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ORIGINAL RESEARCH ARTICLE **OPEN ACCESS**

ARGINASE-1, FIBRONECTIN, IL-13, TISSUE INHIBITOR OF METALLOPROTEINASE-1 AS INDICATOR HEPATOCELLULAR CARCINOMA ON SPRAGUE-DAWLEYRATS INDUCED BY DIETHYLNITROSAMINE AND N-MORPHOLINE

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) characterized by stromal fibrosis develops in 90% of cases of liver cirrhosis, during the clinical course of chronic liver inflammation. Arginase-1 (Arg-1), fibronectin (FN), IL-13, and the inhibitor of metalloproteinase-1 (TIMP-1) are important regulators of proliferation, differentiation, tumorigenesis.. This study was conducted to investigate the progression of liver fibrosis (cirrhosis) to HCC by inducing diethyl-nitrosamine (DEN) and N-nitrosomorpholine (N-Mor) in Spraque-Dawleyrats.

Methods: This study was the experimental design with randomized groups. A total of fourty*Sprague- Dawley*(SD) rats, male, were divided into a control group of ten SD rats, and the treatment group was induced resulting in fibrosis of the liver, and HCC. Statistical analysis was performed by multiregression analysis and Mann Whitney test.

Results: This study showed Arg-1, FN, IL-13 and TIMP-1 have strongly and significantly correlate with stromal fibrosis by histopathology (r = -0.870, r = -0.791, r = 0.932, r = 0.808, p = 0.000 respectively). We found that there are significantly difference level of Arg-1 and FN (p=0.041; p 0.002, respectively) between cirrhosis and hepatocellular carcinoma, meanwhile markers of IL-13, TIMP-1 was not significant.

Conclusions: The serology marker of Arg-1 or FN could be used as the indicator of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death, which initiated by the clinical course of chronic liver diseases that cause fibrosis of the liver (Mittal, 2013). In HCC, DNA ploidy, proliferating activity of tumor cells, tumor suppressor and promotor genes, cells cycle controllers, proteinases that degrade extracellular matrix, adhesion molecules, angiogenic factors, metabolic genes, have been regarded as biomarkers for the malignant phenotype of HCC, and related to progression (Lin-Xiu, 2002). During tumour progression the malignant hepatocytes and activated hepatic stellate cells are accompanied by cancer-associated fibroblasts, myofibroblasts and immune cells generally called tumour stromal cells (El Fatah *et al*., 2016). The genomic point of view on alterations of cellular microenvironment in liver cancers, particularly the stromal tissue, emphasizing the importance of the crosstalk between tumor cells and stromal cells, notably activated hepatic stellate cells (HSC), in tumor onset and progression (Lin-Xiu, 2002). Difficulty in describing the process of stromal fibrosis progress to stromal cancer, with conventional clinical laboratory tests that general and specific, ultrasound, histopathology. Inconsistent criteria and measurement errors such methods, as well as different cut-off limits influential in the evaluation of progressivity HCC. Hepatocellular Carcinoma originates from chronic necroinflammation which is a key element of HCC occurrence

that caninduce chromosomal mutations and eventually malignant transformation of proliferating hepatocytes (Lin-Xiu, 2002). Several facts support that the role of liver stellate cells (HSC) are interrelated with markers arginase-1 (Arg-1), cytokine IL-13, extracellular matrix fibronectin (FN), and Tissues Inhibitor of Metalloproteinase-1 (TIMP-1) in the pathogenesis of liver fibrosis and schirrous HCC (Wynn, 2004; Yan *et al*., 2010). Direct seromarker of Arg-1, FN, IL-13, and TIMP-1 in this study focused as a marker in the follow-up of liver fibrosis (progression) to cirrhosis and HCC, especially the role ARG-1 at necroinflammation persistent, increased cytokine IL-13 activation of fibroblasts, deposition FN and the changing balance of fibrogenesis and fibrinolysis with increased TIMP-1 due to increased expression of mRNA and the gene of TIMP -1 (Lichtinghagen *et al*., 2003; Feng *et al*., 2009; Pinzani *et al*., 2005). In this research, thematerial of carcinogen DEN and N-Mor came to the substance along with SD rats as a modelthat causes inflammation, destruction and fibrosis until the HCC (Lin-Xiu Qin, 2002; Chuang *et al*., 2009). The aims of this research is to find out the model of serology marker circulating Arg-1, FN, IL-13, activities of TIMP-1 as the indicator of the progressivity in the growth of chronic hepatitis disease/fibrosis which developed into the cirrhosis and HCC.

This study is an analytical observational with randomized group experimental design, conducted from August to November 2014, located in Departement of Clinical Pathology and Laboratory Medicine, Anatomical Pathology at Medical Faculty of GadjahMada University, Clinical Laboratory Installation of Prof. Dr. Sardjito General Hospital and Integrated Research of Testing Laboratories of GadjahMada University, Yogyakarta, Indonesia. The subject consist of 30 SD rats treatment and 10 SD rats control, divided in 6 SD treatment rats and 2 SD control rats in each treatment group (4^{th}, 8^{th}, 10^{th}, 12^{th}, and 14^{th} week).

Hepatitis cirrhosis was made using the induction of 100 mg/kg BB of diethyl-nitrosamine intraperitoneal carcinogen for one distribution and 140 ppm of N-nitrosomorpholine intodrinking water in every day. Score assessment system of Metavir provides an indication of activity or the number of inflammation and is the number of fibrosis or scar tissue. The scores of fibrosis is also given the number ranging from 0 to 3 in which (0) no scar tissue, (3) Minimal scar tissue, (6) scar tissue occurred and spread outside of liver containing blood vessel,(9)Fibrosis spread and connecting to other area containing fibrosis and (12) Cirrhosis, (15), HCC. [(10)]The SD rats were fed with standard food of AD II with the composition

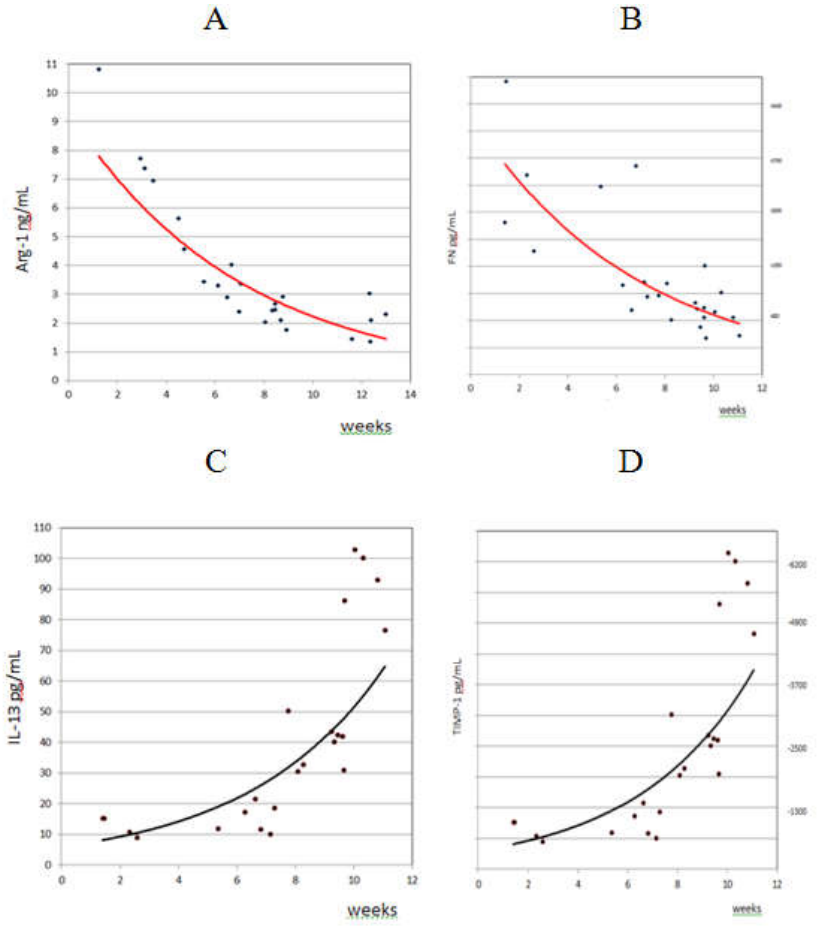

Figure 1. Profile of the tendency Arg-1, FN, IL-13, and TIMP-1, during the progression of liver fibrosis to occurs liver cirrhosis appear: A). Arg-1 serum showed a decline. B). serum FN-1 also showed a decline. C). serum IL-13 showed an increasing. D). serum TIMP-1 showed a increasing.

MATERIALS AND METHODS

This study was approved by Medical and Health Research Ethics Committee Faculty of Medicine GadjahMada University, Yogyakarta, Indonesia.

of maximum 12% of water, minimum 15% of unrefined protein, 3-7% of unrefined fat, maximum 6% of unrefined fibre, maximum 7% of ash, 0.9 to 1.1% of calcium, 0.6 – 0.9% of phosphor antibiotic, and coccidiostate. A histological examination was conducted in each 2 or 4 weeks to determine

the liver fibrosis, HCC, and to measure level of Arg-1, IL-13, FN, TIMP-1 in serum (Kilkenny *et al*., 2010). Serum and liver tissue from 6 male Sprague-Dawley (SD) rats done sampling on eachtreatment group (4^{th}, 8^{th}, 10^{th}, 12^{th}, and 14^{th} weeks). Two SD rats were used as the controller for each group in which all of rats had standard food of AD II. The serum was stored in each distribution 2 or 4 weeks in an -80°C. The measurement level of Arg-1 was done using the quantitative method of enzyme linked immunoassay (Qusabio, USA), and FN, IL-13, TIMP-1 using enzyme linked immunoassay (Booster, USA). Meanwhile, the staining of liver tissues by Haematoxylin Eosin and Malory Aniline Blue were performed for matrix extracellular. *Statistical methods*: The results were performed using statistical Program for Window Evaluation Version 15.0 (www.spss.com). Statistical analysis by univariate and multivariate analys is with models multiple regression used to assess liver fibrosis. Analysis indicator of progressivity fibrosis of the liver by Mann Whitney test, judging appearance, determination, and changes in blood chemistry and immunology to cirrhosis, and HCC.

RESULTS

The pattern of level of Arg-1, FN serum with mild fibrosis until cirrhosis, had diminishing correlation with profile of pathology anatomy, meanwhile the pattern of level of IL-13, and TIMP-1 serum the subject of rats from hepatitis and fibrosis to cirrhosis its opposite. (Figure 1.)

Cirrhosis refers to the presence of macro-nodular or in combination with the micro-nodular type. Whereas significant cellular changes to grow into dysplastic nodules (high or low levels) of hepatocellular carcinoma, after induction with DEN and N-Mor until week 14, the subjects of the mouse undergo a prenoplastic change (Ishak, 2000). After induced by DEN and N-Mor until the 14th week, the SD rats has been changed into preneoplastic rat. It was found that the levels of Arg-1 and FN at week 14^{th} has changed significantly, Arg-1 ($p = 0.041$) and FN ($p = 0.002$), while the level of IL -13 and TIMP-1 was no changed to HCC. (Table 1, Figure2)

DISCUSSION

Liver cancer progresses in a large percentage of cases during the clinical course of chronic fibro-inflammatory liver diseases leading to cirrhosis. Therefore, HCC development is regarded as the result of different environmental risk factors each involving different genetic, epigenetic- and chromosomal alterations and gene mutations. During tumour progression, the malignant hepatocytes and the activated hepatic stellate cells are accompanied by cancer-associated fibroblasts, myofibroblasts (Berasain, 2009). In the past 10 years, a number of inflammatory mediators have been shown to contribute to the progression of chronic liver disease,14, 15 many of which are either targets or activators of nuclear factor-κB (NF-κB). NF-κB is a key transcriptional regulator of the inflammatory response, 16, 17 and plays an essential role in the regulation of inflammatory signaling pathways in the liver.

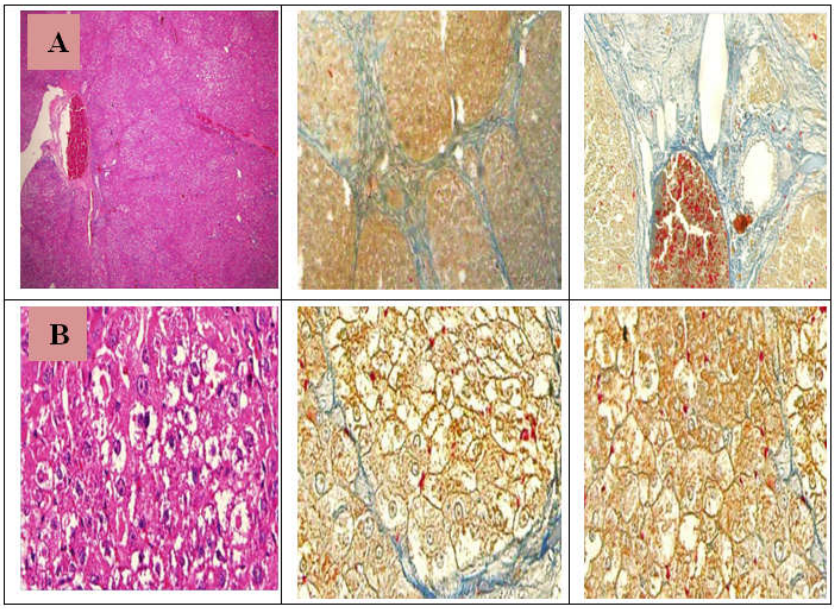

Figure 2. Association between liver fibrosis, schirrous HCC and Arg-1, FN, IL-13, TIMP-1 levels in serum. A. Liver cirrhosis with severe fibrosis B. Dysplastic changes in hepatocytes cells (preneoplastic)

Table 1. Profile of seromarker ARG-1, FN, IL-13, TIMP-1 in liver cirrhosis transformed into HCC

	Cirrhosis	Scirrhosis with HCC	p
ARG-1	2.00±0.62	2.87±0.50	0.041
FN	614.44±57.44	1523.38±192.71	0.002
IL-13	83.60±29.04	92.91±29.04	0.483
TIMP-1	6247.92±216.54	6393.73±128.33	0.180

By multiregression analysis to see exponential trendline graph of Arg-1, FN, IL-13, TIMP-1 obtained respectively : r=-0.870 (95%CI:10.05-13.490), r=-0.791 (95%CI:9.27-13.73), r=0.932 (95%CI:2.34-4.67), r=0.808 (95%CI: -23.86- -6.61)p = 0.000 (Figure 1).

NF-κB regulates multiple essential functions in hepatocytes, Kupffer cells and hepatic stellate cells (HSCs). Genetic inactivation of different NFκB signaling components results in liver phenotypes that include spontaneous injury, fibrosis and carcinogenesis suggesting that NF-κB makes an essential

contribution to liver homeostasis and wound-healing processes (Shiha, 2011; Inokuchi *et al*., 2010; Bettermann *et al*., 2010; Maeda *et al*., 2005). NF-κB inhibition by deletion of inhibitory κB kinase -2 (IKK2) in hepatocytes resulted in increased liver tumor formation in the genotoxicdiethylnitrosamine (DEN) tumor model (He *et al*., 2010; Chrzanowska *et al*., 2009). Arginase-1 (previously known as "arginase liver") is the core enzyme of the urea cycle and occurs in the periportal and hepatocyte. Besides urea, arginase participates in ornithine biosynthesis main compound for proline (for collagen), glutamate (for glutamine) and polyamine (for regulating the cell proliferation and regeneration). Involved in so manyessentialbiochemical pathways, arginase might be the key point in the development of any pathological processes (Yan *et al*., 2010). The immunohistochemical studies examining the expression of Arg-1 in a variety of neoplasms and normal tissue samples showed that the sensitivity and specificity of this marker for HCCs are 96.0% and 99.6%, respectively . In contrast, found that the sensitivity and specificity of HepPar-1 are 84.1% and 96.3%, respectively. It seems that the sensitivity of Arg-1 is consistently superior to that of HepPar-1 within each grade of HCC. The identification of Arg- 1 as an immunohistochemical marker of hepatocellular neoplasms may lead to its developmentas a useful diagnostic adjunct in routine surgical pathology practice (Hart, 2010). This study found the level of seromarker Arg-1, its lowest in cirrhosis and increased markedly in HCC (Table1).

Fibronectin produced by the tumor, endothelial, and Ito cells. Regarding their localization patterns in relation to the histological pattern of tumor, although present in the extracellular matrix of the subendothelial space sinusoid in each histologic pattern, the localization of these components in the intercellular spaces between tumor cells are most marked for hepatocellular carcinoma with solid (Reif *et al*., 2003).In activated HSC downstream integrin signalling, via the focaladhesion kinase (FAK)-phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway, promotesECM deposition (Levental *et al*., 2009). Increased ECM stiffness in vitro enhances integrin expression and activity and focal adhesion formation (Rybak *et al*., 2007), with subsequent activation of downstream integrin signalling within the hepatocyte that may nurture the growth and survival of precancerous cells.

As shown in the research of Rybak *et al*. in 2007 the domain EDA fibronectin was characterized as the vascular marker that is neovascularisation in primer and metastases tumour (Moustafa *et al*., 2012). In week 14th of this study with the induction of DEN and N-Mor, the level of FN increased due to the change in liver cell into preneoplastic (Table 1). The research of Moustafa*et al*. in 2012 compared the fibronectin plasma to the hepatitis fibrosis by 234.4 122 µg/ml; while in HCC, the level of FN plasma increased by 322.6 80,57 µg/ml (Deng *et al*., 2015). Interleukin- (IL-) 13 is a cytokine secreted by several cell types, including eosinophils, mast cells, basophils, epithelial cells, smooth muscle cells, fibroblasts, macrophages, and T cells, especially Th2 cells. IL-13 participates in asthma, tumorigenesis, and parasitic diseases.In previous study, this performed a large case-control study that determined the association between SNP sin the IL-13 gene and the presence of chronic hepatitis B and HBV-related HCC. The rs1800925 CT and TT genotypes were not associated with risk in either the chronic hepatitis B patients or the HBV-related HCC patients. Although the IL-13 rs20541 SNP does not appear to have a significant association with the risk of chronic hepatitis B, we found a significant relationship between the rs20541 SNP and the risk of HCC (Deng *et al*., 2015). The other study by Song *et al.* found that TIMP-1 is aberrantly up-regulated in 76.6% of HCC cells, which are associated with worse prognosis (Song *et al*., 2015). Intriguingly, our findings of TIMP-1 expression in HCC samples are consistent with results from Lempinen's have significantly better overall survival than those with high concentrations group showing that HCC patients with low concentrations of serum TIMP-1 of serum TIMP-1. Thus, TIMP-1 merely functions as abiomarker for HCC progression and contributes to accelerating cancer progression, which implies that it may serve as an important HCC therapeutic target. The data in the study of Song *et al.* reveal that aberrant over expression of TIMP-1 in HCC cells initiate transformation from liver fibrosis (LFs) to carcinoma-associated fibroblasts CAFs (Song *et al*., 2015). In this study showed levels of Arg-1 and FN seromarker, significantly associated with the development of liver fibrosis becomes HCC, contrary to seromarker IL-13 or TIMP-1. Meanwhile the previous studies by immunohistochemical liver tissue indicate Arg-1, FN, Il-13 and TIMP-1 have a relationship with the development of HCC. Conclusion: This finding suggests that determination of Arg-1, or FN serum are useful as an indicator for assess the progression of stromal fibrosis of cirrhosis become ScirrhousHCC.

Disclosure/conflict of interest:

The author declare no conflict of interest.

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