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Cell-free DNA Levels Serum Patients with Benign and Malignant Epithelial Ovarian Tumor

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ABSTRACT
Background: *An elevated level of cell-free DNA (cfDNA) in the blood circulation has detected in cancer patients in comparison with healthy controls. CfDNA circulation in plasma and serum extensively studied and the results are highly variable due to many factors influence the test results that was preanalytic factors as well as analytic factors. Objectives: Is there any difference in the concentration of serum DNA among patients with benign epithelial ovarian tumors and malignant epithelial ovarian tumors? What is the clinicopathological variable that influences the cfDNA circulation? Method: Venous blood drawn with plain vacutainer, centrifuged at 1,000 rpm for 30 minutes, serum kept in -80^0 C freezer. The cfDNA extracted used NaI method.Results: Collected 30 cases of the benign ovarian tumor and 54 cases of malignant ovarian tumors. The average level serum cfDNA of benign epithelial ovarian tumors and malignant epithelial ovarian tumors were 24.6 ng/mL and 22:29 ng/mL respectively and statistically was not significantly different (p = 0.64). In multivariable analysis with linear regression, there were no clinicopathological variables that statistically significant influence the cfDNA levels in patients with epithelial ovarian tumors where p > 0.05. Conclusion: Concentration of cfDNA circulation of benign epithelial ovarian tumors a little bit higher than malignant epithelial ovarian tumors, but statistically was not significantly different. There was no clinicopathological variable influence the concentration of cfDNA circulation of ovarian tumors.*
Keywords*: cell-free DNA, circulation, benign, malignant, ovarian tumor*

I. Introduction

The concentration of cell-free DNA (cfDNA) in plasma and serum extensively studied and the results are highly variable due to many factors influence the test results was preanalytic factors [1,2,3,4,5,6] as well as analytic factors.[1,5,6,7,8] The first time of the discovery of the extracellular DNA in the blood was in 1948.[9] Basically cfDNA can be detected not only in plasma or serum of cancer patients or other diseases but can also be found in healthy individuals.[10] Serum cfDNA, in general, is a double-stranded molecule with a molecular weight in the range of 0.18 kB up to 21 kB. In healthy individuals cfDNA plasma derived from cells undergoing apoptosis rather than necrosis process.[11] Several studies have shown that all living cells can actively release fragments of DNA. It suggested that the spontaneous release of DNA fractions with a lower molecular weight than the genomic DNA associated with the so-called metabolic DNA portion. This metabolic DNA appears to have a role in cellular functions such as transcription to be RNA. The DNA can also form complexes with glycoproteins and is released in the blood circulation to act as a messenger with the function of signaling between cells and tissues. The release of the active fraction of DNA derived from all the normal and abnormal cells.[11,12] It is estimated that plasma cfDNA in healthy individuals primarily derived from the hematopoietic cell. Plasma or serum cfDNA in cancer patients comes from the process of apoptosis and necrosis as the characteristics of tumor cells with rapid cellular metabolism. Both of these mechanisms simultaneously contribute to the production of plasma cfDNA in cancer patients due to apoptosis which is common in DNA fragments with low molecular weight while the characteristic of cell necrosis process with a greater molecular weight that can detect in serum and plasma patient.[11,12,13]CfDNA presence in serum or plasma derived from tumor has sparked a lot of studies to explore the possibility of cfDNA has the clinical potential be more accurate tumor markers for diagnosis, prognosis and early detection of cancer. However, because this circulation cfDNA few and a small fragment so that the possibility of a variety conditions will cause the loss of some DNA fragments into small pieces during the isolation process. CfDNA fragment from apoptosis results has about 180 bp or multiples while cfDNA fragments of necrosis generate bigger particles than from apoptosis.[14,15] To be said that cfDNA levels associated with clinicopathological variables where high levels frequently found in cancer patients than in healthy patients.[12,16] Proportion of cfDNA tumor related to total cfDNA and influenced by several factors such as the type of tumor, stage, degree of differentiation, tumor location and size. However, none of the clinicopathological variables consistently associated with the concentration cfDNA. This condition may be the cause of considerable differences in the levels of cfDNA were obtained in plasma or serum that reported in numerous research in the literature.[17] This study will measure the concentration of serum cfDNA

benign tumors and malignant epithelial ovarian tumors. Is there a difference in the level of serum DNA among patients with benign epithelial ovarian tumors and malignant epithelial ovarian tumors? What is the clinicopathological variable that influences the level of cfDNA circulation?

II. Research methodology

This study has approved by Joint Institutional Review Board (JIRB) Faculty of Medicine, Universitas Gadjah Mada No: KE/FK/730/FC. Patients diagnosed with an ovarian tumor will be asked consent if willing to participate in the study and signed an informed consent for blood sampling. Blood was processed to be taken the serum within a maximum of six hours after blood drawn. Venous blood was taken with plain vacutainer for about 5 ml, and then centrifuged at 1,000 rpm for 30 minutes, then serum to be extracted in microtube container and kept in -80^0 C freezer. If the results of the histopathological examination were benign or malignant epithelial ovarian neoplasm with histopathological type is serous and mucinous, the serum will measure the concentration of cfDNA using NaI method.

DNA serum isolation with Sodium Iodide (NaI) method

After the serum thawed at room temperature the serum to be vortex and spin. Aliquot 500 µL of serum add 500 µL of 1x enzyme reaction solution (1.43% SDS; 7,15mM EDTA; 14,3mM Tris, pH 8) and 35 µL proteinase K (10mg/mL) incubated at a temperature of 56^0 C for 2 hours. Add 1.5 µL of glycogen (20 mg/mL) and then to be a vortex, incubated at room temperature for 3 minutes. Add 1,000 µL of 9 M NaI and vortex briefly then added 2,000 µL Isopropanol and incubated at room temperature for 15 minutes. Centrifuged 10,000 x g for 25 minutes at room temperature, then the supernatant was discarded. Wash pellet with 3,000 µL of 45% isopropanol. Centrifuged 9,000 x g for 25 minutes at room temperature and then the supernatant was discarded. Wash pellet with 75% ethanol and vortex. Centrifuged 9,000 x g for 25 minutes at room temperature and the supernatant was discarded. Dry the pellet at room temperature 10 minutes and then add 50 µL of TE solution. Incubation at 56^0 C for 30 minutes. Moving DNA solution into microcentrifuge 1.5 mL. Preparation of DNA solution by dilution of 50 times; 98 mL DNAH2O/RNase free water plus two µL solutions of DNA, interfering with the vortex 5 seconds, the measurement of the concentration of DNA with fluorophotometer (Gene Quan II Pharmacia Biotech RNA/DNA calculator).

III. Research Result

Collected 30 cases of benign ovarian tumor composed of 17 serous cystadenoma and 13 mucinous cystadenoma of the ovary, 54 cases of malignant ovarian tumors consisting of 35 serous cystadenocarcinoma and 29 mucinous cystadenocarcinoma of the ovary. Several clinicopathological characteristic were recorded such as; age, body weight, body length, histologic type of the tumor, the limited spread of the tumor in the ovary or spread outside of the ovary, grade of differentiation, blood CA125 level, the age of menarche, the status of menopause and parity. In the bivariable analysis the grouping of spreading, benign ovarian tumors and malignant ovarian tumor stage I included in one group as the tumor confined to the ovary. Likewise for grouping the degree of differentiation of benign ovarian tumor contained in the well-differentiated group. The cfDNA serum of all cases were measured the minimum level was 0.60 ng/mL, and maximum level was 76.0 ng/mL, and the mean of all measurement was 22.92 ng/mL with standard deviation was 16.81 ng/mL (Table 1). The average level serum cfDNA of benign epithelial ovarian tumors and malignant epithelial ovarian tumors were 24.6 ng/mL and 22.29 ng/mL respectively and statistically was not significantly different (p=0.64) (Table 2). In the bivariable analysis with independent t-test showed that there was no significant difference in the level of cfDNA serum in each group of clinicopathological variables where $p > 0.05$ (Table 2). In multivariable analysis with linear regression, there were no clinicopathological variables that statistically significant influence the cfDNA levels in patients with epithelial ovarian tumors where $p > 0.05$ (Table 3). However, at the variables of menopause status with $p = 0.08$ seem that it still has an impact on the level of cfDNA circulation.

Table I. Cell-free DNA serum meseurement in epithelial ovarian Tumor(serous and mucinous type)

Mesuarement	***Epithelial ovarian tumor***		***Total***
Cell-free DNA (ng /mL)	***Benign***	***Malignant***	
No. of cases	30	54	84
Mean	24.06	22.29	22.92
Std. Deviation	18.57	15.89	16.81
Median	14.20	16.75	15.65
Minimum	1.50	0.60	0.60
Maximum	57.10	76.00	76.00
Range	55.60	75.40	75.40

Table II. Bivariable analysis of Clinicopathological characteristic with Serum Level of cell-free DNA

ClinicopathologicalVariable	***n***	***Cell-free DNA***		***p***
		Mean (ng/mL)	*SD (ng/mL)*	
Malignancy status:				
Benign	30	24,06	18.57	0.64
Malignant	54	22.29	15.89	
Age group:				
< 50 yo	56	22.61	16.61	0.80
≥ 50 yo	28	23.55	17.47	
BMI group:				
< 25	65	23.58	17.76	0.41
≥ 25	18	20.52	12.83	
Histology type:				
Serous	42	23.17	17.90	0.91
Mucinous	52	22.77	16.27	
Tumor spread:				
Limited in ovary	50	22.43	16.83	0.74
Outside of ovary	34	23.65	17.00	
Grade of diff:				
Well diff	64	21.99	15.73	0.36
Mod + poor diff	20	25.91	20.03	
Level of CA125:				
< 70 U/ml	42	20.99	15.18	0.29
≥ 70 U/ml	42	24.85	18.26	
Age of menarche:				
< 16 yo	67	22.84	16.89	0.92
≥ 16 yo	17	23.26	16.99	
Menopauze status:				
Not menopauze	47	25.07	17.23	0.18
Menopauze	37	20.19	16.06	
Parity:				
< 3	57	20.14	16.24	0.53
≥ 3	27	24.59	18.16	0.53

Table III. Multivarable analysis of linear regression of DNA serum with Clinicopathological characteristic

Regression Model	***B***	***95% CI of B***	***p***
Constant	17.311	-11.29 - 45.91	0.23
Malignancy status	-5.44	-15.94 - 5.05	0.30
Age group	4.62	-4.78 - 14.02	0.33
BMI group	-3.47	-12.70 - 5.75	0.45
Histology type	3.44	-5.46 - 12.34	0.44
Tumor spread	-2.02	-11.67 - 11.27	0.97
Grade of diff	5.70	-5.64 - 17.06	0.32
Level of CA125	6.79	-2.52 - 16.11	0.15
Age of menarche	-3.15	-13.52 - 7.22	0.54
Menopauze status	-7.97	-17.02 - 1.07	0.08
Parity	4.04	-4.77 - 12.86	0.36

IV. Discussion

The mechanism of the presence of cfDNA in the blood circulation both in normal and pathological conditions have not clearly understood. It is estimated that cfDNA in the blood in healthy individuals primarily derived from hematopoietic cells. Several studies have shown that all living cells can actively release fragments of DNA. Apoptosis generates DNA fragments of about 180 bp, while necrosis was resulting larger particles than apoptosis. The average quantity of plasma cfDNA in normal individuals varies between 10 ng/mL to more than 1500 ng/mL. In healthy individuals cfDNA circulation levels are low, as most cells death soon phagocytosis by

phagocytes cells. The study results also get a total average concentration cfDNA blood in healthy male was higher (1030 ng/mL of blood) than in healthy women (430 ng/mL).[18,19,20] It said that cfDNA levels in cancer patients are higher than normal individuals.[17, 21,22] In epithelial ovarian cancer cfDNA concentration in plasma was significantly higher than patients with benign tumors and in healthy women.[22,23] Median concentrations cfDNA in plasma patients with solid tumors is 17 ng/mL (with a range of 0.5 - 1600 ng/mL) in which 3-fold higher than healthy volunteers.[17] In this study found that mean of cfDNA level of benign ovarian tumor patients was greater than in patients with ovarian cancer, although statistically no significant different (p=0.64). No et al. found the concentration of cfDNA ovarian epithelial benign tumors higher than the concentrations cfDNA in epitelial ovarian cancer although not significantly different.[24] Jung et al., also reported cfDNA serum levels in patients with hypertrophy of the prostate was higher than in prostate cancer patients.[25]

This study showed that the concentration of cfDNA not influenced by various clinicopathological factors with the multivariable analysis $p > 0.05$ (Table 3). However, this considered results found the menopausal status of the patients with ovarian tumors apparently might have affected the concentration of cfDNA as the probability value was almost significant p=0.08. Other researchers also found that the levels of cfDNA not influenced by other prognostic factors such as age, stage of disease, histopathological types,[22,24,26] residual tumor, recurrence,[24] levels of CA125, volume of tumor.[26]

For producing output levels of cfDNA reliable, sample analysis should be more controlled and standardized as the condition of processing requirements.[27] Plasma and serum samples widely studied to measure levels of cfDNA circulation and both can use.[28,29] However, it was found that serum concentrations of cfDNA were 3-24 times higher than in the plasma.[3,5,28,29] Preferably the processing is done six hours after blood was taken.[30] The different extraction method is also the most important reason that causes different results of the total cfDNA concentrations of plasma or serum. Extraction method with a spectrophotometer less sensitive than the method of fluorometric or various methods of PCR target to different DNA fragments.[31,32] Fong et al. said the fluorometric method is recommended as the preferred ways to determine the levels of total serum cfDNA because the process is simple, powerful and with a detection limit of about one ng/mL is sensitive enough to detect low concentrations of serum cfDNA. Extraction procedures with phenol-chloroform (PCI - glycogen), methods of sodium iodide (NaI method), and the kit QIAamp blood DNA then measurement assessed by fluorescent the DNA harvested higher significantly. The discovery of rising DNA fragments obtained with NaI and the PCI-glycogen procedure is also shown on an agarose gel. Interestingly, a significant number of small DNA fragments were found by these two methods, whereas the other methods are not obtained. Comparing approach to PCI-glycogen and NaI method, it was found that the NaI method is not only superior regarding quantity of DNA, but also simpler, faster, and cheaper.[33]

Although cfDNA found since more than half a century ago, the cancer diagnostic tests based cfDNA circulation have not been developed for clinical applications because it still requires in-depth evaluation. But in the field of fetal medicine cfDNA successfully used for Rhesus D genotyping of fetuses,[34] and for the detection of paternally inherited genetic diseases.[35]

V. Conclusion

The study found that the concentration of cfDNA circulation of benign epithelial ovarian tumors a little bit higher than malignant epithelial ovarian tumors with mean level were 24.6 ng/mL and 22.29 ng/mL respectively, but statistically was not significantly different ($p = 0.64$).There was no clinicopathological variable influence the concentration of cfDNA circulation of ovarian tumors. However, the variable of menopause status seem still possible has to impact the level of cfDNA circulation ($p = 0.08$).

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Conflictsofinterest

The author declares that this study no conflict of interest

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