miR-21 Expression in Grade I, II, and III Breast Cancer

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ABSTRACT

Background: Breast cancer is the second largest cancer in the world.The gold standard for breast cancer diagnosis and prognosis is grade assessment (mitotic, nuclear pleiomorphism, tubular formation), based on breast tissue histopathology. However, this assessment has limitation in that it is subjective and semi quantitative. A genetic biomarker known as microRNA (miRNA) may regulate human gene expression by targetting mRNA. One of the miRNA, i.e. miR-21, is able to suppress tumor suppressor mRNA, resulting in increased cellular proliferation andneoplastic transformation, and decreased apoptosis. This study aimed to investigate the expression of miR-21in breast cancer with histopathologic grade I, II, and III.

Subjects and Method: This was a descriptive study with cross-sectional design involving paraffin block (FFPE) samples of ductal-type breast cancer tissue. Sixty four samples were obtained from the department of Anatomical Pathology, Haji Adam Malik Hospital, Medan. Patients history were obtained through medical records followed by sample processing (Total RNA Isolation, cDNA Synthesis, and Examination of miR-21 Expression using Real-Time qPCR). MiR-21 expression from every hystopathologic grade was obtained using Fold Change Calculation.

Results: Breast cancer occurred more frequently in age group of 30-49 years old. Based on the histopathologic grade, most breast cancer was found in grade II. Analysis of miR-21 expression showed that miR-21 expression was higher in grade III compared to other grade.

Conclusion: Breast cancer occured more frequently in the productive age group.Most breast cancer were found in grade II. Increased miR-21 expression in grade III may suggest that miR-21 is an oncomiR, which plays an important role in the development of malignant breast cancer.

Keywords: breast cancer, histopathology grade, miRNA, miR-21.

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BACKGROUND

Breast cancer is the second most common cancer in the world (Ferlay et al., 2010; Kementerian Kesehatan Indonesia, 2013). According to the data from Haji Adam Malik Central Hospital, Medan, Indonesia, there were 200 new patients diagnosed with breast cancer during 2012. Histopathology examination of breast tissue is a Gold Standard for the diagnosis enforcement, and also for prognosis prediction through histopathology grade. Histopathology is an assessment toward the degree of cancer differentiation (cell mitosis, pleomorphism of cell nucleus, and tubulus formation),

which is related to patients' prognosis prediction (Tavassoli and Deville, 2003; Malhotra et al., 2010). The assessment method is already commonly used (Rakha et al, 2010), however it has limitation since it is subjective and semi quantitative. Therefore other more objective biomarkers are needed to complement prognosis. A relatively potential biomarker of breast cancer which is also frequently investigated currently is microRNA.

MicroRNA (MiRNA) is a non-coding endogen RNA with 18-22 nucleotides in length, and also plays an important role in the regulation of gene expression. MiRNA works by degrading or impeding the translation of mRNA target; so that miRNA is able to influence protein expression (Calin and Croce, 2006; Cho, 2007), including protein which acts in the development of breast cancer. MicroRNA-21 (miR-21) is a micro RNA which experience increasing expression in breast cancer, by suppressing or impeding protein translation of tumor suppressor (Ioro et al, 2005). It results in the uncontrolled cell proliferation (can be assessed through grading component that is cell mitosis calculation). The excessive expression of miR-21 in breast cancer patients is suspected to be related with poor prognosis (Lee et al., 2011).

In Indonesia study on microRNA and its association with cancer is still very few, therefore this study aims to investigate the expression of miR-21 toward histopathology of patients' breast cancer tissue, especially in the city of Medan, Indonesia.

SUBJECTS AND METHOD

Research license has been obtained fro the Committee of Research Ethics of Faculty of Medicine, University of North Sumatra Medan Indonesia. The research design was cross sectional. The type was descriptive. The subject used was paraffin block (FFPE) of invasive ductal breast cancer tissue, with 64 subjects obtained from Pathology Anatomy Department of Haji Adam Malik Hospital Medan. Subject determination was conducted in purposive sampling, helped by Pathological Anatomist. The studied variable was the expression of miR-21 (independent) and the histopathology grade of breast cancer tissue (dependent). Data of research subject sampling was conducted in Haji Adam Malik Hospital Medan. The grade assessment was obtained from medical records data of breast cancer patients. miR-21 examination was conducted in Molecular Biology Laboratory of Faculty of Medicine UGM, through phases of RNA isolation from FFPE subject, examination on RNA concentration, cDNA synthesis, and analysis on miR-21 expression by using Real Time qPCR. Using Fold Change formulation, miR-21 was obtained in every grade of histopathology, subsequently, normality test and descriptive analysis were conducted.

Total RNA Isolation of Paraffin Block (*FFPE*)

Paraffin block was sliced by using microtome with 7 μm of thickness, afterwards it was put into 1.5 ml centrifuge tube. The Total RNA isolation process was conducted based on the manual of TiangenRNAprep Pure FFPE kit. Transfer 1ml of Xylene into the tube, vortexed and centrifuged at 12.000 rpm, afterward, supernatant was aspirated and disposed. Insert 1ml of ethanol, using similar phase as Xylene. Dry the pellet of ethanol. Insert buffer and proteinase K into the tube and vortexed. The tube was incubated in temperature 55° C and 80° C. The tube was centrifuged and supernatant was transferred into new tube. Buffer was put inside new tube, and vortexed. Ethanol was added into the tube, and vortexed. The

mixture was transferred into Spin Column CR3, and centrifuged, afterward the liquid inside the tube was disposed. DNAse I solution was prepared, and put inside Spin Column CR3, then incubated for 15 minutes. Buffer RW1 was put inside Spin Column CR3, centrifuged, and disposed the liquid inside the tube. Insert buffer RW into Spin Column CR3, incubated for 2 minutes, centrifuged and disposed the liquid inside the tube. Collection tube was centrifuged for 2 minutes, and disposed the liquid inside the tube. Spin Column CR3 membrane was dried up. Spin Column CR3 was put in a new centrifuge tube, put RNAse-freeH_2O into membrane. The tube was closed, incubated and centrifuged until total RNS was obtained. The centrifuge tube with RNA Total inside and the tube were closed, and put inside refrigerator. Subsequently, total RNS concentration was examined by using NanoDrop Spectrophotometer.

CDNA Syntesis of Total RNA

The materials used were RNA sample, Reaction Buffer, Nuclease-Free Water, Spike in (UniSp6, as internal control), and Mixed Enzyme. The steps were conducted based on the manual cDNA synthesis kit from miRCURY LNA™ Universal RT microRNA PCR (Exiqon). RNA sample was slowly defrosted in room temperature, vortexed, and spun-down. Removed 5x Reaction Buffer, Nuclease-free water and Spike in (UniSp6) from refrigerator, de-frosted in room temperature, vortexed and spun-down. Removed Mix Enzyme from refrigerator to be homogenized, to make Master Mix, after ward it was vortexed and spun-down. Master Mix was distributed to every PCR tube, then RNA sample was inserted into PCR tube, and spun-down. PCR tube subsequently was put inside RT-PCR instrument, operate it according to its

program. cDNS result was finally stored within refrigerator.

Analysis miR-21 with Qrt-PCR

The materials used were cDNA, SYBR Green Master Mix, primary miR-21 (has-miR-21), and primary UniSp6. The steps were conducted based on the manual kit from miRCURY LNA™ Universal RT microRNA PCR (Exiqon). cDNA was defrosted slowly in room temperature, vortexed, and spun-down. cDNA was diluted in advance inside 1.5ml centrifuge tube, vontexed, and then spun-down. Removed SYBR Green Master Mix, Primary set miR-21 and Primary Spike in (UniSp6) from refrigerator to be homo-genized by being thawed and vortexed to make Master Mix for has-miR-21 and Spike in control. The Master Mix was put into every qPCR tube, and then added with cDNA sample. Every tube was labeled and put into qRT-PCR machine, set the program into Real Time qPCR, and operate it according to its program.

Statistical Analysis

The data obtained is in a form of miR-21 expression (based on Fold Change formula from the obtained value of Cq miR-21 and UniSp6 by using qRT-PCR), and followed by conducting normality test, Kolmogorov-Smirnov and descriptive analysis.

RESULTS

A descriptive analysis research had been conducted on the description of miR-21 expression toward histopathology grade of breast cancer, by using 64 subjects of paraffin block (FFPE) of invasive ductal type breast cancer tissue. Data analysis was conducted as follow:

A. Characteristics of Research Subjects

Table 1 presents the characteristics of research subjects obtained based on the age. It can be observed from Table 1 that the

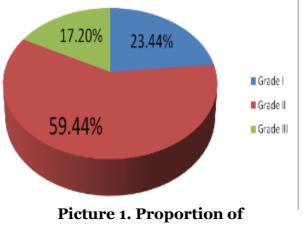research subjects are mostly come from age group 30-49 year old which is 36 subjects (56.30%), compared to age group of 50-78 year old.

Table 1. Distribution of research subjects based on age group

Age Groups (Year)	Diagnose						Total	
	Grade I		Grade II		Grade III			
	n	%	n	%	n	%	n	%
30 – 49	6	40.00	28	73.70	2	18.20	36	56.30
50 – 78	9	60.00	10	26.30	9	81.80	28	43.80
Total	15	100	38	100	11	100	64	100

B. Proportion of Histopathology Grade of Research Subjects

According to the entire research subject, proportion of each histopathology grade of breast cancer was obtained as it is seen in the following picture 1:

Picture 1. Proportion of histopathology grade of breast cancer of research subjects

From Picture 1, it can be observed that the biggest proportion comes from grade ii which is 38 subjects (59.4%), meanwhile the smallest proportion is grade III which 11 subjects (17.2%).

C. Measuring miR-21 Expression in Breast Cancer Tissue of Research Subjects

miR-21 expression was measured by using qRT-PCR instrument, it was conducted to the entire research subjects. Based on the value of Cq miR-21 and UniSp6 from qRt-PCR, the value was put into Fold Change Formula ($2^{-\Delta Cq}$) and obtained miR-21 expression data. Data normality test and descriptive test were conducted toward the data as it is presented in Table 2.

From Table 2 it can be found out that miR-21 expression data is distributed normally. The average of miR-21 expression was obtained from the result of descriptive test, and miR-21 expression in grade III is the highest that is -0.49 ± 1.28. Whereas, the average of miR-21 expression on grade II is the lowest that is -0.86 ± 1.09. The average of miR-21 expression in grade I is as much as -0.66 ± 1.45.

Table 2. MiR-21 expression toward histopathology grade of breast cancer

Diagnose	miR-21					p
	Median	Mean	SD	95% CI		
				Lower	Upper	
Grade I	-0.29	-0.66	1.45	-1.46	0.14	0.200
Grade II	-1.11	-0.86	1.09	-1.21	-0.49	
Grade III	-0.51	-0.49	1.28	-1.35	-0.37	

DISCUSSION

In this research, the number of breast cancer patients who came from 30-49 year age group were a lot more than those who came from 50-78 year age group. It was estimated as the impact of the exposure of estrogen or progesterone hormone toward breast tissue, since the beginning of menstruation up to menopause (Hulka and Moorman, 2001). The onset during first menstruation and during menopause influences the period of hormone exposure. Proliferation of breast epithelia can be induced by estrogen (Aguas et al., 2005), and its active form that is estradiol (E2) can trigger the process of neoplastic transformation on human breast epithelia (Russo et al., 2006; Fernandez et al., 2009).

In post-menopause women, breast cancer is estimated influenced by estrogen synthesis which is related to adiposity. During menopause, ovaries no longer can produce estrogen hormone. Estrogen is obtained from androgenic precursors of adrenal glands which experience aromatization to become estrogen in adipose tissue (Kluttig and Pokrzywnak, 2009). Menopause women with obesity are having a high risk to suffer from breast cancer as the impact of high level of estrogen especially estradiol (Hulka and Moorman, 2001; Key et al., 2003). Based on the research conducted by McTiernan et al., (2003), the increasing estrone and estradiol was found along with the increasing parameter of adiposity. In post-menopause women with obesity and breast cancer, there are is increasing parameter of adiposity which is related to the increasing level of hormones that cause tumor.

The proportion of histopathology grade of the research subjects showed the most proportions came from grade II (59.44%), followed by grade I (23.44%), and the smallest proportion came from grade III (17.20%). It was similar with the study made by Yan et al., (2008), however it was different with the study made by Qian et al., (2009) where the most proportion came from grade III and the smallest was from grade I.

An analysis on miR-21 expression toward the entire research sample was conducted. Based on the comparison of average miR-21 expressions between grade, it was obtained that the highest miR-21 expression came from grade III, followed by grade I and the lowest expression was from grade II. The increasing expression of miR-21 in grade III from the research result was in accordance with the research result of Qian et al., (2009) and Lee et al., (2011).

A soaring MiR-21 expression in grade III caused an increasing regulation in a form of translation or degradation pressure by miR-21 toward tumor mRNA target tumor suppressor (among others are Network p53 and PDCD4 (Chen et al, 2012). Consequently, the function of tumor suppressor protein was extremely decreasing in term of control on cell proliferation and neoplastic transformation. The process of cell proliferation, cell differentiation, and neoplastic transformation kept on going and it was hard to control. The impact was seen from the description of grade III which were extensively varied formation of tubulus and nuclear pleomorphism, as well as excessively increasing cell mitosis, compared to grade I and II.

Otherwise, low expression of miR-21 in grade I and II caused regulation effect of miR-21 toward Mrna target tumor suppressor was also low. Consequently, cell proliferation and neoplastic transformation were still in control by tumor suppressor protein. It was in accordance with the description of grade I and II, which was the formation

pattern of tubulus, nuclear pleomorphism, and cell mitosis relatively close to the description of normal breast tissue.

MiR-21 is known to have some target mRNA tumor suppressor, among others are PDCD4 and Network of p53 (Frankel et al., 2008; Papagiannakopoulos et al., 2008). PDCD4 is tumor suppressor protein which functions to control cell transformation process, is able to hamper c-Jun and AP-1 transactivation so that malignancy progressivity can be lowered (Yang et al., 2001; Bitomsky et al., 2004; Jansen et al., 2005). Frankel et al., (2008) found that mRNA PDCD4 could be hampered by miR-21. If miR-21 suppressed PDCD4 expression, thus would impact in the increasing of cell proliferation and transformation.

In addition to PDCD4, another tumor suppressor is p53 that functions to minimize genetic breakdown so that carcinogenetic process can be prevented. Abnormal cell proliferation can be controlled through cycle cell arrest or apoptosis, it depends on the level of DNA breakdown. In doing its functions, p53 protein needs other protein to help stabilizing and activating p53. The proteins meant are among others *HNRPK* (Moumen et al., 2005), *DAXX* (Li et al., 2007), *TOPORS* (Lin et al., 2005). MiR-21 can suppress protein expression which is important for stabilization and activation of p53 as well as proteins which become the target of p53 transcription (Papagiannakopoulos et al., 2008). The hampered protein expression by miR-21, will impact in the increasing of cell proliferation and the decreasing of apoptosis.

Breast cancer is suffered more by productive age group, and grade II is the largest proportion of histopathology grade. By finding an escalating miR-21 in grade III (compared to grade I and II), it is even more confirming the assumption that miR-21 is an oncomiR which plays an important role in the process of malignancy development on breast cancer.

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