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The Correlation between Mammaglobin a mRNA Concentration in Peripheral Blood and Clinical Stages of Breast Cancer Patients at Sanglah General Hospital

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Abstract: ***It's difficult to determine the state of breast cancer after complete treatment by the clinical finding alone. Mammaglobin A mRNA is one of the most simplest promising method in follow-up post-operative due to it's specific overexpression for breast cancer. Methods: The study design was cross sectional analytic study. The samples consist of 60 breast cancer patients were analysed the peripheral blood by qRT-PCR for mRNA Mammaglobin A. Results: The average concentration of mRNA Mammaglobin A is 1902.1287 µ g/mL. The ANOVA test showed a significant differences of mean concentration of mRNA Mammaglobin A between metastatic and non-metastatic (Average difference concentration 285.71328 µ g/mL; 95% CI = 28.20960 – 543.217). Conclusion: Mammaglobin A mRNA concentration was different in metastatic and non metastastatic. Micrometastases could detect as early as possible and quantification of CTC can be done before clinically manifested.***

Keywords: Mammaglobin A mRNA, qRT-PCR, Clinical Stages Breast Cancer

1. Background

Breast cancer is still the main health problem among women worldwide.

In Indonesia, the incidence of breast cancer was ranked second and still become a serious public health problem. Breast cancer is a type of malignancy with second largest incidence in women after cervical cancer in Indonesia. In Bali, the incidence of breast cancer is still unknown, but more than 70% of breast cancer patient that treated at Sanglah General Hospital were already in the advanced stages (stage III and IV)[(1)]

The main cause of death in people with cancer is metastatic. The metastatic process occurs through the release of cancer cells from the primary tumor then follows lymphatic pathway and spread hematogenously to distant organs. Detection of metastasis with conventional methods such as imaging and clinical examination were unable to detect the presence of early metastases accurately. When the examination is positive, the disease already in the late stage and the treatment merely is palliative care, which means the end result is unsatisfied[(2)].

Cancer cells that circulate in the circulation known as the Circulating Tumor Cells (CTC), so early detection against the CTC in patient with solid tumors have an important implications for prognosis and outcome of the therapy [(3)]. There are two challenges in tracking the CTC, first; the CTC has heterogeneous characteristics, second; the numbers of CTC are very rare. Because of those characteristics, it needed a sophisticated, sensitive, and specific technology [(4)].

More than 10 years ago, many specific and sensitive criteria has been found for qPCR, one of them is Mammaglobin A [(5)]. Human Mammaglobin A mRNA (hMAM) is a specific marker and sensitive for breast cancer [(6)].

Mammaglobin A mRNA as a protein specific somatic type will become over expressed on the breast tissue especially in breast malignancy. Cancer cells containing this mRNA is a viable cell and will through shedding process into the circulation to become CTC (Circulating Tumor Cells) and the DTC (Disseminated Tumor Cells) in the bone marrow [(7)].

Mammaglobin could become an ideal biomarker for micrometastatic cells detection in people with breast cancer [(6B, 8)]. Therefore, the aim of this study will focus on tracking mRNA of Mammaglobin A in the peripheral blood of the breast cancer patient associated with different clinical stage. Blood samples were selected because it can be checked at more frequent intervals than a sample from bone marrow and relatively less of pain [(9)].

Molecular diagnostics, for the first time, has been integrated into the TNM system classification of breast cancer. The staging system commonly will underestimate the severity of the disease [(10)]. Information from pathology and molecular biology will increase the accuracy of the staging and as the base of most clinical trials. This study also examines the differences of the average concentration of Mammaglobin

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mRNA in the peripheral blood of breast cancer patients with TNM clinical stage.

2. Method

This research used cross sectional analytic study design. Peripheral blood sample from breast cancer patient was taken for detection of Mammaglobin A mRNA. Then looked for the differences of average concentration of Mammaglobin A mRNA toward breast cancer patient clinical stage. The samples were breast cancer patient that treated in Sanglah General Hospital during the time period of the study. The criteria of inclusion, namely; new breast cancer patient that was treated at Sanglah General Hospital, breast cancer patient who have a complete medical record, only have one kind of cancer (breast cancer), have Karnofsky score > 70%, and breast cancer patient who did not undergo a surgical therapy, chemotherapy, radiotherapy, or hormonal therapy yet. Breast cancer patient who refuse to participate and have no clinical data, imaging, and histopathology were excluded.

Subject's clinical aspects were recorded before undergo the treatment. Those clinical aspects include age, employment (social economy), breast cancer's risk factors, menstrual status, and history of taking hormonal drugs. Minimum samples needed in this study was 56 samples.

After undergo biopsy and imaging examinations for identification the stage of breast cancer, data concerning aspects of clinical, pathological, and stage of the tumor were also obtained.

All registered breast cancer patients who meet the requirement's criteria, then 10 cc of their peripheral blood were taken and stored in vacutainer containing EDTA. This blood samples then immediately sent to the Laboratory of Molecular Biology Medical Faculty Udayana University.

QRT-PCR examination in the Laboratory of Molecular Biology Medical Faculty Udayana University pass through 4 stages. The first stage, buffi coat was isolated to remove erythrocytes and unucleated cells. Then the samples was stored in a refrigerator at a temperature of minus 80℃. The second stage, mRNA isolation using Invitrogen® was also isolated. The third stage was processed reverse into cDNA (iScript® cDNA synthesis). The fourth stage was running process on qRT-PCR assay using specific primer and Evagreen® probe. Mammaglobin was measured by real time PCR assay that use specific primer from primer3 online software as well as Evagreen probe by forward primer 5' GCCCCTTATTGGAGAATGTGAT 3' and a reverse Primer 5' CACCTCAACATTGCTCAGAGTTTC3. Ct Result of qRT-PCR was then converted by using standard curve into µg/mL.

Table 1: The Characteristics of Subject (n=60)

Characteristics	Frequency	Percentage
Age		
21-30 years	3	5,0
31-40 years	19	31,7
41-50 years	26	43,3
51-60 years	6	10,0
> 60 years	6	10,0
Tumor Status:		
T0	0	0
T1	2	3,3
T2	13	21,7
T3	10	16,7
T4	35	58,3
Node Status		
N0	16	26,7
N1	22	36,7
N2	13	21,7
N3	9	15,0
Stage		
I	1	1,7
IIA	12	20,0
IIB	5	8,3
IIIA	8	13,3
IIIB	17	28,3
IV	17	28,3
Metastasis:		
Visceral	14	82,4
Bone	1	5,9
Contralateral breast	2	11,7
Mammaglobin A:		
Positive	55	91,7
Negative	5	8,3

3. Results

1. The Characteristics of Subject

The average age of the patients diagnosed with breast cancer in this study was 44,4 years, from 27 years to 65 years old. Incidence of breast cancer was most commonly occur in premenopausal women (76,7%) compared to menopausal women (23,3%). Almost all of the sample (98,3%) did not have any history of breast cancer in their families and the mean menarche occur at age of 13,8 years old.

Based on the location of the breast cancer, the most common breast cancer in samples were found on the lateral upper quadrant (51.7%) followed by central quadrant (21,7%), medial upper (13.3%), lower lateral quadrant (3.3%) and lower medial (1.7%).

Based on histology properties of the cancer, 83.3% of the cases was intraductal carcinoma and more than a half (51%) was found at grade 3. Most of the patients (50%) was on LABC stadium, size more than 5 cm wide, and palpable lymph nodes. The rest was found at EBC stadium (21,7%) and MBC (28,3%).

In Mammaglobin A mRNA examination, 91.6% of peripheral blood samples showed positive result and only 8.3% was negative. The average concentration of Mammaglobin mRNA is 1902.1287 µ g/mL, with the lowest concentration of 1.53 µ g/mL, and the highest 2808.46 µ g/mL.

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Table 2: The differences of Mammaglobin A mRNA average concentration (µg/mL) from peripheral blood toward clinical stage, node status, tumor size, and metastasis

Variable	Amount/ Number of patient	Mammaglobin Average Concentration	Std. Error mean	95% *Confidence Interval* for mean		P value
				Lower border	Upper border	
STAGE						
EBC	14	1768.5257	212.29645	1309.8871	2227.1643	p =0.78
LABC	27	2123.5504	84.55414	1949.7469	2297.3539	
MBC	14	2288.0357	86.70570	2100.7194	2475.3520	
NODE						
Positive	42	2175.0374	57.62784	2058.6556	2291.4192	p = 0.112
Negative	13	1752.0115	241.63740	1225.5289	2278.4942	
Primary Tumor Size						
T1-T2-T3	25	1979.0904	132.94113	1846.1493	2112.0315	
T4	30	2155.0153	80.07024	2074.9451	2235.0855	p=0.264
METASTASIS						
META	14	2288.0357	86.70570	2100.7194	2475.3520	
NON META	41	2002.3224	93.65098	1813.0467	2191.5981	p=0.03

In this study, 14/55 (25.5%) Mammaglobin was positive in EBC stadium, 27/55 (49,1%) on LABC stadium and 14/55 (25.5%) on MBC. There was no significant differences in average concentrations of Mammaglobin A mRNA between stadium EBC with LABC or MBC in peripheral blood samples, p = 0.78

The proportion of positive axillary nodal metastasis found 42/55 (76,4%) compared to no axillary metastasis 13/55 (24%). There was no significant difference in average concentration of Mammaglobin A mRNA between samples with positive axillary lymph node metastasis and those without metastasis, p = 0,112

The combined proportion of T1, T2 and T3 discovered on 25/55 (45.5%) and T4 30/55 (54.5%). Non parametric test found there was no significant difference between the average concentration of Mammaglobin A mRNA in peripheral blood samples in primary tumor that show no infiltration any structure compared to the primary tumor which is already infiltrating into the skin or chest wall, and p = 0,264

The same result was found while measuring mean concentration of mRNA based on metastasis status. Statistic analysis by ANOVA showed a significant differences (p=0,03) of mean concentration of Mammaglobin A mRNA between group of metastatic and non-metastatic (average difference 285.71328 µ g/mL; 95% CI = 28.20960 – 543.217).

4. Discussion

Mammaglobin A is one of gene from uteroglobin family which has a specific characteristic in breasts [(11)]. These Mammaglobin gene expression was found limited in normal breast epithelial and amplified neoplastic. Mammaglobin A mRNA expression can be found in blood of breast cancer patient's detected using nested-RT-PCR method. If mRNA carried out in the peripheral blood, those mRNA can predict hematogen dissemination of tumor cells. Because of that reason, Mammaglobin mRNA can be a potential biologic marker to detect present of micrometastasis in breast cancer[(12)].

In this study found high level of mRNA detection (91,7%) in the peripheral blood of breast cancer patient by using qRT-PCR method and negative expression in the healthy individual. Xie et al 2008 proved that RT-PCR method have high specificity and potential to identify hidden cancer cells. Incidence of positive mRNA Mammaglobin A in the peripheral blood found 34%, meanwhile in tumor tissue found 95% by using RT-PCR method. Negative results found in healthy group and in benign breast tumor group[(13)].

There's no significant differences between the average concentration of Mammaglobin A mRNA in the peripheral blood toward TNM clinical stadium. This result proved that CTC can be found in early stage of breast cancer and its quantity can be calculated with tracing Mammaglobin A mRNA. Ntoulia et al (2006) reported Mammaglobin mRNA positive 13.9% in early stage breast cancer patient, 64,3% among those patient were having relapse during follow up period [(14)].

There's no significant difference found in average concentration of Mammaglobin A mRNA between positive axillary node and negative axillary node. This showed CTC can be found not only in positive axillary node but also in negative axillary node. This data also showed that role of hematogenous dissemination were important as well as lymphogenic dissemination.

No significant difference found in average concentration of Mammaglobin A mRNA between tumor size that already infiltrate to skin and size of tumor that not infiltrated yet. This result more likely support the result of mRNA Mammaglobin A concentration toward breast cancer clinical stage. Based on immunohistochemistry, there is significant Mammaglobin expression in breast cancer [(15)].

No relation was found among Mammaglobin A expression, disease progression, primary tumor extension, tissue metastasis, and grade of tumor histology. Meanwhile

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hormone receptor and Mammaglobin A both commonly have positive result [16].

In this study, significant differences between average concentration Mammaglobin A mRNA in the peripheral blood of breast cancer patient that have distant metastatic 41/55 (74,5%), and non metastatic breast cancer patient 14/55 (25,5%) was found. The differences of mean concentration of Mammaglobin A mRNA was in 28,20960 – 543,217 µg/mL with 95% confident interval.

Five samples have negative Mammaglobin mRNA expression. Instability of in vitro RNA, cell in these patients may become one of the causes. The amount of mRNA copy could change during the duration of storage and transportation in room temperature. These would be the influencing factors toward counting process of certain gene transcript, especially if there is a limited amount of mRNA target. There were significant decrease of B-actin, *GAPDH*, cytokeratin-19 (*CK-19*) and *HER2* amount. *CK-19 and HER2* amount decreased after 4 hours, b-actin amount decrease after 6 hours and *GAPDH* decreased after 24 hours. Mammaglobin expression still stable in 6 hours. The expression of vascular endothelial growth factor (VEGF) increasing after 24 hours since patient's blood taken. Blood sample and RNA must be process or stabilize 3 hours after blood taken to avoid gene expression because of ex vivo stress response [17].

5. Conclusion

Mammaglobin A mRNA concentration was different in metastatic and non metastastatic. Micrometastases could detect as early as possible and quantification of CTC can be done before clinically manifested.

Because the concentration of Mammaglobin A mRNA can be known quantitatively and have a specific characteristic for breast cancer, so Mammaglobin A mRNA could be a micrometastatic biomarker and can be clue for tumor with hematogenous spread. It can help to monitor the disease, make decision for therapy, and know the prognosis of breast cancer patient.

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