Association of Fibrosis with the Proliferation Index of Liver Malignancy

Indra Yacob*, Delyuzar, Joko S Lukito

Department of Anatomical Pathology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia. *Corresponding author

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Abstract- Myofibroblast is a contractile cell type that has a high capacity to release ECM which will eventually cause fibrosis. TGF-β regulates the development of epithelial-mesenchymal transitions (EMT) and promotes fibrogenesis. TGF-β acts to encode proteins that regulate cell proliferation, differentiation, and growth and play a role in the main pathway of the myofibroblast signaling which will ultimately lead to fibrosis. TGF-β can also act as a tumor promoter through epithelial-mesenchymal transition (EMT) regulation. This study used tissue samples to analyze the correlation between fibrosis and the proliferation index in liver malignancy. Tissue paraffin block from 20 patients with liver malignancy was stained Massons trichrome then performed an assessment of the percentage of fibrosis area then performed re-cutting and Ki-67 immunohistochemical staining to assess the proliferation index and then searched for the extent of fibrosis with proliferation index on liver malignancy by testing spearmen test. Fibrosis was assessed semi-quantitatively by calculating the percentage of the area of fibrosis. we found 35% of cases were in the range of 30-50% fibrosis. And 65% of cases have fibrosis <30%. Most cases show a high proliferation index, in 80% of cases, we found Ki-67 expressed more than 20% in the nucleus of the tumor cells. analysis of the spearman test there was no significant correlation between the fibrosis with the proliferation index of liver tumor cells.

Index Terms fibrosis, liver, proliferation index.

I. Introduction

During the wound healing process, the injured tissue undergoes an inflammatory process, which then progresses to the pro-fibrotic phase. Fibroblasts or other mesenchymal cells such as liver stellate cells (HSC) are activated by a disrupted tissue microenvironment, wherein infiltration of cells involved in wound healing reactions secretes cytokines and differentiates into myofibroblast.[1] Fibrosis also plays a major role in tumorigenesis. Most solid tumors are fibrotic because of a desmoplastic reaction to tumor development. Fibroblasts in tumors are known as cancer-associated fibroblasts (CAF), which have many images of myofibroblasts in organs with fibrosis but have very different phenotypes.[2]

Myofibroblast is a contractile cell type that has a high capacity to release ECM which will eventually cause fibrosis. TGF-β regulates the development of epithelial-mesenchymal transitions (EMT) and promotes fibrogenesis. TGF-β acts to encode proteins that regulate cell proliferation, differentiation, and growth and play a role in the main pathway of the myofibroblast signaling which will ultimately lead to fibrosis. TGF-β can also act as a tumor promoter through epithelial-mesenchymal transition (EMT) regulation.[1,3,4]

Proliferation marker Ki-67 (MCI67) located at 10q26.2 and served to encode antigens on proliferating cells. During the G1, S, and G2-M phases. Ki-67 was found to correlate with tumor growth rate, histological stage, and tumor recurrence. A high Ki-67 index indicates rapid progress and a poor prognosis in patients with hepatocellular carcinoma (HCC). Ki-67 expression is associated with TGFB1 expression in liver cancer tissue

TGF-β is responsible for encoding proteins that regulate cell proliferation, differentiation, and growth and play a role in the main pathway of myofibroblast signaling which will ultimately lead to fibrosis. TGF-β can also act as a tumor promoter through epithelial-mesenchymal transition (EMT) regulation. Therefore, we wanted to find out the association of fibrosis with the proliferation index of liver malignancy, which was assessed by Ki67 immunohistochemical expression in liver malignancy.

II. Material and Methods

Sample selection

This study is an analytic study with a cross-sectional design that aims to analyze the broad relationship of fibrosis with the proliferation index in liver malignancy. This research was conducted at the Anatomic Pathology Laboratory of the Faculty of Medicine, University of North Sumatra, Jalan Universitas No.1, Medan, and the Pathology Unit of the Anatomic Hospital of H. Adam Malik Hospital, Jalan Bunga Lau No.17 Medan.

The research sample was obtained through data storage software and obtained by a label/block paraffin from tissue that was diagnosed histopathologically as a malignancy with inclusion and exclusion criteria. The inclusion criteria in this study were all paraffin blocks from tissue diagnosed as liver cancer histopathologically and found fibrosis after examination with Masson's trichrome staining.

Immunohistochemistry protocol and interpretation

The area of fibrosis is identified by calculating the percentage of fibrosis identified by Masson's trichrome staining and then compared with the total area of tissue on the slide at 200x magnification: (1) score 0 for <5% fibrosis, (2) score 1 for 5% – 10% fibrosis, (3) score 2 for 10% –20% fibrosis, (4) score 3 for 20% -30% fibrosis, (5) score 4 for 30% -50% fibrosis, (5) score 5

for > 50% fibrosis. The proliferation index is identified by the presence of brownish granules which are colored in the cell nucleus by the Immunohistochemical Ki-67 staining (Scy Tek, Logan, Utah, America.) Ki-67 Antigen; Polyclonal (Ready-To-Use), Rabbit Synthetic peptide from 62 base pair region of the human Ki-67 antigen. Percentage is assessed from 1000 cells identified at 400x magnification, then categorized based on the modified criteria made by Xuhui Ma, et al with the following criteria: 34 1 (1) score 0 for <5% positive cells, (2) score 1 for 5 % –10% positive cells, (3) score 2 for 10% –20% positive cells, (4) score 3 for 20% -30% positive cells, (5) score 4 for 30% - 50% positive cells, (6) score of 5 for> 50% positive cells**.**

Statistical analysis

Statistical analysis was performed using SPSS software package version 22.0 (SPSS Inc., Chicago). Spearman test was applied to find out the association of fibrosis with the proliferation index of liver malignancy. The p-values < 0.05 were considered significant.

III. RESULT

Patients' characteristics

The mean age for liver malignancy in this study was 45.65 years, the youngest age was 15 years and the oldest was 66 years. In distribution by sex, we found 13 (65%) cases of men and 7 (35%) cases of women. the ratio of men and women in this study was 1.3: 0.7

Table 1. Characteristic of liver malignancy patients

Characteristics	Number of cases	Percentage (%)
age, mean	45,65	
sex		
male	13	65 %
female	7	35%

Fibrosis distribution

In this study, the largest area of fibrosis was found in score 4, on the range of 30-50% fibrosis, in 7 (35%) cases. While for the fibrosis area less than 5%, we found in 5 (25%) cases. Median (Min-Max) value of the fibrosis in this study was 18 (1-45)

Table 2. Frequency distribution of fibrosis

fibrosis	Score	f	%
Mean±SD= 21,1±14,94			
Median (Min-Max)= 18 (1-45)			
<5% fibrosis	0	5	25%
5% - 10% fibrosis	1	0	0%
10% - 20% fibrosis	2	6	30%
20% - 30% fibrosis	3	2	10%
30% - 50% fibrosis	4	7	35%
> 50% sel fibrosis	5	0	0%

Proliferation index

We assessed the proliferation index using the Ki-67 immunohistochemical staining and then assessed its expression in 1000 tumor cell nuclei. The highest proliferation index was found in score 3 and score 4, in the range of expression of Ki-67 20-30% and 30 -50%, respectively in 7 (35%) cases. And the lowest proliferation index we found in score 0, in the range of expression Ki-67 <5%, in 1 (5%) case. From the data, we also found the Median (Min-Max) Proliferation index frequency was 29.2 (4.70-89.30)

Table 3. Frequency distribution of Proliferation index

Proliferation Index	Score	f	%
Mean±SD			
31,97±20,07			
Median (Min-Max)			
29,2 (4,70-89,30)			
<5% sel positif	0	1	5%
5% - 10% sel positif	1	2	10%
10% - 20% sel positif	2	1	5%
20% - 30% sel positif	3	7	35%
30% - 50% sel positif	4	7	35%
> 50% sel positif	5	2	10%

Association between the fibrosis and Proliferation index

Table 4. Spearman correlation test for fibrosis with proliferation index.

Variable	Fibrosis		
	n	r	p
Proliferation Index	20	-0,107	0,474

The Spearman correlation test in 20 cases of liver malignancy in this study, it was found that fibrosis statistically did not show a correlation to the proliferation index. This was indicated by the Spearman correlation coefficient (r) of -0.107 and the P-value of 0.474

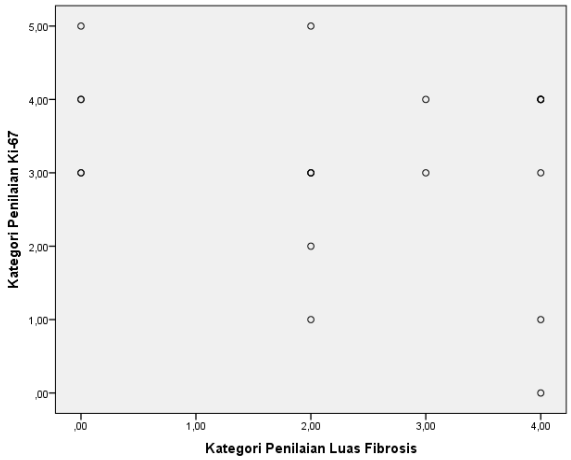

Figure 1. Scatter plot graph

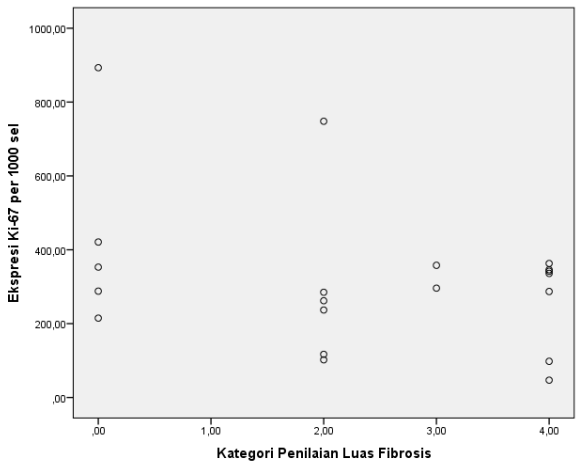

Figure 2. Scatter plot graph.

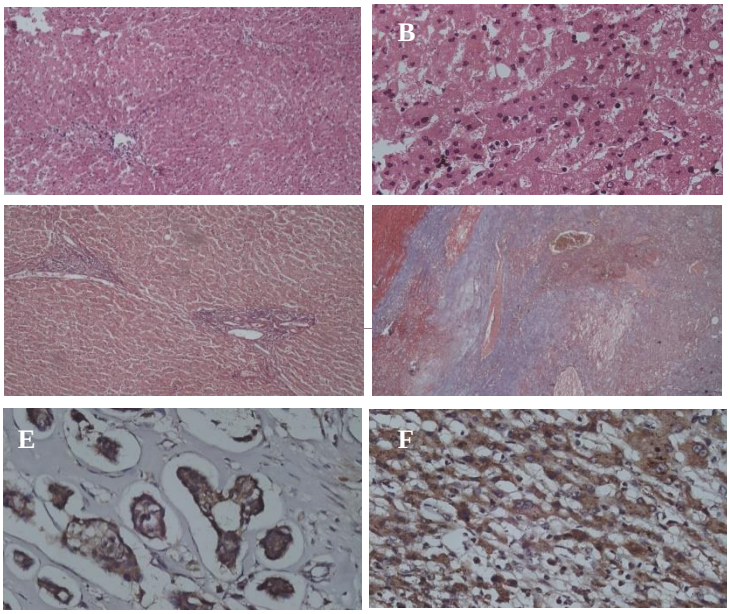

Figure 3. (A) Hepatocellular carcinoma. H&E, 40X. (B) Hepatocellular carcinoma H&E. 400X (C) Minimal portal fibrosis appears blue with Masson's Trichome staining, 40X. (D) Extensive fibrosis appears blue with Masson's Trichome, 40X. (E) Immunohistochemical Ki-67 on the breast tissue (Control) 400X. (F) Immunohistochemical Ki-67 expression on HCC 400X

IV. DISCUSSION

HCC is the most common primary malignant tumor of the liver and attacks men 3 to 4 times more often than women. HCC is the fifth most common malignancy in men and the ninth most common in women.[5] Data from the Surveillance, Epidemiology, and End Result (SIER) cancer statistics database shows that the annual incidence rate for men is 6.7 per 100,000 years compared to 2, 0 per 100,000 years for women. The peak incidence in women occurs 2 decades later than in men which is 4.4 per 100,000 years at the age of 65 to 69 years and 4.2 per 100,000 years at the age of 45 to 49 years.[6] In this study, it was found that the average age of sufferers of liver malignancy was 45.65 years and men were more common than women. The ratio between males and females in this study was 1.3: 0.7. This is not much different from other studies.

Fibrosis quantity calculation system can be done by measuring tissue thickness using a micrometer that has been calibrated at 400x magnification.[7] In this study fibrosis was assessed semi-quantitatively by calculating the percentage of area of fibrosis in the entire field of view of the slide which had previously been diagnosed histopathologically as a case of malignancy in heart, we have not used computerized systems analysis to get more accurate results. Furthermore, the results obtained are categorized into 6 values, namely: (1) Value 0 for fibrosis <5%, (2) Value 1 for fibrosis 5% -10%, (3) Value 2 for fibrosis 10% -20%, (4) Value 3 for fibrosis 20% -30%, (5) Value 4 for fibrosis 30% -50%, (6) Value 5 for fibrosis> 50%.

Toyoda H et al. reported that the degree of liver fibrosis was an important factor related to the recurrence of HCC in patients who had previously undergone curative liver resection.[8] In other studies, according to Toyoda et al., prognostic factors for HCC such as tumor development, liver function, and liver fibrosis significantly influence the survival of HCC patients. There are variations of these prognostic factors. Tumor development factors have a strong impact in the short term and liver fibrosis has a strong impact and long after diagnosis. Then, liver function has a constant prognostic effect on patient survival after diagnosis.[9] In this study, most patients had fibrosis in the range of 30-50% of the tumor tissue area observed, in 7 (35%) cases.

In the development of cancer, unlimited cell proliferation is believed to have an important role. In addition, programmed cell death called apoptosis, regulated by several oncogenes and tumor suppressor genes has become an important pathway for carcinogenesis.41 Dong Sup Yoon et al. Conducted a study of cell proliferation index and expression of p53 and Bcl-2 in tumor lesions. and non-tumor hepatocellular carcinoma and metastatic liver cancer. In the research, he found that the expression of Ki-67 was equally increased in both primary liver cancer and metastatic liver cancer.[10]

In this study, the results of various Ki-67 expressions were found, but most of them showed a high proliferation index where 80% of samples showed Ki-67 colored more than 20% in the nucleus of tumor cells with a median of 29.2. This study divides the assessment of the proliferation index into six categories referring to research conducted by Xuhui Ma, et al, the categories were: (1.) score 0 for <5% positive cells, (2.) score 1 for 5% – 10% positive cell, (3.) score 2 for 10% -20% positive cell (4.) score 3 for 20% -30% positive cell, (5.)score 4 for 30% -50% positive cell, (6.) score 5 for> 50% positive cells.34 This study has not used a calculation system with computer analysis to calculate the Ki-67 expression. The calculation is done manually on 1000 cells carried out at 400X magnification then counted the number of colored cell nuclei and assessed the percentage.

The prognosis of patients with liver malignancies is determined by various factors. Immunohistochemical Ki-67 is a fast, simple and sensitive detection technique for the activity of liver cancer cell proliferation, and overexpression (> 10%) can predict recurrence of liver cancer after surgical treatment, which is associated with death of liver cancer patients.41 Chengkun Yang et al. investigated the expression of Ki-67 proliferation

markers associated with TGF beta 1 can predict the prognosis of patients with hepatocellular carcinoma associated with hepatic B viruses. he found that the level of Ki-67 expression has an association with TGF beta 1 expression in liver cancer tissue and HepG2 cells.[4]

Several reports on cell proliferation and apoptosis in the development of human liver disease have been published, but the area of fibrosis and its relationship to the cell proliferation index in liver malignancy have not been discussed. In this study, we sought the relationship of fibrosis with the proliferation index in liver malignancy by using statistical analysis to obtain data as a reference for the prognosis of liver cancer based on the area of fibrosis associated with the proliferation index in the liver malignancy. From the Spearman correlation test conducted on 20 samples of liver malignancies in this study, it was found that the extent of fibrosis statistically did not show a correlation with Ki-67 immunohistochemical expression. This is indicated by the Spearman correlation coefficient (r) of -0.107 and the P-value of 0.474.

Noda et al. Evaluated the effects of various levels of fibrosis as a prognosis in non-viral HCC patients, and found no significant association between liver fibrosis and overall survival in HCC patients (p = 0.1185).[11] in other studies that conducted by Suh et al who examined the effect of liver fibrosis as a prognosis in HCC with Child-Pugh grade A and HCC with a single lesion <5 cm found that there was no significant difference in survival rates between mild and severe fibrosis (P = 0.267).[12] However, according to Toyoda et al, liver fibrosis can indicate the potential for the development of HCC and increase the impact of liver fibrosis in the long term after being diagnosed as HCC. And it also indicates the potential factor for the development of new HCC. In another study, Kaibori et al., Reported that the degree of liver fibrosis was an important factor associated with HCC recurrence in patients long after curative liver resection.[13] Fibrosis is likely to further influence the progression of liver malignancy by engaging in the process of tumorigenesis, but less influencing cell aggressiveness. liver cancer cells and do not have a significant relationship with the proliferation index.

V. CONCLUSION

There is no significant association between fibrosis and the proliferation index.

COMPETING INTERESTS

The authors have no relevant financial interest in the products or companies described in this article.

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ETHICAL APPROVAL

Health Research Ethical Committee, Universitas Sumatera Utara, Medan, Indonesia approved this study.

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AUTHORS

First Author – dr. Indra Yacob, Resident of Department of Anatomical Pathology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia, **email ID**: dr.indra.y@gmail.com

Second Author – DR. dr. Delyuzar, M.Ked(PA), Sp.PA(K), Department of Anatomical Pathology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia.

Third Author – dr. Joko s Lukito, Sp.PA(K), Department of Anatomical Pathology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia

Correspondence Author – dr. Indra Yacob, Resident of Department of Anatomical Pathology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia, **email ID:** dr.indra.y@gmail.com