperlukan penemuan marker baru yang dapat membantu penentuan stadium, prognosis dan kemungkinan terapi target yang baru. Sel tumor yang berproliferasi berlebihan akan menyebabkan peningkatan ekspresi CXCR4, suatu reseptor kemokin. Aktivasi CXCR4 akan meningkatkan pelepasan proteinase MMP13 melalui jalur MAPK/ERK. MMP13 akan mendegradasi matriks ekstraseluler sehingga menyebabkan migrasi atau metastasis sel tumor.

Metode: Studi *cross sectional*, dilakukan pada 32 sampel adenokarsinoma kolorektal. Sampel dibagi menjadi 4 grup; stadium Dukes A, B, C dan D. Dilakukan pulasan imunohistokimia dengan antibodi CXCR4 dan MMP13, ekspresi keduanya dinilai menggunakan *immuno-reactive score* (IRS) dan dianalisis secara statistik.

Hasil: Didapatkan korelasi positif bermakna antara ekspresi CXCR4 dan MMP13 dengan stadium Dukes, dengan r_s = 0,628 dan r_s = 0,597. Ekspresi CXCR4 berkorelasi positif dengan ekspresi MMP13 dengan r_s = 0,670 (p = 0,05).

Kesimpulan: Ekspresi CXCR4 dan MMP13 terbukti berkorelasi dengan kedalaman invasi dan migrasi sel ganas pada adenokarsinoma kolorektal. Keduanya dapat dipertimbangkan sebagai marker prognostik namun tidak dapat digunakan sebagai marker prediktif stadium. *Targeted therapy* untuk kedua protein ini menarik untuk dilakukan investigasi lebih jauh.

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BACKGROUND

Colorectal cancer is the third most common cancer and the fourth leading cause of cancer death in the world.[1] There is 9.89% incidence in Indonesia.[2] Data from the anatomical pathology department in RSUD Dr. Soetomo Surabaya also shows increasing numbers in newly diagnosed cases of colorectal cancer every year.

Most cases were present in late stage, in which 40% were cases with nodal metastases and 20% were cases with distant metastases. Surgical treatment, chemotherapy and radiotherapy have been widely applied however the 5-year survival rate in advanced stage remains very low. Biomarkers like CEA and Ca19-9 from the blood serum are mainly used for detection and monitoring. Even though MSI (micro-satellite instability), KRAS and BRAF mutation from the tumor tissue are important for tailored therapy, those are sophisticated measurement that may not be feasible for many centers. It is necessary to find new markers that can be used for prediction of stages, prognostic markers, and possibility of new targeted therapies for more optimum management planning.[3]

CXCR4 is one of the extensively studied new markers. It is a chemokine receptor, which is overexpressed in > 23 types of tumors, including colorectal adenocarcinoma. CXCR4 activation will further activate various signaling pathways that contribute in tumor cells proliferation, angiogenesis, survival and metastasis, one of them is MAPK/ERK pathway which will activate MMP13. MMP13 is a proteolytic enzyme and has roles in degradation of extracellular matrix and basement membrane. Detached tumor cells then will migrate to surrounding tissues, flow in the blood and lymphatic system and reach lymph nodes or distant sites to proliferate and form metastases.[4,5]

In this study, CXCR4 and MMP13 expression in colorectal adenocarcinoma was performed to evaluate whether they have correlation with the depth of tumor invasion and migration processes, thus determining the patient stadium and prognosis.

METHODS

This is a cross sectional retrospective study, conducted on colorectal adenocarcinoma cases obtained from Anatomical Pathology Laboratory of Dr. Soetomo General Hospital archives, from January 2014–March 2017. There were 235 cases, narrowed down to 111 cases. The inclusion criterias were minimum 12 nodes found in resection, with good quality of paraffin blocks. Cases with lymphomas and mesenchymal tumors in review were excluded. The remaining 111 cases were grouped into four groups; Dukes A, B, C and D. Eight cases were randomly sampled from each group. Total samples used were 32 samples.

Immunohistochemistry with antibody against CXCR4 and MMP13 was performed in all samples. This study used monoclonal antibody for CXCR4 (4G10): sc-53534 and monoclonal antibody for MMP13 (MM0019-12E10: sc-101564, both from Santa Cruz Biotechnology with 1:50 dilution. Positivity was evaluated in cytoplasm of tumor cells. Both expressions were then assessed for intensity and percentage of stained tumor cells. Strong intensity is scored 3, moderate intensity is scored 2 and weak intensity is scored as 1. For percentage, no positive cell stained is scored 0, < 10% positive cells is scored 1, 10–50% positive cells is scored 2, 51–80% positive cells is scored 3 and > 80% positive cells is scored 4. Final immunoreactive score (IRS) is the multiplication of intensity and percentage score, ranged 0–12.[6]

SPSS version 16.0 was used to calculate the stastitical analysis in this study. CXCR4 and MMP13 expressions were analyzed with Kruskal Wallis and Mann Whitney test. Correlation between CXCR4 and MMP13 in various Dukes stages was analyzed with Spearman correlation test, with significance of $p < 0.05$. This study had been reviewed and approved by Institutional Ethical Committee of Dr. Soetomo General Hospital (493/Panke. KKE/VIII/2017).

RESULTS

Characteristics of all patients are summarized in Table 1. Age mean was 55.25 ± 10.51 years old. There was slight predominance in male gender. Majority of tumors, 24 from total 32 cases, were located in left-sided colon (2 cases in colon descendens dan 22 cases in rectosigmoid). Based on histopathology, 28 (87.5%) cases had adenocarcinoma with well differentiation, 2 (6.25%) cases with moderate differentiation and 2 (6.25%) cases with poor differentiation.

Kruskall-Wallis test found significant over-expression of CXCR4 in various Dukes stage of colorectal adenocarcinoma in general, with $p = 0.003$ ($p \leq 0.05$). But if the stage was compared one another, Mann-Whitney test showed that there were no significant differences of CXCR4 expression in Dukes stage A, B and C. The CXCR4 expression was only significantly different in Dukes D. Details are shown in Table 2.

Table 1. Clinicopathological characteristics of patients and tumors (n = 32)

Parameter/characteristics		Dukes stage				Total	
		A	B	C	D	Quantity	Percentage (%)
Age (y.o)	<40	1	0	0	0	1	3.12
	40–49	2	2	4	2	10	31.25
	50–59	3	3	1	5	12	37.5
	60–69	1	1	3	1	6	18.75
	70–79	1	1	0	0	2	6.25
	≥80	0	1	0	0	1	3.12
Gender	Male	3	6	5	3	17	53.12
	Female	5	2	3	5	15	46.88
Tumor location	Left-sided	7	7	5	6	25	78.125
	Right-sided	1	1	3	2	7	21.875
Differentiation	Well	8	8	6	6	28	87.5
	Moderate	0	0	1	1	2	6.25
	Poor	0	0	1	1	2	6.25

Table 2. CXCR4 expression in various Dukes stages

Dukes stage	n	IRS score of CXCR4					p
		Mean	SD	Median	Minimum	Maksimum	
A	8	5.63	2.56	6.0[a]	2	9	0.003*
B	8	6.00	2.27	6.0[a]	3	9	
C	8	7.88	2.03	8.0[a]	6	12	
D	8	10.38	1.77	10.5[b]	8	12	

*significant in α = 0.05 (Kruskal-Wallis test)
[a, b] same superscript showed no difference between groups (Mann-Whitney test)

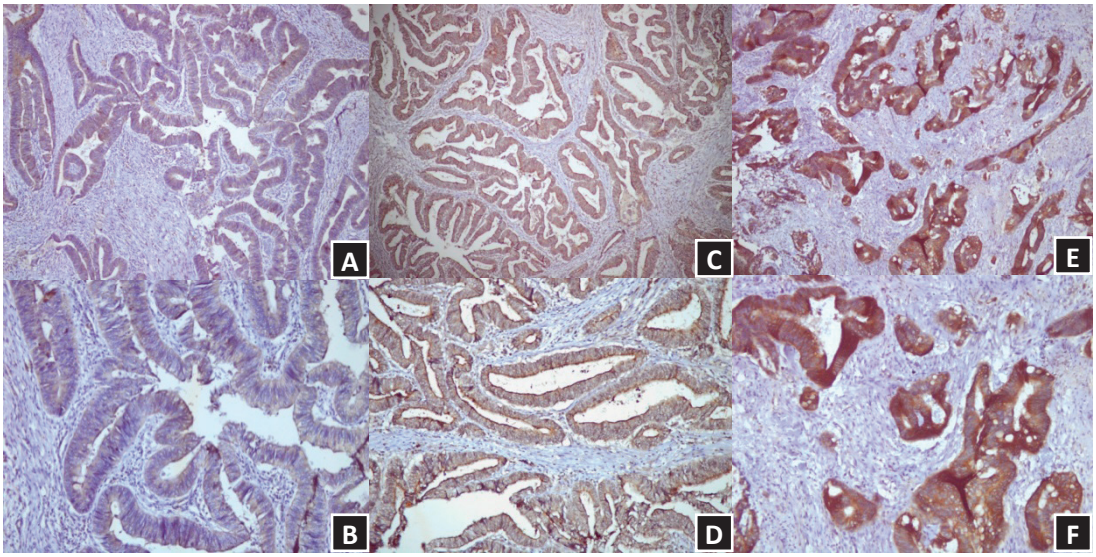

Figure 1. CXCR4 was positively stained in tumor cells cytoplasm with weak intensity (A and B: 100x and 200x magnification), moderate intensity (C and D: 100x and 200x magnification), and strong intensity (E and F: 100x and 200x magnification)

Table 3. MMP13 expression in various Dukes stages

Dukes stage	n	IRS score of MMP13					p
		Mean	SD	Median	Minimum	Maksimum	
A	8	4.88	3.18	4.5[ab]	0	9	0.005*
B	8	4.63	2.72	5.0[a]	0	9	
C	8	7.13	1.55	6.0[b]	6	9	
D	8	9.63	2.20	9.0[c]	6	12	

*significant in α = 0.05 (Kruskal-Wallis test)
[a, b, c] same superscript showed no difference between groups (Mann-Whitney test)

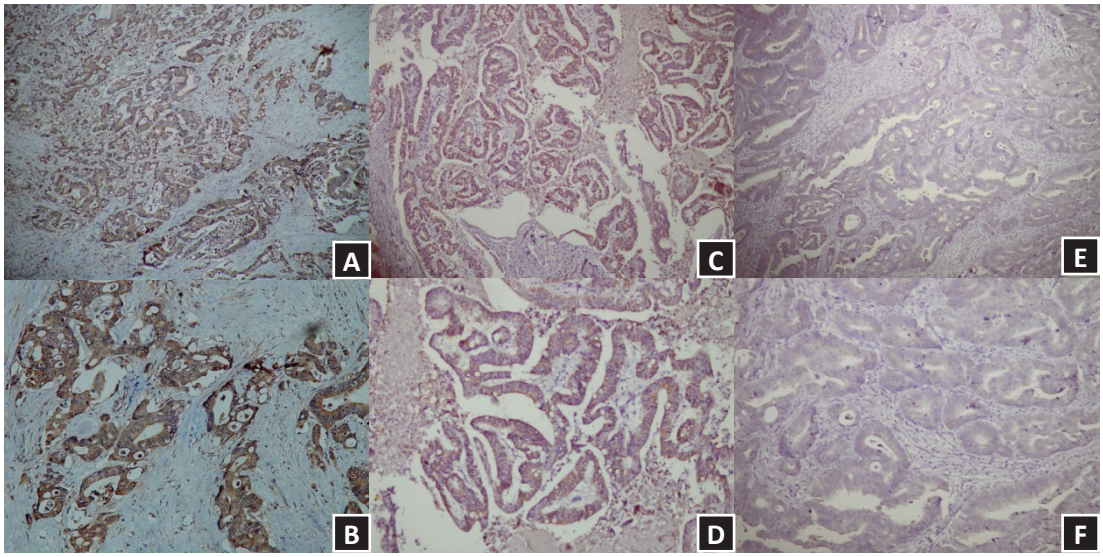

Figure 2. MMP13 was positively stained in tumor cells cytoplasm with weak intensity (A and B: 100x and 200x magnification), moderate intensity (C and D: 100x and 200x magnification), and strong intensity (E and F: 100x and 200x magnification)

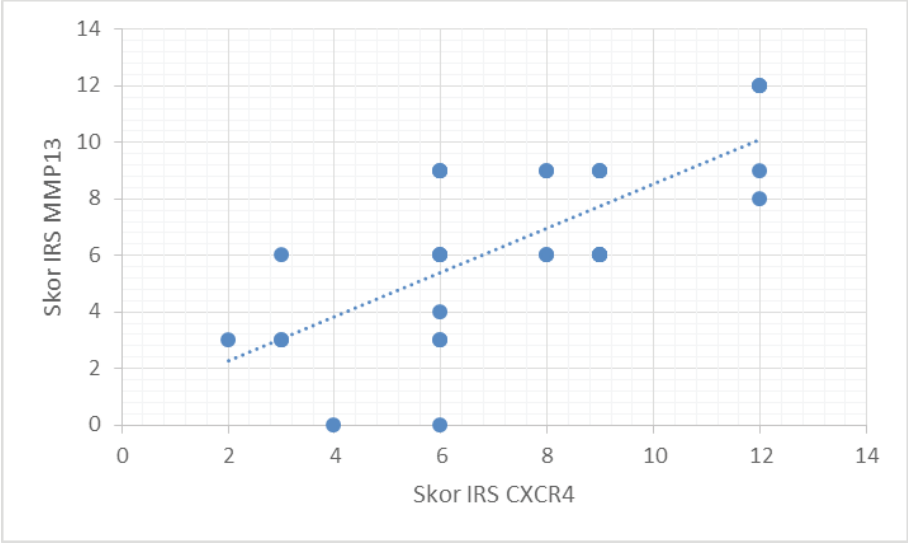

Figure 3. *Scattered plot* of correlation between CXCR4 and MMP13 expression in various Dukes stage in colorectal adenocarcinoma

Kruskall-Wallis test found significant over-expression of MMP13 in various Dukes stage of colorectal adenocarcinoma in general, with p = 0.005 (p ≤ 0.05). In terms of between groups difference, Mann-Whitney test found significant difference of MMP13 expression in Dukes B, Dukes C and Dukes D. Details are shown in table 3.

Spearman correlation test found there was significant correlation between CXCR4 expression and Dukes stage (r_s = 0.628), also there was significant correlation between MMP13 expression and Dukes stage (r_s = 0.597), p ≤ 0.05. This means the higher Dukes stage is, the higher CXCR4 and MMP13 expressions are.

Correlation between CXCR4 and MMP13 expression in various Dukes stages were also analysed with Spearman test. There was positive significant correlation between CXCR4 and MMP13 as shown in Image 3, r_s = 0,670 (p ≤ 0.05).

DISCUSSION

Characteristics of samples

This study used 32 paraffin blocks of colorectal adenocarcinoma patients. Adenocarcinoma was chosen because it is the most common subtypes of colorectal cancer, > 90%.[7] Dukes staging was preferred because it could represent overall stage of disease, including invasion depth, presence of nodal metastases and distant metastases.

In this study, age mean was 55.2 ± 10.5 years old, concordant with the literature facts that colorectal adenocarcinoma was mostly occurred in age > 50 years old, only 9% of cases occurred below 50 years old.[7-9] There was 53.12% cases in male in this study. Literatures also state that the incidence of colorectal adenocarcinoma is higher in male.[7,8,10] This disparity could be caused by different risk factors exposure such as smoking, alcohol consumption, and sexual hormone.[11]

Most tumors in this study were located in rectosigmoid, fitted the predilection location of colorectal adenocarcinoma.[7] The most frequent tumor differentiation was well differentiation (87.5%), this was concordant with previous meta-analysis studies.[12]

CXCR4 expression in colorectal adenocarcinoma

In normal condition, CXCR4 is constantly expressed in most hematopoietic cells, vascular endotels, thymus, brain tissue (neuron, microglia and astrocytes), and embryonic stem cells.[13] CXCR4 is not expressed or weakly expressed in normal colon mucosa and non-neoplastic lesions, but over-expressed in adenocarcinoma.[3,14,15]

CXCR4 staining in this study showed the same median IRS score in group Dukes A and B, increased score in group Dukes C, and highest score in group Dukes D. There was significant difference of CXCR4 expression in overall Dukes stages, but there were no significant difference if the expression were compared between group Dukes A, B and C. If compared to other stages, significant difference in expression was only found in Dukes D. This result implies that CXCR4 expression cannot be used as predictive markers for disease stage. Previous studies also reported the same result, that CXCR4 could be over-expressed even in early stage (I-II), and could be under-expressed in late stage.[16,17] While the reason to this finding remains unclear, another study also reported that over-expression of CXCR4 in early stage was an independent risk factor to higher recurrency of disease and liver. A large-scale cohort study with multicenter setting is needed to validate this finding.[16]

MMP13 expression in colorectal adenocarcinoma

MMP13 is synthesized as latent proenzyme, and will be activated if peptide N-terminal is cleavaged. In this study, the median IRS score of MMP13 showed increasing tendency of over-expression, with the highest score in Dukes D group. Statistical analysis found significant difference of MMP13 expression in overall Dukes stages, however there was no significant difference of expression if Dukes A were compared to Dukes B or C. While there were significant difference of MMP13 expression in Dukes B, C and D. This result implies that MMP13 expression cannot be used as predictive markers for disease stage. This result is not concordant with previous study by Leeman that stated there was significant difference of MMP13 expression in favorable prognosis group compared to unfavorable prognosis group of colorectal adenocarcinoma cases.[18,19] Nevertheless, in this study, there was quite different mean score in group without metastases (Dukes A dan B) compared to group with metastases (Dukes C dan D).

Correlation between CXCR4 expression and Dukes stage in colorectal adenocarcinoma

Spearman correlation test found positive significant correlation between CXCR4 expression and Dukes stage, r_s 0.628 with p = 0.000 (p < 0.005). The higher Dukes stage, the higher CXCR4 expression. CXCR4 expression correlates with tumor proliferation, and migration or metastases to other sites,[3,14,15,21] especially liver,[16] overall will result in worse prognosis and survival rate.

CXCR4 facilitates the proliferation and metastases of tumor cells through various molecular mechanisms. The first mechanism is by giving signals to pathways that related to cell proliferation. CXCR4/CXCL12 axis will activate MAPK, PI3K/AKT, Src/ERK1-2, NF-κβ and STAT3 pathways that induce cell proliferation. Crosstalk of CXCR4 with stem-cell related pathways i.e Notch, Wnt and SHH pathways also have roles in cell tumor proliferation.[20] CXCR4/CXCL12 also suppresses apoptosis by activation of NF-κβ pathway which suppresses apoptotic signals, induces the Bcl-2 gene regulation by direct inhibition of pro-apoptotic BAD (Bcl-xl/Bcl-2-associated death promoter).[21] The second mechanism is by promoting angiogenesis. CXCR4-CXCL12 axis activates PI3K/Akt pathway, recruiting endotel progenitor VEGF to generate more microvessels thus increasing density of microvessels in cancer.[20]

The third mechanism is recruiting immune cells. Recruited dendritic cells suppress antitumor immunity by inactivation of cytotoxic T-cells, promoting tumor growth.[20]

The fourth mechanism is by inducing EMT (epithelial to mesenchymal transition), by activating ERK1/2 pathway, increasing MMP activities to surrounding ECM.[21] CXCR4/CXCL12 axis also influences Wnt signaling, decreasing the expression of E-cadherin and stimulates β-catenin phophorilation. This will decompose the E-cadherin/β-catenin complex which is an important mediator for intercellular adhesion, thus facilitates detachment and eventually metastases of tumor cells.[14,22]

Correlation of MMP13 expression and Dukes stage in colorectal adenocarcinoma

Spearman correlation test found significant correlation between MMP13 expression and Dukes stages, r_s 0,597 with p = 0,000 (p < 0,005). The higher Dukes stage, the higher expression of MMP13. Previous studies also reported significant correlation between MMP13 expression and invasion depth, nodal metastases, and post operative relaps rate in colorectal adenocarcinoma.[23]

In normal condition, MMP13 is not expressed in normal colon tissue. In malignancy, MMP13 is secreted by tumor cells and also by surrounding stromal cells. It has central role in MMP activation cascade. MMP13 activation could occur automatically by autoproteolytic process, and could also be catalyzed by other MMPs. Active MP13 will further activate MMP2, MMP3, and MMP9.[18] All MMPs then will degrade surrounding ECM, basal membrane in particular, thus facilitates the invasion and metastases of tumor cells. MMPs also have intricating roles in creating condusive microenvironment for tumor cells,[24] one of them is by influencing angiogenesis. MMP13 could stimulate VEGF-A secretion, a potent inducer for angiogenesis and lymphangiogenesis by fibroblast and endotel cells.[25]

Correlation between CXCR4 and MMP13 expression in Dukes staging of colorectal adenocarcinoma

Spearman correlation test found positive significant correlation between CXCR4 expression and MMP13 expression, r_s 0.670 with p = 0.000 (p < 0.005), which means the higher CXCR4 expression, the higher MMP13 expression is.

There is no previous study analyzing the correlation between CXCR4 and MMP13 in colorectal adenocarcinoma however positive correlation between these two proteins had been reported in laryngeal and hypopharynx squamous cell carcinoma,[26] in squamous cell carcinoma of oral cavities, and in facial basal cell carcinoma.[27]

CXCR activation will trigger various downstream signaling pathways. CXCR4 that was activated will dissociate to α and βγ subunits. βγ subunit will activate phospholipase C-β (PLC-β) and PI3K. PLC-β will cleave phosphatidylinositol to IP3 (inositol (1,4,5) triphosphate and diacylglycerol (DAG). IP3 will induce the intracellular calcium release. Acting in conjunction with calcium, DAG activates kinase C protein and MAPK/ERK pathway.[13] MAPK/ERK pathway then induces the transcription and translation of pro MMP13 to MMP13.[4]

Since this is a cross sectional study, we could only investigate rough starting data of CXCR4 and MMP13 expression in colorectal adenocarcinoma. A larger scale of cohort study is needed to further investigate these two proteins for new targeting therapy possibilities.

CONCLUSION

CXCR4 and MMP13 expression were proven to correlate with the depth of invasion and migration of tumor cells in colorectal adenocarcinoma however both could not be used as predictive marker for staging. There was significant positive correlation between CXCR4 and MMP13 expression in colorectal adenocarcinoma. Tailored therapies targeting these two proteins could be an interest for further investigation.

CONFLICT OF INTEREST STATEMENT

The auhors disclose no conflict of interests.

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