Description of immunohistochemically p53 gene expression and pattern of blood-edta absorbance of rat (*rattus norvegicus)* induced with hepatocarcinogenic agents

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Abstract. This study aimed to investigate spectrometric method to detect hepatocarcinogenesis in rats. *Rattus norvegicus* (n = 21) as a subject induced with hepatocarcinogenic agent $FeSO_4$ 3.5% and CCl_4 0.1 ml/ kg BW. Subjects are divided into seven groups each group induced for 0, 3, 6, 9, 13, 16, and 21 weeks, respectively. The observation on each group consists of ZPBC pattern and blood parameter; and also an observation on the liver preparat (stained using hematoxylin-eosin technique). The pattern of EDTA-blood spectrum is analysed using deterministic and stochastic approaches via specific software program and continued by one-way Anova (MATLAB program in Microsoft Windows XP). The results show that the parameters of EDTA-blood spectrum pattern (k6, k7, Pid, and Ppred) in the group treated with $FeSO_4$ and CCl_4 for 21 weeks are significantly different from the control group and groups treated with $FeSO_4$ and CCl_4 for 16, 13, 9, 6, 3 weeks, i.e.: p = 0.0007, 0.0044, 0.0009, and 1.11022e-016 for k6, k7, Pid, and Ppred, respectively. Hence, spectrometric method used in the detection of hepatocarcinogenesis. The alteration pattern of ZPBC in rats that experienced hepatocarcinogenesis via $FeSO_4$ and CCl_4 treatments is very specific. Compared to the normal subjects (100%), the average of Ppred in 21 weeks of treatment is 30.8%; whereas the average Ppred in 16, 13, 9, 6, and 3 weeks of treatments are 43.5%, 50.8%, 63.7%, 74.3%, and 83.4%, respectively.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the malignant disorders whose prevalence tends to increase throughout the world, e.g.: in the regions of China and Africa an average of between 20 and 100 per 100,000 people [1,2], whereas in the Asian region there are more than 120 per 100,000 people [3]. In general, patients with HCC are treated in an advanced stage; these patients are difficult to be detected

at an early stage, which is sometimes discovered by chance when general check-ups are conducted or when patients with chronic hepatitis are monitored regularly using ultrasonography (USG). Approximately 50% to 80% of the occurrence of HCC is caused by the influence of the hepatitis B virus and chronic hepatitis C, and further consequences of other chronic liver diseases, especially cirrhosis [4]. In addition, the occurrence of HCC can also be caused by the presence of excessive iron accumulation [5-7] and exposure to CCl_4 [8,9].

Studies on animals and humans show that excessive iron mineral administration is one of several important risk factors for liver cancer. Excessive iron accumulation causes damage to liver cells, which can develop into the occurrence of cirrhosis. The trending of cirrhosis development into HCC is very high, which is 200 times higher than non-cirrhosis [2], but recent developments show that around 50% of cases of HCC occur without first undergoing the cirrhosis stage [3,4].

Carbon tetrachloride (CCl_4) is a hepatotoxin substance that can also cause liver cancer. CCl_4 is often used as an inducing agent (inductor) for the occurrence of liver damage in experimental animals; which is analogous to liver damage in humans [8]. The exposure to CCl_4 causes loss of integrity of lysosomes in liver cells, which can progress into fibrosis [10]. Liver cell damage begins with oxidation of CCl_4 by cytochrome P-450 to become CCl_3, the formation of oxygen radical and lipid peroxide compounds [11,12]. In induced 15-day-old male mice (via injection) using 10 µl/grBB of 10% CCl_4 solution in olive oil 3 times a week for 4 months it was found that there were HCC. HCC is also found in the induction using diethylthitrosamine (DEN) of 10 mg/kgBB in mice. The occurrence of HCC due to CCl_4 induction requires shorter time than induction using DEN (ages 6 to 12 months for CCl_4 and 10 to 18 months for DEN). In addition, the chance of HCC due to CCl_4 induction is also greater than induction using DEN (> 88% for CCl_4 and > 75% for DEN) [9]. A study using mice given excessive iron mineral (3.5%) and CCl_4 of 0.1 ml/kgBB for 3 weeks turns out to find 1,N6-ethenodeoxyadenosine adduct (εdA). The εdA is still present when time intervals (pauses) for 2 weeks after 3 weeks of treatment were given, which showed that the DNA repair system was unable to cope with DNA damage due to exposure to iron and CCl_4 [6].

In this study, the process of HCC formation is examined in experimental animals exposed to hepatocarcinogen compounds. Changes in the composition of EDTA-blood in the formation of liver cancer cells will influence the occurrence of changes in potential zeta blood cells (PZBC) in EDTA-blood resulting in a spectrum in a GEI spectrometer. Changes in composition (number and type) of collector components in EDTA-blood result in changes in the electrical interactions between the surface of negatively charged cell membranes and ions in the EDTA-blood [13,14], and changes in the balance of ion transport from plasma into blood cells, which results in a decrease in the zeta potential value on the cell's surface [15]. In conditions of repulsive forces between blood cells in the EDTA-blood no longer able to overcome van der Waals interactions, the aggregation of blood cells will continue to form deposits [16,17]. The occurrence of the change in EDTA-blood process above will give a response change in the GEI spectrometer [18]. Thus, the description of the stages of interaction between blood cells at the very top of EDTA-blood can be detected by the GEI spectrometer by installing a light sensor at the top of the EDTA-blood sample. The interaction between cells in the EDTA-blood will be described in the form of a spectrogram, which is a printout of the GEI spectrometer.

2. Experimental

The subjects of this study are experimental animals, which are induced by hepatocarcinogenic agents ($FeSO_4$ 3.5% and CCl_4 0.1 ml/kgBB). This study is conducted in i) Biochemistry Laboratory, Faculty of Medicine, Universitas Gadjah Mada, ii) Experimental Animal Care Unit, Universitas Gadjah Mada, and iii) Clinical Pathology and Internal Disease Unit Laboratory, Sardjito General Hospital.

The materials used are i) basal-a self-made feed consisting of 54.0% maizena, 40.0% skim milk, 1.5% $CaCO_3$, 0.5% KH_2PO_4, 0.3% NaCl, 0.1% $MgSO_4$, 0.3% vitamin B complex, 0.2% vitamin D, 0.0001% vitamin K, 2.4% corn oil, 0.1% $FeSO_4$, 0.012% vitamin E, 0.1% vitamin A, ii) basal-b feed produced by PT Japfa Comfeed Indonesia, consisting of maximum of 12% water, minimum of 19%

coarse protein, minimum of 14% coarse fat, maximum of 5% coarse fiber, maximum of 6.5% ash, 0.9% to 1.1% calcium, and 0.7% to 0.9% phosphor, iii) EDTA solution, iv) reagent to examine the histological liver tissue of rats: 10% formalin, 99%, 90%, 80%, and 70% alcohol, methyl benzoate, benzol, paraffin, glycerin, hematoxyline-eosin (HE), and histological balsam, and also v) reagent to imunohistochemically examine the p53 gene expression pattern: rabbit polyclonal antibody, avidin-biotin-peroxidase complex kit (ABC kit) from Vector Laboratories Inc., Burlingame, CA. 3,3'-Diaminobenzidine obtained from Sigma Chemical Co., St. Louis, MO.

The equipment used is GEI spectrometer consisting of photocell apparatus containing a light source (5 watt lamp with λ of 550 nm to 600 nm) and a cuvette of 10 mm diameter and 75 mm in height. The distance between the light sources with the sample is 40 cm, a light dependent resistor (LDR) sensor that is 3 mm in diameter. The distance of the cuvette and the light sensor is about 0.1 mm; the analog to digital converter-bio cycle interface (ADC) is made of biological materials sensitive to cycle information to convert analog signals from photocells to digital signals that will be forwarded to a portable computer; and portable computers (note-books) that are equipped with special software that will automatically record data from the photocells into a hard disk. Besides that, a set of Sysmex SE-9500 device; a BX51TF Olympus Optical model tool. Co. LTD microscope; U-MDOB3 and Nikon Eclipse E600 Nikon digital NET Cameras made in Japan are used.

The study was carried out by taking 3 ml of orbital venous blood, inserted into a tube containing 300 uL EDTA used for examining the EDTA-blood spectrum using a GEI spectrometer. This study was continued by painting HE and examining the liver tissue p53 gene expression patterns. Subjects were divided into 5 groups: group I: controls, subjects without treatment; group II: subjects were induced with $FeSO_4$ and CCl_4 for 3 weeks; group III: given a gap of 2 weeks after being induced for 3 weeks; group IV and group V: each subject was induced for 9 and 16 weeks of treatment, respectively. The EDTA-blood spectrum from each group was analyzed using deterministic and stochastic approaches. Differences in blood parameters and differences in the values of representative deterministic parameters, representative stochastic parameters, and general parameters of the EDTA-blood spectral over time in each group were tested using the one-way ANOVA test, $P < 0.05$.

3. Results and Discussion

The results of HE liver tissue staining in experimental animals without exposure to hepatocarcinogenic agents show that the lobular arrangement of liver cells for all subjects in the control group (group I) is within normal limits, monotonous cells with round nuclei of varying size, the difference in nucleus size is not too striking. In this group there are fine chromatin, the nucleus is quite clear but not too large, the nucleus ratio of cytoplasmic cells appears normal. In group II (3 weeks of treatment) there is a rather irregular (intermittent) lobular arrangement. Cells are less monotonous, there are quite a lot of double nucleated cells, round cell nuclei and polygonal with a size variation that is quite large (there are very small ones and also big ones), the nuclei are also large, the cytoplasmic nucleus ratio appears abnormal, a polymorphism. There also appears to be rough chromatin and a lot of fatty liver (steatosis). There is a degenerative area, but the portalis structure is still normal, the central vein is normal, and the nucleus is rather large. Group III (treatment for 3 weeks with a gap of 2 weeks) is generally the same as in group II, but steatosis is lighter compared to group II. In group IV (treatment for 9 weeks) it is found in subject no. 10 a large cell nucleus with a rough, winding and atypical surface of the cell; the lobular structure is less normal, all chromatins are rough, also multiple nuclei is found, which shows that in this subject dysplasia occurs. In group V the condition of the subject no. 13 as a whole is still normal, only a lot of steatosis is found, which is much more than in group V. Conditions in subjects no. 14 and no. 15 are very similar to subject no. 12 (group IV). In examining the pattern of p53 gene expression by immunohistochemistry in all groups it appears that the colour between the cell nucleus and cytoplasm are not distinguished (Figure 1), which shows that the subjects in this group are not found to have p53 gene mutations (Xiao-Mou et al., 1998).

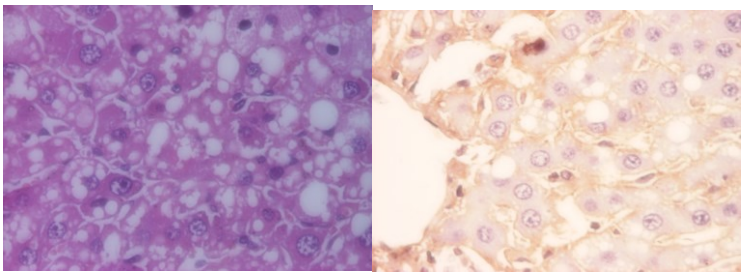

Figure 1. Results of painting HE (A) and examining the patterns of p53 gene expression by immunohistochemistry (B) (400x magnification) from subject no. 14 (begin induced by $FeSO_4$ and CCl_4 for 16 weeks).

Changes in the response of the GEI spectrometer with respect to time in the EDTA-blood sample are analysed based on the deterministic and stochastic approaches [26-30], which produce 4 parameters, i.e. parameter-6 (k6), parameter-7 (k7), ideal parameter (Pid), and prediction parameter (Ppred). The results of one-way ANOVA analysis show that k6, k7, and Pid values, for the exposures of $FeSO_4$ and CCl_4 for 16, 13, 9, 6, 3 weeks and control are significantly very different ($p = 0.0007$, 0.0044, 0.0009, and 1.11022e-016 for k6, k7, Pid, and Ppred, respectively). 3-dimensional scatter diagram of k6, k7, and Ppred can be seen in Figure 2.

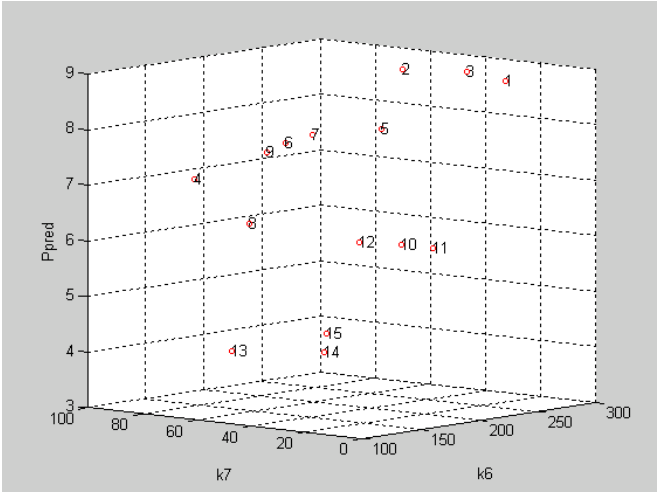

Figure 2. 3-dimensional scatter diagram of k6, k7, and Ppred of the experimental animal model study. I: control, subject no. 1-3. II-VII: treatment using $FeSO_4$ and CCl_4; II: 3 weeks of treatment, subject no. 4-6; III: 6 weeks of treatment, subject no. 7-9; IV: 9 weeks of treatment, subject no. 10-12; V: 13 weeks of treatment, subject no. 13-15; VI: 16 weeks of treatment, subject no. 16-18; VII: 21 weeks of treatment, subject no. 19-21.

From Figure 2 it appears that there are seven groups that are well separated; the top group in the diagram is the subjects of group I (subject no. 1-3); the group below that is subjects of group II (no. 5 and 6); the next is the subjects of group II (subject no. 4) and group III (no. 7-9); and so on until the subjects of group VII is at the bottom of the diagram. A more detailed analysis can be seen in the Dendrogram Cluster graph for Predictors in Figure 3.

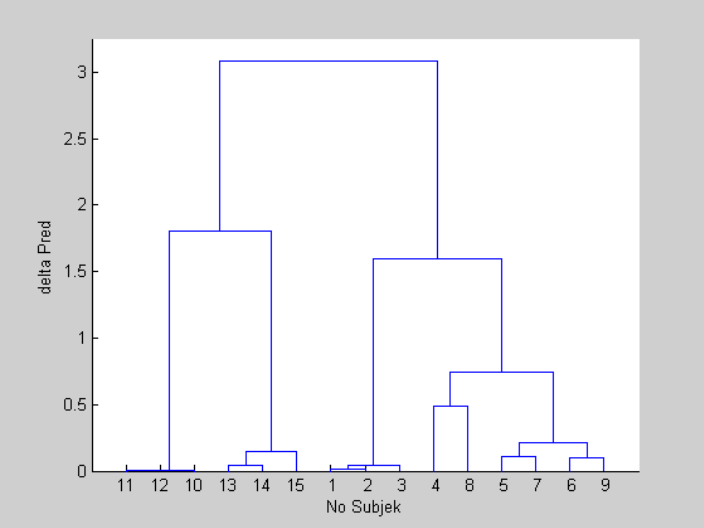

Figure 3. Graph of the Dendrogram Cluster of Ppred of the experimental animal models. I: control, subjects no. 1-3. II-VII: treatment using multiple inductors of FeSO4 and CCl4; II: 3 weeks of treatment, subjects no. 4-6; III: 6 weeks of treatment, subjects no. 7-9; IV: 9 weeks of treatment, subjects no. 10-12; V: 13 weeks of treatment, subjects no. 13-15; VI: 16 weeks of treatment, subjects no. 16-18; VII: 21 weeks of treatment, subjects no. 19-21.

From Figure 3 it appears that in the difference of the value of Ppred = 1.0 there are 4 clusters; the right most cluster (cluster-1) contains 6 subjects: no. 4-9 (groups II and III); the left cluster (cluster-2) contains 3 subjects: no. 1-3 (group I); next (cluster-3): subjects no. 13-15 (group V); and at the left most cluster (cluster-4): subjects no. 10-12 (group IV). It appears that all groups are well separated, except in groups II and III, which show that the administration of iron overload (3.5%) and CCl_4 0.1 ml/BB in rats for 3 weeks have formed an adduct. An addition of time lag for 2 weeks after treatment also forms an adduct, which shows that the DNA repair system is unable to overcome DNA damage due to exposure to iron and CCl_4 (Yang et al, 2000). The occurrence of these adducts does not mean the occurrence of the p53 gene mutation, which can be proven by examining the pattern of p53 gene expression by immunohistochemistry in group V (exposure to inductors up to 16 weeks), which shows that the colour between the cell nucleus and cytoplasm is not distinguishable (Xiao-Mou et al., 1998).

Thus it can be observed that the occurrence of damage stages to liver cells due to the administration of hepatocarcinogen inductors is reflected in the description of changes in ZPBC in EDTA-blood. The average Ppred value in rats given exposure to hepatocarcinogen inductors for 16 weeks is the lowest, followed by administration of 9 and 3 weeks exposures. Thus, the Ppred of the ZPBC changes description in EDTA-blood can be used as a measure to determine the journey of rats undergoing the process of HCC formation due to exposure to $FeSO_4$ and CCl_4 inductors of hepatocarcinogen agents.

4. Conclusions

The change in composition in the blood due to the process of HCC formation can be seen indirectly by analysing the description of changes in ZPBC in the EDTA-blood. The description of changes in ZPBC, which is a reflection of interactions between cells in EDTA-blood due to changes in ZPBC cause by changes in composition in the blood can be analysed using deterministic and stochastic approaches. From this study, a new parameter has been found in the changes description of ZPBC in EDTA-blood, i.e.: Ppred, as a parameter used for initial guidance on the search for HCC early detection method. In general, it can be concluded that the spectrometry method can be used to detect the process of HCC in experimental animals. Description of changes in ZPBC in rats undergoing the process of HCC formation due to exposure to hepatocarcinogen agents of 3.5% $FeSO_4$ and 0.1

ml/kgBB CCl_4 are specific: i) the Ppred value in rats given exposure to hepatocarcinogen inductors for 21 weeks is the lowest. When compared to normal subjects, the average Ppred value for giving exposure for 21 weeks is 30.8%, and 43.5%, 50.8%, 63.7%, 74.3%, and 83.4 % at 16, 13, 9, 6, and 3 weeks of treatment (normal = 100%), respectively, and ii) the sensitivity and specificity of the prediction of liver damage due to exposure to hepatocarcinogen agents is very high (p = 1.11022e-016). Further study is needed for determining the feasibility of the spectrometry method as an early detection method for HCC. The study is conducted using human subjects that look normal but have parameter values of ZPBC changes similar to those of abnormal animal parameters in this study.

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