# The Difference Expressions of EBNA-1 in Epstein-Barr Virus Infection in Low and High Grade Colorectal Carcinoma

*Epistel Pangujian Simatupang*, Marcellus Simadibrata***
*Rino Alvani Gani***, Dyah Ratna Budiani*****
* Department of Internal Medicine, Siloam Hospital Kebon Jeruk, Jakarta
** Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine University of Indonesia/Dr. Cipto Mangunkusumo General National Hospital, Jakarta
*** Division of Hepatology, Department of Internal Medicine, Faculty of Medicine University of Indonesia/Dr. Cipto Mangunkusumo General National Hospital, Jakarta
**** Department of Clinical Pathology, Faculty of Medicine University of Sebelas Maret/Dr. Moewardi General District Hospital, Surakarta

## ABSTRACT

***Background**: Colorectal carcinoma is a common malignancy with the highest mortality rate. Epstein-Barr virus (EBV) as the virus that most commonly infect humans, also can infect the body in a latent and induce the occurrence of malignancy. This study aimed to prove an association between EBV virus infection with degree of colorectal carcinoma by examining the main EBV oncogene expression, namely Epstein-Barr nuclear antigen-1 (EBNA-1), in low grade and high grade colorectal carcinoma.*

***Method**: Cross-sectional study was performed in 14 colorectal cancer patients in Moewardi Hospital, Surakarta between July 2011 and January 2012. The biopsy specimens were stained for EBNA-1 expression using immunohistochemical technique. Statistical analysis was performed using T-test and Mann-Whitney by SPSS software version 19.0 for windows.*

***Results**: Of the 14 patients, there were 7 patients with low grade colorectal carcinoma and 7 patients with high grade colorectal carcinoma. EBNA-1 expression was found in epithelium of low grade and high grade colorectal carcinoma with p = 0.01; CI = -5.24-0.88. We also assessed the expression of EBNA-1 on lymphocytes B of low grade colorectal carcinoma and high grade colorectal carcinoma with p = 0.043.*

***Conclusion**: Significant differences in the expression of EBNA-1 was found in association with EBV infection either in low grade and high grade colorectal carcinoma. The role of EBNA-1 as tumor initiator needs to be elucidated further.*

***Keywords:** carcinoma colorectal, Epstein-Barr virus, EBNA-1*

## ABSTRAK

***Latar belakang**: Karsinoma kolorektal merupakan keganasan yang sering ditemukan dengan laju angka mortalitas yang terbanyak. Virus Epstein Barr merupakan virus yang paling sering menyerang manusia dan dapat menginfeksi tubuh secara laten, serta menginduksi terjadinya proses keganasan. Penelitian ini bertujuan untuk membuktikan adanya asosiasi antara infeksi virus Epstein Barr terhadap tingginya derajat karsinoma kolorektal dengan meneliti ekspresi onkogen utama virus Epstein Barr, yaitu Epstein-Barr nuclear antigen-1 (EBNA-1) pada karsinoma kolorektal derajat rendah dan tinggi.*

***Metode**: Penelitian potong lintang dilakukan pada 14 pasien kanker kolorektal di Rumah Sakit Moewardi Surakarta pada Juli 2011-Januari 2012. Pewarnaan imunohistokimia dilakukan pada 14 spesimen biopsi untuk melihat ekspresi dari EBNA-1. Analisis statistik dilakukan dengan uji T dan Mann-Whitney menggunakan SPPS 19.0.*

***Hasil**: Dari sejumlah 14 sampel, diperoleh 7 sampel biopsi karsinoma kolorektal derajat rendah, dan 7 sampel biopsi karsinoma kolorektal derajat tinggi. Didapatkan ekspresi EBNA-1 pada epitel baik pada karsinoma kolorektal derajat rendah dan tinggi dengan p = 0,01; CI = -5,24-0,88. Selain itu dilakukan penilaian ekspresi EBNA-1 pada limfosit B pada karsinoma kolorektal derajat rendah maupun tinggi, dan didapatkan p = 0,043.*

***Simpulan**: Didapatkan perbedaan bermakna atas ekspresi EBNA-1 yang berkaitan dengan infeksi EBV pada karsinoma kolorektal derajat rendah maupun tinggi. Namun peran EBNA-1 sebagai tumor inisiator masih perlu diteliti lebih lanjut.*

***Kata kunci:** karsinoma kolorektal, virus Epstein Barr, EBNA-1*

## INTRODUCTION

Colorectal carcinoma is the world's third largest malignancy and the second largest cause of malignancy mortality in the United States. In Western countries this disease gained approximately 1 million new cases per year, resulting in up to 500,000 deaths annually. Epstein-Barr virus (EBV) is the most common virus infecting humans and is found around the world. Epidemiological events show that most humans are infected by this virus at some time in their life. In the United States, 95% of adults aged between 35-40 years have been infected by this virus. In children, viral infections may occur soon after maternal immunity is lost. Patients infected with this virus will complain of symptoms such as fever, dysphagia and swelling of cervical lymph nodes. However, due to nature of the disease, symptoms of this disease often resolve on its own after 1-2 months. This viral infection is often overlooked by us, and then the virus will persist throughout life and are latent in the human host body.[1-3]

EBV have been long identified as oncogenic viral, but only recently, specifically EBV has been identified as the initiator of malignancy in human epithelium, arising in gastric, breast, and colon region. The carcinogenesis incidence of colorectal carcinoma can be caused by the presence of oncogenic viral infections, such as Epstein-Barr virus. This virus gains entrance via the oral route through the infected saliva, which then spreads to the entire gastrointestinal tract. It can also induce malignancy process through hematogenic or lymphogeneous route.[4-6]

Epstein-Barr nuclear antigen-1 (EBNA-1) is one of the core proteins secreted by EBV. EBNA-1 plays an important role in the regulation of EBV, especially in gene regulation, extrachromosomal replication, and maintaining the presence of EBV episome through the regulation of gene promoter virus. EBNA-1 with latent membrane protein (LMP)-1 also plays important role in the insertion of the EBV genome information in the host DNA, thus allowing EBV to take over the function of cells and viral replication. EBV infection in patients with colorectal carcinoma on existing literature is correlated with worsening of sensitivity to chemotherapy. In patients with high grade colorectal carcinoma, EBV is a predisposing factor leading to severe dysplasia and insensitivity towards chemotherapy.[7-9] Based on the above, the objective of this study was to prove that there are significant differences in the expression of Epstein-Barr virus infection, EBNA-1, in low grade and high grade colorectal carcinoma.

## METHOD

An analytical observational cross-sectional study was conducted at Moewardi Hospital, Surakarta for obtaining biopsy of colorectal cancer specimens. Further, pathological grading evaluations for histopathology and immunohistochemical staining was conducted in the Laboratory of Anatomical Pathology and Laboratory of Biomedical Research, University of Sebelas Maret, Surakarta. The study was conducted between July 2011 and January 2012. Inclusion criteria were samples obtained from new cases of colorectal carcinoma in patients who never had any previous chemotherapy, while exclusion criteria was any damaged specimen samples.

Fourteen patients were recruited consecutively using purposive quota sampling. There were 7 samples for high grade colorectal carcinoma, and 7 samples for low grade colorectal carcinoma. Each sample will be assessed for the level of EBNA-1 expression by immunohistochemical technique, attained from Genway Biotech, Rockford, USA. A pathologist will evaluate each biopsy slide with an objective scoring system, namely cytology score (CS).[10,11]

Level of expression of EBNA-1 is characterized by a reddish blue color on the cell membranes, whereas plain blue color stain shows lack of EBNA-1 expression on the biopsy tissue. To validate this study, we were interested in studying the EBNA-1 expression on the lymphocytes B membrane using the same biopsy slides. A light microscope Olympus® BX-41 series was used to observe the color intensity of those slides with magnification of 400 x. The histology score of the positive staining was assessed from each field of view and the mean was calculated for each slide. Histological scores obtained were then used in determining the level of expression of EBNA-1. The higher the histological score, the stronger is the expression. The scores from each slide were tabulated into a table. T-test and Mann-Whitney, using SPSS 19 for windows, were used for statistical analysis.[10,11]

## RESULTS

The technique of immunohistochemical staining was applied properly to the 14 biopsy slides. EBNA-1 expression was considered positive in the appearance of reddish blue color stain on the epithelial membrane of colorectal carcinoma or on the membrane of lymphocyte B. The stain towards blue color indicated weaker or negative expression.

Six out of seven slides of high grade carcinoma and 2 out of 7 slides of low grade carcinoma showed the EBNA-1 expression on the epithelia, ranging from negative to weak positive. Strong EBNA-1 expressions were noted more on the slides of high grade carcinoma (Figure 1), while weaker expressions were noted on the slides of low grade carcinoma (Figure 2). Table 1 tabulates the cytology scores and expressions of the 14 slides. EBNA-1 expression in colorectal carcinoma epithelium is shown in Figure 3.

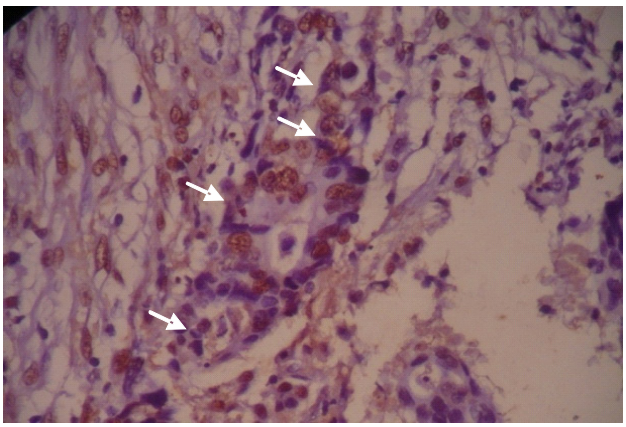

**Figure 1. Strong EBNA-1 expression is observed on a biopsy slide of high grade colorectal carcinoma, note the reddish blue color of the cell membrane**

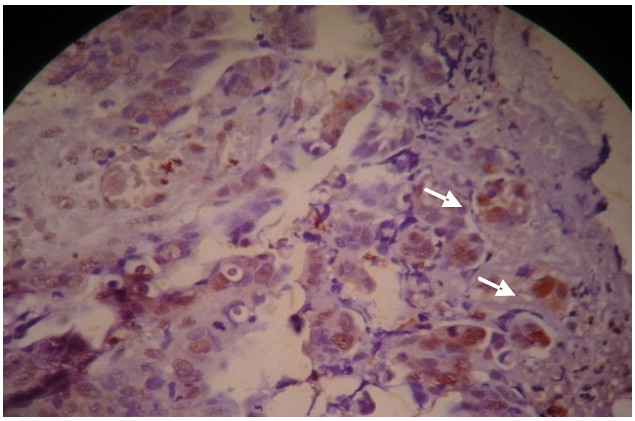

**Figure 2. EBNA-1 expression is observed weak on a biopsy slide of low grade colorectal carcinoma. The arrow points to the reddish blue color cell membrane**

**Table 1. Histological score of EBNA-1 expression in the epithelial high grade and low grade carcinoma colorectal**

| Grading | No slide | Cytology score | Expression |
|---|---|---|---|
| High grade | 1,448 | 2.78 | Negative |
| | 1,289 | 4.89 | Weak-positive |
| | 1,358 | 3.67 | Negative |
| | 1,449 | 0 | Negative |
| | 943 | 6.12 | Weak-positive |
| | 1,743 | 7.12 | Weak-positive |
| | 696 | 3.78 | Weak-positive |
| Low grade | 1,249 | 0 | Negative |
| | 486 | 0 | Negative |
| | 1,134 | 0 | Negative |
| | 1,322 | 2.23 | Negative |
| | 1,582 | 0 | Negative |
| | 1,060 | 2 | Negative |
| | 1,177 | 2.67 | Negative |

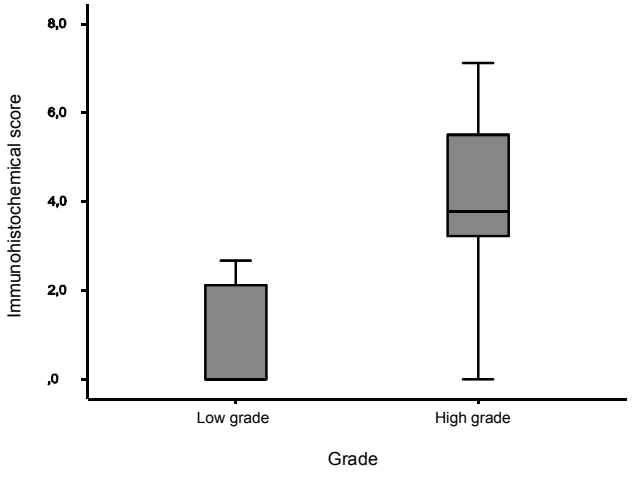


**Figure 3. Box plot diagram of EBNA-1 expression in colorectal carcinoma epithelium**

To validate this finding, EBNA-1 expressions were to be observed on the membrane of lymphocyte B. Table 2 reveals the findings of cytology scores and EBNA-1 expressions on the 14 slides. Six out of seven and 3 out of 7 slides of high grade and low grade carcinomas, respectively, show the appearance of reddish blue color on the lymphocyte B membranes, with cytology scores of negative to weak positive (Table 2). We observe that more lymphocytes B infiltration with stronger EBNA-1 expression are noted in high grade carcinomas, than in low grade carcinomas (Figure 4 and 5). EBNA-1 expression on lymphocyte B membrane of colorectal carcinoma is shown in Figure 6.

**Table 2. Histological score of EBNA-1 expression in lymphocyte B in high grade and low grade carcinoma colorectal**

| Grading | No Slide | Cytology score | Expression |
|---|---|---|---|
| High grade | 1,448 | 1.56 | Negative |
| | 1,289 | 4.34 | Weak positive |
| | 1,358 | 2.56 | Negative |
| | 1,449 | 0 | Negative |
| | 943 | 5.44 | Weak positive |
| | 1,743 | 1.67 | Negative |
| | 696 | 2.22 | Negative |
| Low grade | 1,249 | 0 | Negative |
| | 486 | 0 | Negative |
| | 1,134 | 0 | Negative |
| | 1,322 | 1.22 | Negative |
| | 1,582 | 0 | Negative |
| | 1,060 | 1.1 | Negative |
| | 1,177 | 4.1 | Negative |

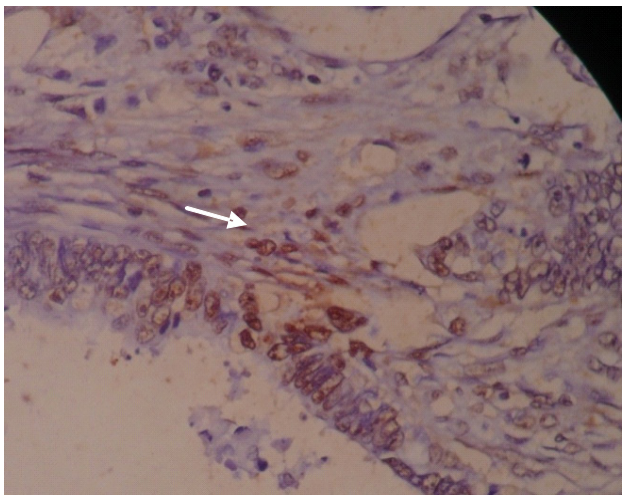

**Figure 4. Lymphocyte B infiltration in low grade colorectal carcinoma, note only small amount of EBNA-1 expression**

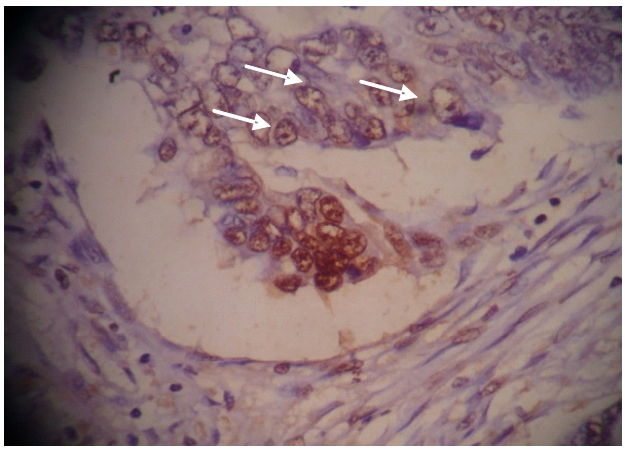

**Figure 5. Lymphocyte B infiltration on high grade carcinoma colorectal, EBNA-1 expression is quite strong**

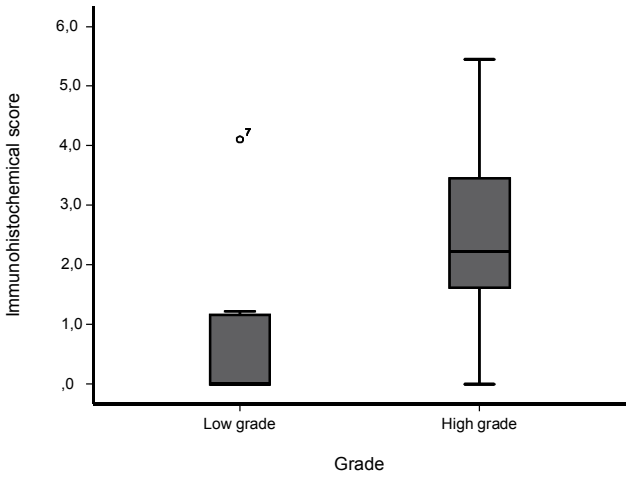


**Figure 6. Box plot diagram of EBNA-1 expression on lymphocyte B membrane of colorectal carcinoma**

The calculated T-test from the above data obtained $p = 0.010$ ($p < 0.05$) for the significant EBNA-1 expression on the epithelia of high grade and low grade colorectal carcinoma, and $p = 0.043$ for the significant EBNA-1 expressions on the lymphocyte B membranes.

This study showed that EBV infection is present in lymphocytes B membranes and epithelia of high grade and low grade colorectal carcinoma which express EBNA-1. High grade carcinoma had higher EBNA-1 expression at the membranes of lymphocyte B and at the epithelia membranes as compared to the low grade carcinomas.

## DISCUSSION

There are significant differences on the expression of EBNA-1 in epithelia of high grade and low grade colorectal carcinoma ($p = 0.01$). EBNA-1 expression in high grade colorectal carcinoma was noted to be higher than in low grade carcinoma.

This is consistent with our initial hypothesis, that the expression of EBNA-1 will increase along with the magnitude of the degree of malignancy that occurs.[12-14] The latent viral infection will induce higher EBNA-1 expression, which is correlated with the latency duration of EBV infection.[15-17]

The need to identify EBNA-1 expression on lymphocyte B membrane in this study is to observe the presence of EBV infection in lymphocyte B containing virus that can infect the epithelium of gastrointestinal mucosa. Study by Liu et al, had shown EBV positive colorectal cancer was present in 26 out of 130 specimen.[12] In this study EBV positive were in 7 out of 12 specimen. This study can prove that the absence of EBNA-1 expressions on lymphocyte B membrane result in the absence of EBNA-1 expression on epithelia of gastrointestinal mucosa. This finding demonstrated that EBV infection can cause malignancy.[18-20]

A study by Mercurio et al, showed that cells infected by EBV would experience malignant transformation.[21] LMP-2A as a protein secreted by EBV during the latent phase of infection will activate αv-integrin, which will induce the synthesis of MMP-9 (matrix metalloproteinase-9), an enzyme that can degrade collagen type II and III, which form the basement membrane of connective tissue in the colonic mucosa. At the time of the occurrence of malignancy, the activated αv-integrin will facilitate the transformation of this malignancy to metastasize.[3,22]

Expression of EBV LMP-2 proteins alters cell endogenous signals, namely the tyrosine kinase. Tyrosine kinases are enzymes that transfer the phosphate chains of the protein into the cell. Through a process of phosphorylation the EBV-infected cells can undergo a mutation, thus losing its growth control; the cells will continue to be in "on" position and continue to proliferate without pause.[23]

Conventional chemotherapy based on Mayo protocol are using leucovorin and 5-fluorouracil, other protocols are also using a protocol similar to the 5-flourourasil and platinum-based combination chemotherapy with capecitabine and bevacizumab. However, the use of these drugs have broad side effects, and the results of local control, incidence of tumor recurrence and the survival rates is still poor.[5,23,24]

A potential chemotherapeutic agent, accurately targeted on certain action of cell signaling, is to be used as adjuvant therapy. After total mesorectal excision (TME) surgery. Chemotherapy drugs that target the receptor tyrosine kinases will be able to provide more promising results. This starts to materialize on a new drug, imatinib. This ATP analog binding drugs competitively bind and inhibit tyrosine kinase, thereby inhibits chromosomal translocations. When used in combination with cisplatin-based chemotherapy, the result would be more promising. Based on phase II clinical trials done by Stahtea et al, imatinib suppressed proliferation of colorectal carcinoma cells, even the expression of proteolytic enzymes matrix metallo proteinase (MMP) of cancer cells was also reduced.[23,25]

## CONCLUSION

Based on the results, we concluded that EBV viral infection plays a role in triggering the process of increasing the degree of histopathological malignancy. Findings of EBNA-1 expressions on lymphocytes B membranes prove that there is a chronic EBV infection in patients with colorectal carcinoma in this study. We would like to emphasize that our colorectal carcinoma patients had latent EBV infection.

## SUGGESTION

Advice from this study is on the need of future study, a prospective study with larger population sample and evaluate the expression of EBNA-1 in serial order to confirm the role of EBV as a tumor initiator, and to determine whether higher EBNA-1 expression correlates with poor chemotherapy response and poor 5 years survival on colorectal carcinoma patients.

## REFERENCES

1. Ahmad A, Stefani S. Distant metastases of colorectal carcinoma: a study of 256 male patients. J Surg Oncol 2006;33:194–7.
2. Morrison JA, Klingelhutz AJ, Raab-Traub N. Epstein-Barr virus latent membrane protein 2A activates beta-catenin signaling in epithelial cells. J Virol 2003;77:12276–84.
3. Traverso G, Shuber A, Levin B. Detection of APC mutations in fecal DNA from patients with colorectal tumors. N Engl J Med 2002;346:311–20.
4. Chao C, Lotz MM, Clarke AC, Mercurio AM. A function for the integrin alpha6beta4 in the invasive properties of colorectal carcinoma cells. Cancer Res 2006;56:4811–19.
5. Chen SY, Lu J, Shih YC, Tsai. Epstein-Barr virus latent membrane protein 2A regulates c-Jun protein through extracellular signal regulated kinase. J Virol 2002;76:9556–61.
6. Cyster JG. Chemokines and cell migration in secondary lymphoid organs. Science 2009;286:2098–102.
7. Hartati ED. Perbedaan skor histologis ekspresi protein EBNA-1 dan LMP-1 pada karsinoma sel transisional kandung kemih berdiferensiasi baik dan buruk [thesis]. Surakarta: University of Sebelas Maret 2005.
8. Heussinger N, Buttner M, Ott G, Brachtel E. Expression of the Epstein-Barr virus (EBV)-encoded latent membrane

protein 2A (LMP2A) in EBV-associated nasopharyngeal carcinoma. J Pathol 2004;203:696–9.

9. Li H, Zhuang H, Wang Q, Pang JC. Epstein-Barr virus latent membrane protein 1 (LMP1) upregulates Id1 expression in nasopharyngeal epithelial cells. Oncogene 2004;23:4488–94.
10. Budiani DR. Penentuan Skor Histologis Tampilan Antigen Hasil Pengecatan Imunohistokimia. Surakarta: UNS Press 2005.p.22-30.
11. Budiani DR. Tampilan LMP1 pada Karsinoma Kolon Suku Jawa Berdasarkan Klasifikasi Duke: Indikasi Adanya Keterkaitan antara Infeksi Virus Epstein-Barr pada Keganasan Kolon. Surakarta: Bagian Patologi Anatomi dan Laboratorium Riset Biomedik, FK UNS 2006.p.1-10.
12. Liu HX, Ding YQ, Li X. Investigation of Epstein-Barr virus in Chinese colorectal tumors. World J Gastroenterol 2003;9:2464-8.
13. Jerome G. The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. Department of immunology, University Descartes, Paris. Cancer Res 2007;67:669-716.
14. Kieff E, Rickinson AB. Epstein-Barr virus and its replication. In: Knipe DM, Howley PM, eds. Fields Virology. 4th ed. Lippincott: Williams & Wilkins Publ 2001.p.2511–74.
15. Knipe DM, Howley PM. Fundamental Virology. 4ed. New York: Lippincott Williams & Wilkins 2004.p.191-206.
16. Hochberg D, Souza T, Catalina M, Sullivan JL, Luzuriaga K, Thorley-Lawson DA. Acute infection with Epstein-Barr virus targets and overwhelms the peripheral memory B-cell compartment with resting, latently infected cells. J Virol 2004;78:5194–204.
17. Lo AK, Liu Y, Wang XH, Huang DP, Yuen PW, Wong YC, et al. Alterations of biologic properties and gene expression in nasopharyngeal epithelial cells by the Epstein-Barr virus-encoded latent membrane protein 1. Lab Investig 2003;83:697–709.
18. Lo AK, Yuen PW, Liu Y, Wang XH, Cheung AL, Wong YC, et al. Down regulation of hemidesmosomal proteins in nasopharyngeal carcinoma cells. Cancer Lett 2001;163: 117–23.
19. Maschler S, Wirl G, Spring H, Bredo DV, Reichmann E. Tumor cell invasiveness correlates with changes in integrin expression and localization. Oncogene 2005;24:2032–41.
20. Rex DK. Colonoscopy: a review of its yield for cancer and adenomas by indication. Am J Gastroenterol 2005;3:353–65.
21. Mercurio AM, Rabinovitz I. Towards a mechanistic understanding of tumor invasion-lessons from the alpha6beta1 integrin. Semin Cancer Biol 2001;11:129-41.
22. Wilson JB, Bell JL, Levine AJ. Expression of Epstein-Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice. Embo J 1996;15:3117-26.
23. Stahtea XN, Roussidis AE, Kanakis I. Imatinib inhibits colorectal cancer cell growth and suppresses stromal-induced growth stimulation, MT1-MMP expression and pro-MMP2 activation. Int J Cancer 2007;121:2808-14.
24. Busson P, McCoy, Gilligan K, Tursz T, Raab-Traub N. Consistent transcription of the Epstein-Barr virus LMP2 gene in nasopharyngeal carcinoma. J Virol 2002;66:3257–62.
25. Yasuda K, Nirei T. Density of CD4 (+) and CD8 (+) T lymphocytes in biopsy samples can be a predictor of pathological response to chemoradiotherapy (CRT) for rectal cancer. Radiation Oncol 2011;6:49.

*Correspondence:*
*Epistel Pangujian Simatupang*
*Department of Internal Medicine*
*Siloam Hospital*
*Jl. Raya Perjuangan Kavling 8 Jakarta 11530 Indonesia*
*Phone/facs: +62-21-53695795*
*E-mail: dr_eps@yahoo.com*