

Research Article

The Expression of LFA-1, MMP-9, and Dicer1 in The Histological Grading of Colorectal Cancer

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Abstract

Colorectal cancer (CRC) remains one of the most common cancers in many Asian countries, alongside lung and breast cancer. Tumor dissemination to the organs, particularly the liver, contributed to the mortality of this disease. The $\beta 2$ subunit of lymphocyte function-associated antigen-1 (LFA-1) was revealed to cause impairments in liver endothelial activation, cytokines release, and tumor cell retention in the liver of CRC in the *in vivo* study. Epigenetic and matrix metalloproteinase (MMP)-9 are essential for CRC progression and poor prognosis. Tumor differentiation grade correlates with TNM staging, prognostic factors, patient survival, and lymph node metastasis. This study aimed to investigate the expression of the $\beta 2$ subunit of LFA-1, MMP-9, and Dicer1 in the histological grading of CRC. A total of 38 formalin-fixed paraffin-embedded (FFPE) CRC samples were classified into low-grade, high-grade, and histology variants. Tissue samples were processed for qRT-PCR to examine the $\beta 2$ subunit of LFA-1 expression and immunohistological staining to evaluate Dicer1 and MMP-9 expression. The results show that the expression of LFA-1 was significantly higher in high-grade compared to low-grade ($p < 0.05$), except for mucinous type adenocarcinoma. In contrast, no significant difference was found in Dicer1 and MMP-9 expressions of all grades of CRC ($p > 0.05$). In addition, no significant correlation ($p > 0.05$) was found between Dicer1 expression and LFA-1 and MMP-9. In conclusion, the expression of LFA-1 is upregulated in high-grade tumors; however, Dicer1 and MMP-9 expressions are not correlated with the histological grade.

Keywords: LFA-1, matrixmetalloproteinase, Dicer1, colorectal cancer.

Ekspresi LFA-1, MMP-9, dan Dicer1 dalam Penilaian Histologis Kanker Kolorektal

Abstrak

Kanker kolorektal adalah salah satu jenis kanker yang paling umum di negara Asia, bersama dengan kanker paru dan payudara. Penyebaran tumor ke organ, khususnya hati, turut berkontribusi terhadap angka kematian akibat penyakit ini. Subunit $\beta 2$ dari lymphocyte function-associated antigen-1 (LFA-1) diketahui menyebabkan gangguan pada aktivasi endotel hati, pelepasan sitokin, dan retensi sel tumor di hati pada studi *in vivo* kanker kolorektal. Faktor epigenetik dan matrix metalloproteinase (MMP)-9 memainkan peran penting dalam progresivitas kanker kolorektal dan prognosis yang buruk. Derajat diferensiasi tumor juga berkorelasi dengan stadium TNM, faktor prognostik, angka harapan hidup pasien, serta metastasis ke kelenjar getah bening. Penelitian ini bertujuan untuk menyelidiki ekspresi subunit $\beta 2$ dari LFA-1, MMP-9, dan Dicer1 dalam kaitannya dengan derajat histologis kanker kolorektal. Sebanyak 38 sampel kanker kolorektal yang telah difiksasi dalam formalin dan dibungkus parafin (FFPE) diklasifikasikan ke dalam kategori derajat rendah, derajat tinggi, dan histologi varian. Pemeriksaan qRT-PCR digunakan untuk menilai ekspresi subunit $\beta 2$ dari LFA-1 serta dilakukan pewarnaan imunohistokimia untuk menilai ekspresi Dicer1 dan MMP-9. Hasil menunjukkan bahwa ekspresi LFA-1 lebih tinggi secara bermakna pada sediaan pasien dengan kelompok kanker derajat tinggi dibandingkan derajat rendah ($p < 0,05$). Ekspresi Dicer1 dan MMP-9 tidak berbeda bermakna pada semua derajat kanker ($p > 0,05$). Selain itu, tidak terdapat hubungan antara Dicer1 dengan LFA-1 dan MMP-9. Disimpulkan, ekspresi LFA-1 meningkat pada kanker derajat tinggi, sementara ekspresi Dicer1 dan MMP-9 tidak berhubungan dengan derajat histologis kanker.

Kata kunci: LFA-1, matrixmetalloproteinase, Dicer1, kanker kolorektal.

Introduction

The incidence of colorectal cancer (CRC) is steadily increasing in developing countries.¹ In most Asian countries, CRC is still the top cancer, along with lung and breast cancer, with an incidence of 1.1 million cases and the third leading cause of all-age cancer deaths.² Dissemination of the tumor to the other organs contributed to the mortality of this disease, with approximately 70% of metastatic CRCs occurring in the liver.^{3,4} The staging system and early detection of the metastatic potential must be addressed in molecular studies of CRC.

Based on the onco-immunological approach, the $\beta 2$ subunit of LFA-1, a member of the integrin family, impairs liver endothelial activation, cytokines release, and tumor cell retention in the liver of CRC in the *in vivo* study.⁵ Matrix metalloproteinase (MMP) is also correlated with the inflammation process and thus contributes to the disease staging and poor prognosis of CRC. Pathological pathways in disease progression and liver metastasis have been studied, highlighting the role of MMP-9.^{6,7} In addition to cancer pathogenesis, epigenetic dysregulations, including microRNA (miRNA), DNA methylation, and histone modification, are potential predictive markers.⁸ MiRNA is predicted to modulate more than 60% of gene activity that encodes proteins.⁹ A previous study showed that Dicer, as one of the main regulators of miRNA, may stabilize DNA methylation and its function in regulating hypoxia signals and interfere with cancer treatment.¹⁰ Thus, the expression of Dicer is associated with cancer progression at an advanced stage with the potential for metastasis.¹¹

Tumor differentiation grade is correlated with the TNM staging, prognostic factors, patient survival, and node metastasis in CRC.^{12,13} Therefore, a new predictive biomarker for liver metastasis is crucial to determine the early prompt treatment for CRC. Thus, this study aims to investigate the expression of $\beta 2$ subunit of LFA-1, MMP-9, and Dicer1 in the histological grade of CRC, regarding the possibility of Dicer1 as a prognostic factor in correlation with $\beta 2$ subunit of LFA-1 and MMP-9.

Methods

Ethical approval was obtained from the Medical Research Ethics Commission, Faculty of Medicine, Udayana University (No. 1606/UN14.2.VII.14/LP/ 2019). A total of 38 samples of formalin-fixed paraffin-embedded (FFPE) CRC tissues were obtained from the surgical specimen tissue archive at the Department of Pathology Anatomy, Prof. I.G.N.G. Ngoerah - Faculty of Medicine, Udayana University, Bali, Indonesia. To minimize interobserver variation, the conventional adenocarcinoma was divided into low-grade (well and moderate differentiation), high-grade (poorly differentiated), and histology variants of CRC. Tissue samples were processed for qRT-PCR to examine the $\beta 2$ subunit of the lymphocyte function-associated antigen-1 (LFA-1) expression and immunohistological staining to assess Dicer1 and MMP-9 expression.

Ribonucleic acid (RNA) extraction was performed from the FFPE tumor tissue from CRC patients using the RNeasy FFPE tissue kit according to the manufacturer's instructions. The LFA-1 (ITGB2) primers used to target are ITGB2 forward: 5'-CATTGGCTTCGGGTCCTTCGT-3' and ITGB2 reverse: 5'-ACTGGTTGAGTTGTTGGTCA-3'. The amplification conditions were optimized for the MyGo Mini (IT-IS Life Science, UK). PCR amplifications were performed using the SensiFAST SYBR No-ROX One-Step Kit (Bioline, UK). The reaction mixture contained 10 μ l of the supplied SensiFAST SYBR No-ROX One-Step Mix, 0.8 μ l of each primer (10 μ M each), 0.2 μ l of reverse transcriptase, 0.4 μ l of RiboSafe RNase Inhibitor, and 7.8 μ l of the template (50 ng RNA). The cycling conditions were as follows: cDNA synthesis at 45°C for 10 minutes, initial denaturation at 95°C for 2 minutes, amplification for 45 cycles, with denaturation for 10 seconds at 95°C, annealing for 10 seconds at 62°C, and extension for 15 sec at 70°C. Melting curve analysis was performed according to the manufacturer's instructions. The standard curve was generated from serial dilution of a known concentration of purified PCR product of ITGB2.

Immunohistochemistry was performed on the tissue samples. The primary antibodies used were polyclonal anti-Dicer1 and anti-MMP-9 protein (Bioss, USA), as referred to in the manufacturer's instructions. Cancer cells that expressed Dicer1 and MMP-9 have cytoplasmic staining and were assessed in five visual fields using a light microscope (400x magnification). Semi-quantitative scoring systems using HSCORE as follows: $HSCORE = \sum P_i (i + 1)/100$, where i = staining intensity, with a value of 0-3 that referred to no staining, weak, moderate, and strong, respectively. P_i = the percentage of each CRC cell's cytoplasmic staining intensity.¹⁴

The numerical data obtained from immunohistochemistry and qRT-PCR examination were analyzed using SPSS v.26. All of the data were not normally distributed and not homogeneous, hence Kruskal-Wallis and Mann-Whitney tests were used to compare differences in expression between three independent groups and two independent groups, respectively. Spearman

correlation was used to analyze the correlation between Dicer1 and MMP-9 and the $\beta 2$ subunit of the LFA-1.

Results

The expressions of $\beta 2$ subunit of LFA-1 were examined in 38 samples that consist of 28 samples from conventional adenocarcinoma (79% as low grade and 21% as high grade) and 10 samples from histology variants (mucinous and signet ring cell adenocarcinoma). The expression of LFA-1 in low-grade tumor = 4.5 ± 4.1 fg/ul; high-grade = 10.4 ± 6.6 fg/ul; mucinous types = 4.3 ± 3.5 fg/ul; and signet ring cell type = 9.1 ± 10 fg/ul (Figure 1). Statistical analysis was done for 36 samples since the signet ring cell tumors only have two samples. LFA-1 expression was significantly higher in high-grade tumors compared to low-grade tumors ($p = 0.025$). However, no significant difference was observed between mucinous-type and low-grade tumors ($p = 0.963$), nor between mucinous-type and high-grade tumors ($p = 0.053$).

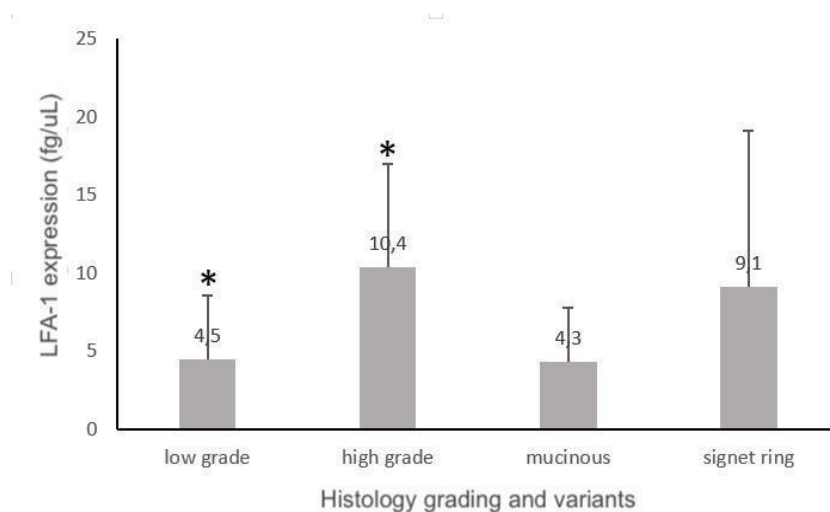


Figure 1. The Expressions of LFA-1 in CRC from Low-Grade, High-Grade, and Histology Variants (Mucinous and Signet Ring Cells)

Dicer1 and MMP-9 expressions were examined by immunohistochemistry. Figures 2 and 3 show the cytoplasmic staining intensity of Dicer1 and MMP-9, respectively. The staining intensity from CRC cells with values 0-3 was referred to as no staining, weak, moderate, and strong, respectively. Dicer1

expressions were assessed in 34 out of 36 samples due to insufficient available FFPE tumors for slide staining. HSCORE for Dicer1 expressions in low-grade tumor = 1.37 ± 0.36 ; high-grade = 1.45 ± 0.49 ; mucinous = 1.48 ± 0.48 .

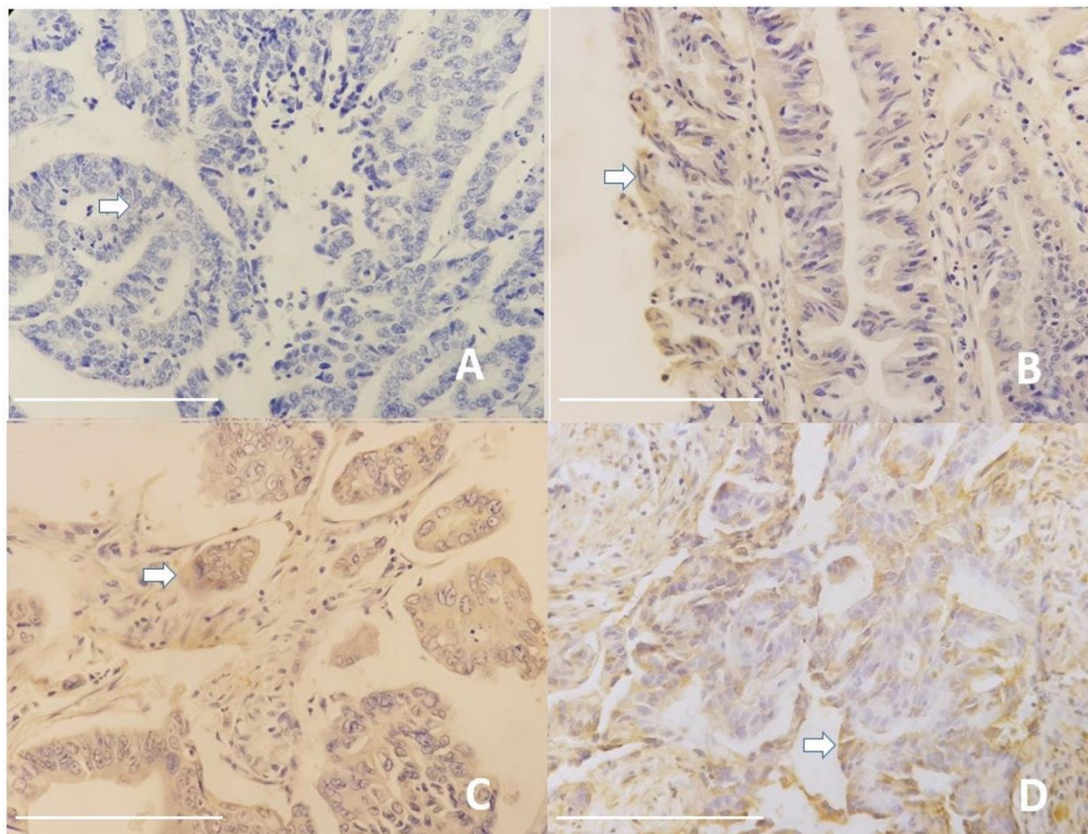


Figure 2. The CRC Cells Showed Immunoreactivity to Dicer1 (Cytoplasmic Staining)
A. No Staining (Score 0); B. Weak Staining (Score 1); C. Moderate (Score 2); D. Strong (Score 3)
Each Figure is Displayed at 400x Magnification. Scale Bar Indicates 100 μ m

The expressions of MMP-9 by HSCORE methods in low-grade tumor = 1.3 ± 0.4 ; high-grade = 1.7 ± 0.7 ; and mucinous type = 1.6 ± 0.5 . The analysis showed no significant difference ($p > 0.05$) in

Dicer1 and MMP-9 expressions between all kinds of CRC. There was no correlation ($p > 0.05$) between Dicer1 expression and LFA-1 and MMP-9.

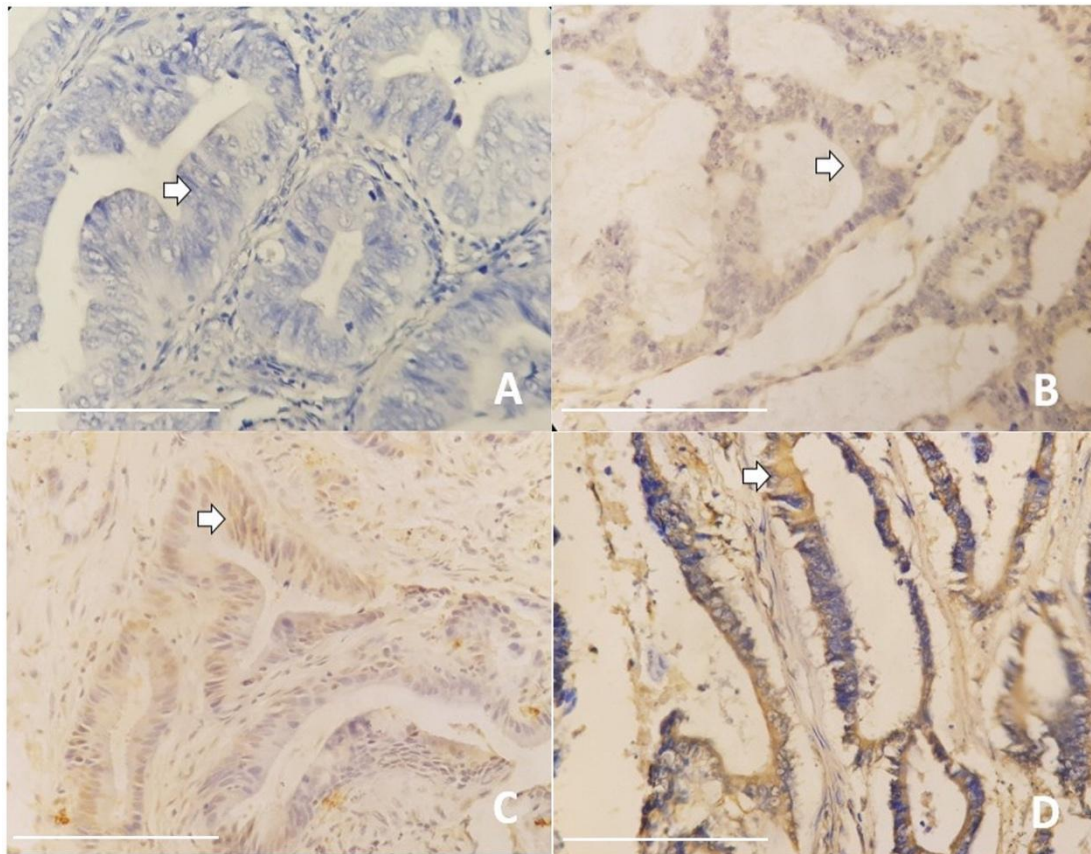


Figure 3. The CRC Cells Showed Immunoreactivity to MMP-9 (Cytoplasmic Staining)
A. No Staining (Score 0); B. Weak Staining (Score 1); C. Moderate (Score 2); D. Strong (Score 3)
Each Figure is Displayed at 400x Magnification. Scale Bar Indicates 100 μ m

Discussion

Histological grading of CRC should be applied only to conventional adenocarcinoma, since some histologic variants may behave as low-grade tumors. However, their morphology is high-grade due to their microsatellite instability status.¹³ Generally, most colorectal patients have conventional adenocarcinoma. In contrast, histologic variants such as mucinous adenocarcinoma are about 10% and signet ring cell adenocarcinoma are about 1%.¹⁵ This study showed a similar result that the conventional adenocarcinoma occurred in most colorectal patients (74%) and most of the histologic variants were mucinous (21%).

The high-grade tumor (poorly differentiated) is related to poor patient survival. The poorer overall survival, prognosis, and lymph node metastasis correlate with increased MMP-9.^{10,16,17} The correlation between MMP-9 and histological grade

in CRC has contradictory results.¹⁸ This study showed that MMP-9 expressions have no significant difference in histological grade (low grade, high grade, and mucinous) of CRC. A similar result found no correlation between differentiation status (well, moderately, and poorly differentiated) and MMP-9 expression.^{10,18} However, another study found that inhibition of MMP-9 reduced the tumor growth in CRC.¹⁹ MMP-9 is involved in the cancer cells' invasion and metastasis process, which degrades the extracellular matrix.¹⁷

Metastatic CRC is a frequent cause of death, with approximately 70% occurring in the liver.^{3,4} In liver metastasis, β 2-subunit of LFA-1 expression may impair liver endothelial activation, cytokines release, tumor cell retention, and development of metastasis in the liver of CRC in the in vivo study.⁵ This study found significant differences in the β 2-subunit of LFA-1 expression between low-grade and high-grade adenocarcinoma, instead of

mucinous type adenocarcinoma. We have to consider other things in determining the cancer progressivity of the mucinous type, such as microsatellite instability (MSI). Mucinous adenocarcinoma, which represents high-level MSI, has decreased the risk of lymph node metastasis or distant organs, while being more aggressive in microsatellite-stable tumors.^{13,20} The mucinous variant usually metastasizes into the peritoneum, instead of liver tissue.²¹ Furthermore, the genomic alterations also differ between mucinous and non-mucinous adenocarcinoma. There are more frequent genomic alterations in the signalling pathway of *PIK3CA*, transforming growth factor- β , and *BRAF* in mucinous adenocarcinoma.²²

In addition to genomic alteration, the epigenetic mechanism, including miRNA, also influences cancer development. Dicer is the main regulator of miRNA biogenesis, and its expressions increase in CRC, especially in stage III, compared to stage II.⁹ This study showed that Dicer1 expressions have no significant difference in low, high, and mucinous adenocarcinoma of CRC. This study also found no correlation between Dicer1 expression and LFA-1 and MMP-9. Despite the previous studies demonstrating the role of Dicer1 in promoting CRC progression.^{10,11} The deletion of Dicer1 allowed the inflammation process leading to CRC progression.²³ Thus, the specific role and pathway of Dicer1 in CRC need to be investigated further.

Conclusion

This study demonstrates that LFA-1 expression is upregulated in high-grade tumors compared to low-grade, but not in mucinous adenocarcinoma. However, Dicer1 and MMP-9 expressions are not correlated with histological grade. Further research is needed in a larger population of CRC to investigate other genetic and epigenetic abnormalities to discover prognostic factors, especially early detection of possible cancer metastasis.

Conflict of Interest

There is no potential conflict of interest related to this article.

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