

The Comparison of *RhoC* and *PI3K* Gene Expression on the Breast Cancer Tissue and Benign Tumour Tissue

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Abstract

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BACKGROUND: The expression of a gene is a process that conveys information of genes to synthesise gene product is functional. Alterations of the molecular biology in breast cancer are very complex because of many factors play a role in the tumorigenesis. *RhoC* is a prometastases gene. The *PI3K* gene is crucial in the regulation of multiple cellular functions, including cell growth, proliferation, metabolism and angiogenesis.

AIM: This study aims to compare of *RhoC* and *PI3K* gene expression on the breast cancer tissue and benign tumour tissue.

MATERIAL AND METHODS: Expression of the *RhoC* and *PI3K* genes was carried out with qPCR. The absolute quantification method was using breast cancer tissue. As a comparison, benign tumours (FATs) tissue was carried out. The standard curves were obtained from cloning target genes, which were inserted into the gGEMT-easy vector from *E. coli*. The gene expression data was carried out by t-test to see the mean difference between the expression of breast cancer tissue and benign tumours (FATs) with a value of $p \leq 0.005$ in *RhoC* and *PI3K* gene expression. And the relationship between expressions was done by Pearson correlation test.

RESULTS: The results showed that it was found that *PI3K* gene expression on breast cancer tissue was higher than the number in a benign tumour (fibroadenoma mammae) as an endogenous control. And also, the expression of *RhoC* is much lower on breast cancer tissue compared with a benign tumour.

CONCLUSION: This study concluded that expression of *RhoC* affects the expression of *PI3K* so that the thing this is what causes the proliferation and began to provide support aggressive cancer cells in the breast.

Introduction

Fibroadenoma, or fibroadenoma mammae (FAM), is one of the most common types of benign tumours that occur in the breast. Fibroadenoma is round with a firm boundary and has a chewy consistency with a smooth surface [1]. The majority of breast disorders are benign lesions; malignant lesions are only 20% of all abnormalities in the breast. The incidence of this benign disorder begins at the age of

the second decade, and the peak is in the fourth and fifth decades of life. A small portion of benign tumours is associated with breast cancer [2].

Cancer is a non-communicable disease characterised by abnormal/continuous and uncontrolled cell growth that can damage the surrounding tissue and can spread to places far from its origin called metastasis. Cancer cells can originate or grow from any cell in the human body. Cancer has become a health problem in the world, including

Indonesia. The type of cancer that many women suffer and fear is breast cancer [3].

Histopathologically most mammary lesions consist of one or more lumps whose shapes and sizes vary greatly. These lumps can be firmly bound or not, single or multiple nodules, soft or hard, can be moved from the bottom or not. This can help distinguish benign lesions or malignant lesions in the breast [4], [1]. However, in molecular biology, there is still a little-known difference in genetic profile between fibroadenoma mammae and Ca mammae (breast cancer). The profile of gene expression in breast cancer has been studied intensively. Gene expression profiling is enabling scientists to understand the heterogeneous nature of breast cancer on a genomic level.

Breast cancer is currently a problem in the health sector because the incidence of breast carcinoma increases from year to year in both developed countries and developing countries like Indonesia. The breast cancer mortality rate also increased sharply [5]. Based on the Globocan estimate, the 2012 International Agency for Research on Cancer (IARC), breast cancer is cancer with the highest percentage of new cases (43.3%) and the highest percentage of deaths (12.9%) in women in the world. Based on data from the Indonesian Ministry of Health (2010), the prevalence of breast cancer in Indonesia reached 0.5 per 1000 women [6].

RhoC is known to be a pro-metastasis gene belonging to the RAS superfamily. RhoC expression increases in gastric cancer, and it will activate the PI3K / Akt pathway to induce the cell invasion. This mechanism is different in some cancers [7]. RhoC is also an effector on the MAPK pathway that increases VEGF, fibroblast growth factors, and regulates the expression of IL-6 and IL-8 [8], [9]. Changes in the expression of RhoC are associated with increased cell proliferation and cause tumours to become malignant [10]. RhoC is a negative mediator from affects the PI3K/Akt and MAPK pathways [11].

PI3Ks are a family of intracellular enzymes that are associated with signal transduction, are involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular traffic and are in turn involved in cancer [12]. The PI3K/Akt pathway is activated by changes in the expression of RhoC protein [13]. On the PI3K line, activating Akt is called the PI3K/Akt line. This pathway contains many activators, inhibitors, effectors and second messengers. Several studies have shown that high activity of PI3K/Akt signals will induce resistance of chemotherapy and HER-2 therapy targets. Activation in the PI3K/Akt pathway will promote cell proliferation [14].

Activation of PI3K/Akt causes interference with cell growth control. If there is a change in expression, it will cause metastasis, angiogenesis and therapeutic resistance and reduce PTEN activity [15].

Thus, the molecular mechanism that passes through the complex pathway into the future is considered to be one of the most attractive targets for the development of anticancer agents [16].

Also, PI3K pathways are stimulated as a result of many growth physiological factors and regulators. Whatever the mechanism, PI3K activation will cause a disruption of control of normal cell growth and cell continuity, which contributes to competitive growth, metastatic ability, frequent resistance to therapy. This pathway is an attractive target for the development of new anti-cancer [13]. The expression profile has the potential to differentiate between Fibroadenoma and breast cancer. So, this can be used as a guide for the diagnosis and future prognosis. Therefore, the RhoC inhibition target is possible as an alternative path in activating PI3K. So PI3K has the potential to target the care of breast cancer people [17], [18]. Changes in the level of mRNA, which will be related to expression can be used as biomarkers to detect disease early and can be intervened at the stage of disease progression.

Material and Methods

Sample

The sample is fresh tissue of breast cancer and benign tumour tissue stored in the BioBank Biomedical Laboratory Tissue. Samples from breast cancer and benign tumour tissue consisted of 30 breast cancer tissues and 30 benign tumour tissues. For breast cancer tissue taken from 30-50 years old. This group was taken because it is the largest population of breast cancer whose tissue is stored in the BioBank Tissue of Faculty of Medicine, Andalas University.

Total RNA isolation

Total RNA from breast cancer tissue and benign tumours (FAM) was isolated using Pure Link Ambion RNA isolation kit (12183018A). The initial stage of sample preparation is homogenised by using the stator-rotor technique to make homogeneous from the tissue. After the isolation process is complete, save the total RNA at -80°C.

cDNA synthesis

The synthesis composition of the total cDNA was 5 µg total RNA, 1 x RT buffer, 20 pmol oligodT, 4 mM dNTP, 10 mM DTT, 40 U SuperScript TMII RTase and H₂O-DEPC enzymes with a reaction volume of 20 µl. Total cDNA synthesis was carried out at 52°C for 50 minutes with the work protocol by the manual kit

(Isd cDNA synthesis, Biorad). Check the success of cDNA synthesis using NanoDrop.

Table 1: Primer design

No.	Primer	Nucleotide Sequence
1.	RhoC	5'- GCCCGGCCCGACCCGACCGCACC-3' 3'- GGTAACCGATCAGAATGACAACA-5'
2.	PI3K/Akt	5'- CGACCCAACCCAAGAATCTATC- 3' 3'- AGGTGGTCACTTGGTCTTATTC-5'

Amplification of the real-time PCR of the target gene

Each gene was amplified with an SYBR Green amplification kit. The PCR program as follows: Predenaturation of 95.0°C for 3 minutes. Denaturation of 95.0°C for 5 seconds, annealing gradient of 50-60°C for 5 seconds, for 39 cycles. Add a melting curve of 65.0°C to 95.0°C with an increase of 0.5°C for 5 seconds. The miRNA is amplified wherein it contains the primary HSA-miR-16-5p LNA PCR set, HSA-MIR-10b-5p LNA™ PCR primer set and HSA-miR-21-5p primary PCR LNA set (Exiqon, Denmark), ExiLENT SYBR Green master (Exiqon, Denmark). The PCR program as follows Predenaturation 95.0°C for 10 minutes, Denaturation 95.0°C for 10 seconds, gradient annealing 55 – 65°C for 1 minute, for 39 cycles. Add a melting curve of 60.0°C to 95.0°C with an increase of 0.5°C for 5 seconds.

Data analysis

The expression of PI3K and RhoC genes is done by absolute quantification examination. Making standard curves from target genes is based on the Whelan et al., 2003 method [19]. Differences in mean expression of PI3K and RhoC between breast cancer tissue and benign tumours were carried out by t-test with 95% significance level at $p \leq 0.05$. Furthermore, the relationship between RhoC and PI3K gene expression was carried out by the Pearson correlation test with a correlation value of 0-1.

Results

qPCR RhoC gene

Plasmids with the insertion of the RhoC gene for standard curves and target genes together, so that the standard curve is obtained, as shown in Figure 1 below. From the results of run qPCR, the melting curve is obtained, as shown in Figure 1b.

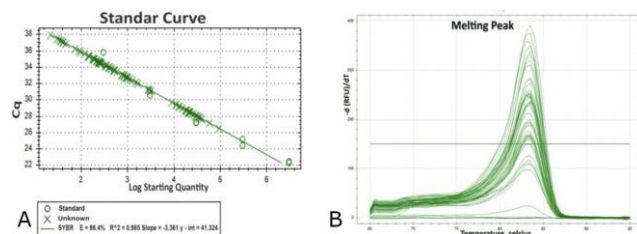


Figure 1: Standard Curve graph of PI3K gene insertion in vector and graph melting peak RhoC gene from Real-time PCR

The results of the expression of the RhoC gene from absolute quantification Real-time PCR were obtained in the form of a copy number, and from this study, it was found that the expression of the RhoC gene in breast cancer tissue was lower than the RhoC gene expression in benign tumour tissue (Figure 2).

Table 2: RhoC gene expression between breast cancer tissue and fibroadenoma tissue

Group	Expression	SD	p
Breast Cancer Tissue	3.701	0.756	0.005
Fibroadenoma Tissue	4.463	0.939	

From this study, there was a significant difference in RhoC gene expression between breast cancer tissue and benign tumour tissue with a value of $p < 0.05$ ($p = 0.0001$)

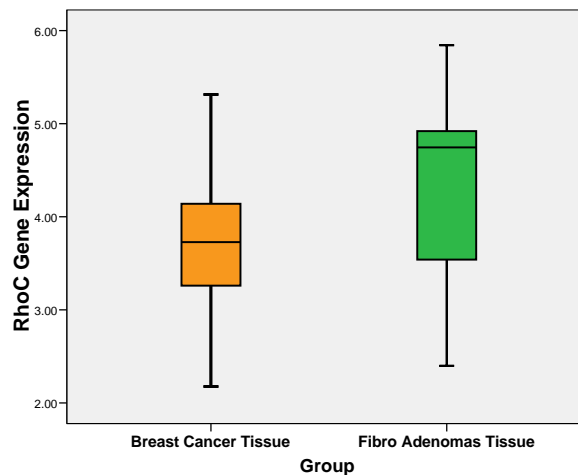


Figure 2: Boxplot graph of RhoC gene expression in breast cancer tissue and fibroadenoma tissue (benign tumour)

qPCR PI3K gene

Plasmids with PI3K gene insertion for standard curves and target genes are concurrent so that the standard curve is obtained, as shown in figure 3a below. From the results of running qPCR, the melting curve is obtained, as shown in Figure 3b.

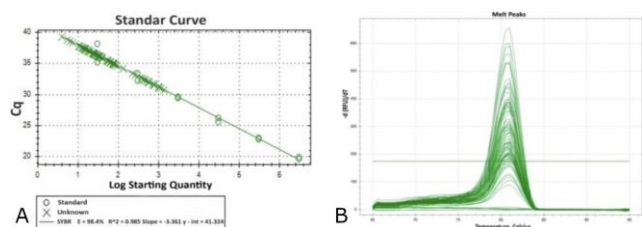


Figure 3. Standard Curve graph of PI3K gene insertion on vector and graph melting peak PI3K gene from Real-time PCR

The results of the expression of the PI3K gene from absolute quantification of real-time PCR were obtained in the form of copy number values.

Table 3: PI3K gene expression between breast cancer tissue and fibroadenoma tissue

Group	Expression	SD	p
Breast Cancer Tissue	4.171	0.569	0.001
Fibroadenoma Tissue	3.620	0.667	

From this study, it was found that the expression of the PI3K gene in breast cancer tissue was higher than the PI3K gene expression in benign tumour tissue.

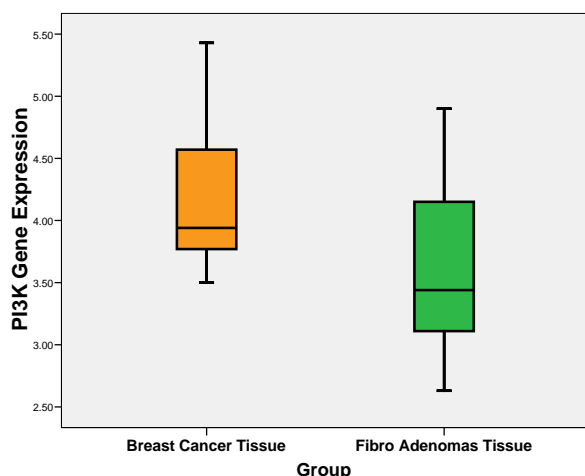


Figure 4: Boxplot graph of PI3K gene expression in breast cancer tissue and fibroadenoma tissue (benign tumour)

Discussion

Molecular changes in the growth of breast cancer are very complex. At first, this change was based on changes in three groups of genes that control cancer growth. The main hereditary gene that influences the growth of breast cancer known today is BRCA1 / 2, which is a suppressor tumour gene. It turns out that this gene only contributes 15% [20].

The expression of the RhoC gene in breast cancer tissue is lower than fibroadenoma tissue

(FAMs). The difference in expression of this RhoC gene between breast cancer tissue and fibroadenoma was significant at $p = 0.0001$ ($p < 0.005$). It is suspected that the breast cancer tissue in this sample is still in the early stages of breast cancer and has not experienced metastasis. Lower expression of the RhoC gene in breast cancer tissue than FAMs is likely because breast cancer tissue is a network of primary breast cancer/primary tumour.

Lower expression of the RhoC gene in breast cancer tissue than FAMs is likely because breast cancer tissue is a network of primary breast cancer/primary tumour. But on the other hand, in other studies on breast cancer tissue that had metastasised an increased expression of RhoC when compared to normal tissue. But it is not stated whether the normal network is the source. Because it can be assumed that it is different between normal networks and FAMs tissue, these results can explain the underlying mechanism and provide new therapeutic targets for inhibiting invasion and metastasis from cancer cells [21].

Conversely, if the expression of RhoC is excessive, this causes degradation and reconstruction of the Extracellular Matrix (ECM), which helps cells escape from their tissues. So that it will increase cell motility and increase the ability to be invasive [22]. Uniquely from this protein RhoC, gene damage (mutation) is not found in RhoC. Changes in the expression are not only caused by mutations, but there are other factors involved, such as the effect of epigenetic miRNA and chromosome modification and can also be caused by disturbances at the post-transcription and post-translational stages.

RhoC functions as a button in the signal transduction of the cascades. RhoC promotes the reorganisation of the cytoskeleton, regulates cell shape and cell motility. RhoC can activate formins such as MDIA1 and FMNL2 to change the shape of the cell cytoskeleton [23], [24]. And also, RhoC affects the PI3K pathway, which is a biomarker of proliferation and initiation of invasion [25]. As is known in human's phosphatidylinositol 3-kinase/AKT is a target of the rapamycin (PI3K / Akt / mTOR) pathway which plays an important role in the intracellular signalling system that guides cell growth and cell defence. It is estimated that 70-75% of breast cancers express the ER-associated with this pathway. Many patients with ER + will be more resistant to chemotherapy. Knowing the state of PI3K expression will help in the prognosis for how long to use endocrine hormone therapy and provide an overview of the use or delay of using chemotherapy [26].

In this study, it was found that the expression of PI3K in breast cancer tissue was higher than that of Fibroadenoma. James Ryan and colleagues also found that PI3K expression in breast cancer tissue was higher than in Fibroadenoma [27].

Activation of PI3K activates Akt, so it is known

as PI3K/Akt, which disrupts cell growth control. This is what happens in breast cancer tissue has higher expression of PI3K compared to FMs tissue. So that if there is a change in expression, it will potentially cause metastasis, angiogenesis and therapeutic resistance. Besides that, the increase in expression of PI3K will reduce PTEN activity [15]. The PI3K/Akt pathway is activated by changes in the expression of RhoC protein. The high intracellular activity on the phosphatidylinositol-3 kinase (PI3K) pathway is common in breast cancer [13]. Thus, the molecular mechanism that passes through the complex pathway into the future is considered one of the most attractive targets for the development of anticancer agents [16]. Activation in the PI3K/Akt pathway will promote cell proliferation [14]. Although the expression of PI3K is higher in breast cancer tissue, when expression of RhoC is still low in breast cancer tissue can be used as a signal that the cancer cell has not yet invaded, because it has not affected the extracellular matrix to change, where changes in the extracellular matrix indicate that the cell cancer will experience invasion and metastasis.

Also, PI3K pathways are stimulated as a result of many growth physiological factors and regulators. Whatever the mechanism, activation of PI3K will cause disruption of control of cell growth and cell continuity, which contributes to competitive growth and metastatic ability. So that resistance often occurs in therapy. This pathway is an attractive target for the development of new cancellers [13]. Therefore, the target of inhibiting PI3K has the potential to target the care of breast cancer people [17], [18]. And it will be even more interesting, the potential for inhibition of genes that are upstream, namely RhoC, will inhibit the process of cancer cells becoming more malignant and undergoing invasion and metastasis. So that RhoC can be used as a good marker to determine breast cancer that is very aggressive and motile and can provide guidance in therapy for intervention before developing into metastasis so that it can identify primary breast cancer cells with breast cancer that have the potential to metastasise [28]. Of research is it can be concluded that an expression of RhoC affect the expression of PI3K so that the thing this is what causes the proliferation and began to provide support aggressive cancer cells in the breast.

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