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Research Article
Profile of miR-10b Expression and Metastases-related Genes in BC and Fibro Adenoma in West Sumatera

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Abstract

Background and Objective: miR-10b is one of oncogenic miRNA first described in promoting metastases in Breast Cancer. The aim of the study was to investigate the expression of miR-10b and the expression of metastases-induced genes in BC (BC) and fibroadenoma (FA) in West Sumatra. **Materials and Method:** LNATM primer enhancer set was used to identify the miR-10b expression as a relative quantification. miR-16 was used as an endogenous control with a relative median expression of miR-10b at $2^{-\Delta\Delta Ct}$. The expression of metastases-related genes was performed by an absolute quantification method. **Results:** The statistical differences between miR-10b and the expression of genes were determined by t-student test to interpret the expression of BC and FA tissue ($p \leq 0.005$). The expression of miR-10b in BC was lower than FA (endogenous control). Relative median of miR-10b expression in BC was 8.51 times lower than FA. Low expression of miR-10b in BC was associated with tissue grading. The expression of metastases-related gene; RhoC, TIMP2 and MMP2 were lower in BC than FA. miR-10b expression differed between BC and FA. **Conclusion:** RhoC, TIMP2 and MMP2 expression caused cancer cell whether to be metastasized or not. miR-10b is a potential marker to predict BC cell which start to be aggressive.

Key words: RhoC, miR-10b, TIMP2, MMP2, qPCR, relative quantification, absolute quantification

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Breast Cancer (BC) is a current issue in health, the incidence of BC increases from year to year as also breast carcinoma in developed and developing countries. The mortality rates of BC also rises sharply[1]. The incidence of BC elevates in developing countries such as in Indonesia, in line with the altered life style. Although it tends to be high in women in developed countries, the mortality rate is 58% lesser than in developing countries. It's caused by an understanding and early detection of BC is better in women in developed country[2].

In West Sumatera, the risk factors of BC are uncommon; most of patients were in the middle to lower economy, 30-45 years old, premenopausal, thin, multiparous, child-rearing, married under 25 years old and not consuming alcohol[3]. This raises a question whether is the factor of BC incidence in West Sumatra.

MicroRNA (miRNA) is a short molecule of non-coding RNA (~22 nucleotides) and acts in regulation of gene[4]. The expression of miRNA in human BC was still debated in the past few decades[5-8]. miR-10b expression was higher in patients with distant relapse, regional relapse and local recurrence[9] while other study found different result[10]. Lund[11] found the upregulation of miR-10b in glioblastoma, anaplastic astrocytomas, primary hepatocellular carcinomas and colon cancer. MicroRNAs act in various important processes of growth, such as differentiation, apoptosis, adhesion and other cellular processes[12]. Iorio and Croce[13] applied microarray to find miRNA-10b. MicroRNA-10b in BC acts in various cellular processes such as proliferation, metastasis, angiogenesis and BC cell invasion[14]. RhoC, is a pro-metastases gene of Ras-superfamily. A growing number of reports focus on RhoC as an essential factor for invasion and metastasis of various types of tumour cells[15-19], the over expression of RhoC was especially linked to aggressive cancers as in inflammatory BC and rapidly metastasizes[20,21]. The changes in RhoC expression are related with cell proliferation and lead to the tumours becomes malignant[22].

In microRNA-involved in invasion and metastasis of BC, RhoC affects the TIMP2 gene[23], triggers the Extracellular Matrix (ECM) to degrade and re-construct, then the cancer cells escape from the tissue, increases cell motility and the ability of cell to be invasive[24]. The TIMP2 is the family of Tissue Inhibitors of Metalloproteinases (TIMPs), an endogenous inhibitor that degrades the membrane of matrix metalloproteinase (MMP) family. TIMP-2 also regulates matrix degradation and acts through the MMP (MT1-MMP) membrane. The MT1-MMP is a key enzyme in angiogenesis and metastatic tumours, hydrolyzes matrix cellular (ECM) component and as a physiologically motor of pro-MMP-TIMP-2 hydrolyzes pro-MMP2 and results in degradation of matrix extracellular[25]. TIMP-2 is assumed to be associated with angiogenesis, invasion and metastasis.

TIMP-2 inhibited angiogenesis *in vitro* and endothelial cell proliferation and migration through MMP-dependent mechanisms and MMP-independent mechanisms mediated by proteolytic and endothelial cells. MMP-2 specifically contributes to the migration of cancer cell by MMP-2 interaction with collagen[26]. There is a positive correlation between BC with lymph node metastasis with MMP2 and TIMP2 ratios, it's caused by an alteration in the expression of TIMP2 and MMP2[27]. The characteristics of BC in West Sumatera are still unknown. The changes in miRNA-level related to the expression of mRNA and the changes are expected to becomes a biomarker to detect disease earlier and the intervention of treatment may be performed at the stage of disease progression, because each miRNA has different target genes and works on different phases. By determining the characteristics of miR-10b and induced-metastases gene, this result is expected as a basis for further research to inhibit tumourigenesis in early stage of BC by investigating the miR-10b and related-genes in the work pathway. Since risk factors of BC in West Sumatra differ in developed countries, this study identified the correlation of miR-10b expression and metastases-induced genes; RhoC, TIMP2 and MMP2.

MATERIALS AND METHODS

Preparation of sample: The samples was fresh frozen tissue consisted of 45 BC tissues and 30 FA tissues (as control), stored in BioBank tissue of Biomedical Laboratories, Andalas University. The samples of patient with 30-50 years old, in premenopausal phase, included in this study. The BC tissue from pregnant or man were excluded. The sampling method was consecutive sampling and sample size is calculated by comparing the two averages[28]. Minimum sample for each group was 27. By considering the lost of follow (DO) minimum 10%, the required sample was 30 samples.

Isolation of total RNA: The RNA of BC and FA was extracted with Pure Link Ambion RNA isolation kit (Thermo Fisher,

USA). The samples were prepared by homogenization of the tissues with stator rotor technique. The extracted RNA then was stored at -80°C.

Isolation of miRNA tissue: Isolation and purification of miRNA used miRNA Isolation Kit GenAid (RMI050) according to the protocol supplied by the company. The quantity of miRNA was determined by Nano Drop (Thermo Fisher, USA) and stored at -80°C. The primers used for amplifying of miR-010b and miR-16 were kit set primer (Exiqon, Denmark). Primer set of RhoC forward: 5'-GCAGTCGATCTCATAGTCTTCCTG-3'. Primer RhoC reverse: 5'-CGTCCCTACTGT CTTTGAGAAC-3'. Primer TIMP2 forward: 5'-AGGGCCTGAGAAGGATATAGAG-3'. Primer TIMP2 reverse: 5'-GGCCTTTCCTGCAATGAGATA-3' Primer MMP2 forward: 5'-GGCACCCATTTACACCTACA-3' Primer MMP2 reverse: 5'-GCAGATCTCAGGAGTGA CAG-3'.

Synthesis of cDNA from mRNA : A total of mRNA was performed in a 20 µL reaction. Synthesis of cDNA total was performed at 52°C for 50 min according to manual kit protocol of Iscript cDNA synthesis (Biorad, USA) and positive cDNA was checked with NanoDrop (Thermo Fisher, USA).

Real-time PCR to determine miRNA expression: Expression of miRNA precursor was investigated by real-time PCR method[29]. HSA-miR-16-5p Primary LNA PCR sets were used as endogenous controls and miRNA target was hsa-miR-10b-5p LNA™ PCR set primer mix (Exiqon, Denmark). The real-time PCR mix used was miRCURY LNA miRNA PCR ExiLENT SYBR Green master (Exiqon, Denmark). The profile of amplification was 15 sec at 95°C and 1 min at 60°C for 40 cycles.

Construction of standard curve for absolute quantitative PCR: The cDNA fragments of PI3K and RhoC were inserted into the pGEMT vector. The positive clone was determined using PCR colonies amplification[30].

Amplification of RT-PCR target genes: Each gene was amplified with a SYBR Green amplification kit. The PCR profile was pre-denaturation at 95.0°C for 3 min, denaturation 95.0°C for 5 sec, gradient annealing 50-60°C for 5 sec with 39 cycles of total reaction.

Research ethics: This study was already passed the ethics clearance and has been approved by the Ethics Committee of the Faculty of Medicine, Andalas University, Padang with registration number: 187/KEP/FK/2016.

Statistical analysis: Expression of miR-10b with normalization of miR-16 is an absolute quantification method. Data were normalized with universal endogenous controls with stable expression during amplification. The relative expression of miR-10b to miR-16 RNA was measured by using a $2^{-\Delta\Delta CT}$ analogue, ΔCT = (CTmiRNA-CtmiR-16RNA). The relative expression of miR-10b was in forming of ratio, representing multiply expressions between miR-10b in BC and FA tissues.

Expression level of RhoC, TIMP2 and MMP2 cDNA was determined by an absolute quantification test. The standard curve was obtained from the cloned target gene inserted into the gGEMT-easy vector of *E. coli*. Preparation of standard curve from the target gene was based on Gou *et al.*[19]. The mean difference of PI3KCA and RhoC expression between BC and FA was calculated by t-test with 95% of CI ($p \leq 0.05$). The correlation of miR-10b expression with the target gene was calculated by performing Pearson correlation test.

RESULTS

Characteristic of sample: Characteristics of subject in this study were presented in Table 1. Based on the characteristics of samples, tissues derived from patients in pre menopausal phase. Metastasis occurred in three samples and most of sample was non metastasis samples (93.3%), with range of age 35-50 years for BC and <35 years for FA. Grade criteria showed that most of sample used was in Grade II (88.9,1%).

A total of 45 BC tissues, three samples were with highest miR-10b expression (metastasis) (Metastasis was referred to advanced clinical status, metastasis and non-metastasis were not included in parameter of study, the result was concluded in according to tracking of clinical pathology data), 42 samples of BC were with miR-10 expression lower than FA and three samples of BC were with high expression level of miR-10 than FA. Tracking of clinical pathology data showed that three samples were positive metastasis and other samples were non-metastasis.

Expression of miR-10b: Based on analysis of melting peaks and curve the isolated miRNA was pure and homogeneous, as illustrated in Fig. 1a, b. The graphic showed the peak of miR-10b was higher (red color), in contrast the peak of miR16 was lower (blue color). The height of peak was inversely proportional to the expression.

The expression level of miR-10b in BC was lower than FA. The ΔCt value of miR-10b in BC was higher than ΔCt in FA,

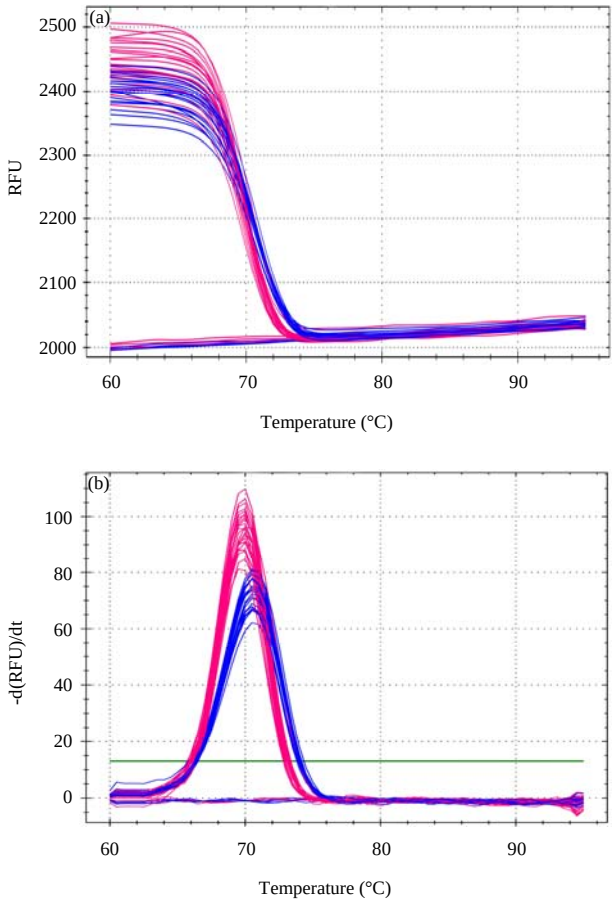

Fig. 1(a-b): Relative expression of miRNA, (a) Melting curve of miR-10b expression (red) was relative to miR-16 (blue) and (b) Melting peaks of miR-10b expression (red) were relative to miR-16 (blue)

Table 1: Characteristics of subject

Criteria	BC tissue (n)	FA tissue (n)
Ages	35-50 years of age	<35 years of age
	50	30
Pathology and anatomy result		
Grade 1	2	-
Grade 2	40	-
Grade 3	3	-
Metastasis	3	-
Non metastasis	42	-

indicated that miR-10b expression in BC was lower than in FA (as controls). Relative median expression of miR-10b2$^{-\Delta\Delta Ct}$) in BC was 8.51 times lower than in FA (Fig. 2). The expression level of miR-10 between BC and FA is significantly different ($p = 0.018$).

Expression of metastases-induced genes: The expression of RhoC gene from absolute quantification of real-time PCR was

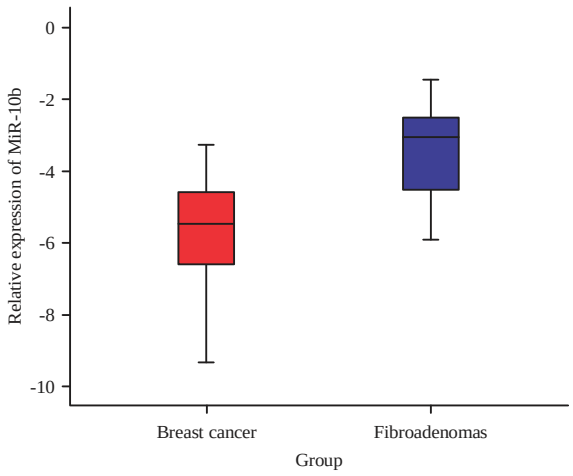

Fig. 2: Relative level of miR-10 expression between BC (red) and FA (blue) as control (Box-plot diagram with median value)
The expression level of miR-10 between BC and FA is significantly different ($p = 0.018$)

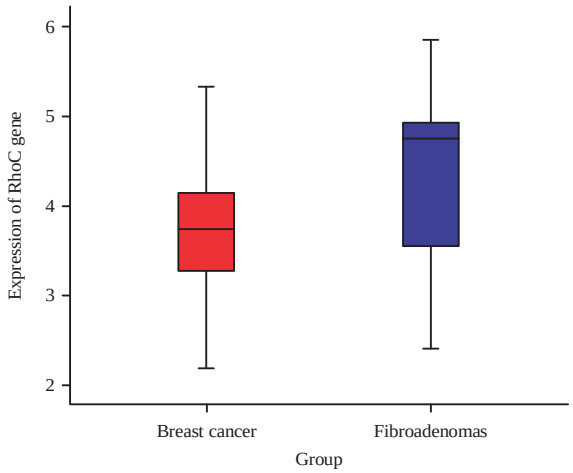

Fig. 3: Expression level of RhoC gene in BC (red) and FA (blue)
RhoC gene expression were significantly different between BC and FA with $p<0.05$ ($p = 0.01$)

obtained from copy number. In this study, the expression of RhoC gene in BC was lower than in RhoC gene expression of FA (Fig. 3). RhoC gene expression were significantly different between BC and FA with $p<0.05$ ($p = 0.0001$). The level expression of RhoC gene was determined from log of qPCR copy number value.

The results of TIMP2 gene expression from the absolute quantification of real-time PCR were obtained in forming of copy number values. The expression of TIMP2 gene in BC was lower than in FA (Fig. 4). Similar procedure was applied for MMP2 gene expression where the expression level was

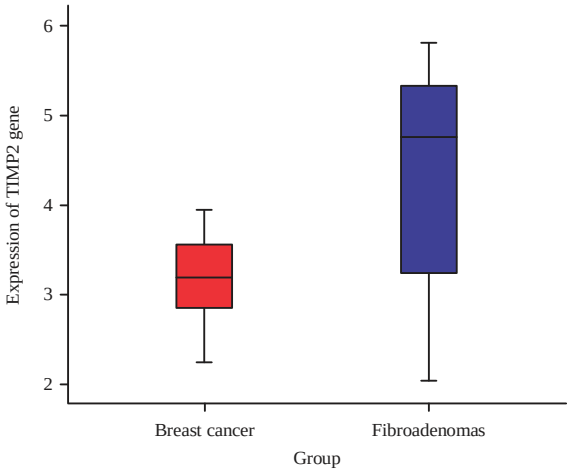

Fig. 4: Expression level of TIMP2 gene in BC (red) and FA (blue)
TIMP2 gene expression were significantly different between BC and FA with (p = 0.001)

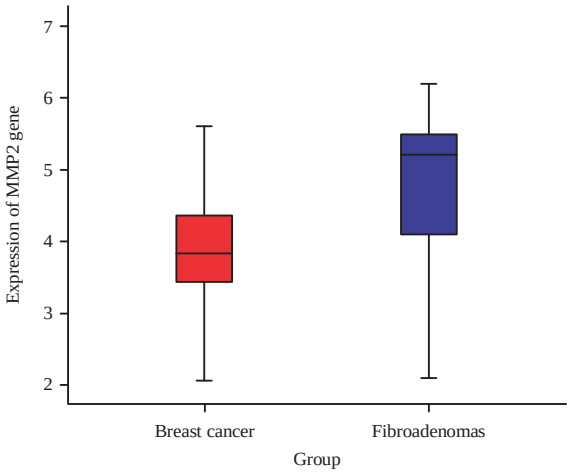

Fig. 5: Expression level of MMP2 gene in BC (red) and FA (blue)
MMP2 gene expression were significantly different between BC and FA with (p = 0.001)

determined by the logarithm transformation of qPCR of copy number. Analysis of MMP2 gene expression was identified by logarithm of qPCR copy number result (Fig. 5).

DISCUSSION

Molecular alteration in developing of BC is very complex. At first phase, the alteration is based on the change of three groups of genes that control the growth of cancer. There are several miRNAs that play a role in the growth of BC and miR-10b is one of miRNA which plays in invasion and metastasis. miRNA is oncogenic[30] and also plays a role as the initiator or progressive growth factor of cancer[31].

Histopathologically, tumour grading is based on the degree of differentiation of tumour tissue and the abnormal conditions of tumour cells, used as an indicator how fast tumour grows and parameters of mitotic activity[32]. Previous study revealed that there was a very significant correlation between histopathological grading and prognosis. Increasing of tumour grade decreases the survival of patient. Histopathological grading has been shown to be a potentially independent prognostic factor in BC patients. Grade II is associated with a moderate prognosis[33].

Tissue samples in this study have not largely undergone metastasis. Based on registration data, three samples have metastasized towards other organs. miR-10b assay with realtime PCR showed that level of ΔCp generally was lower in BC than in FA. This condition leads the expression level of miR-10b in BC tend to be higher than in FA. Positive metastatic tissue was only 6.7% of total BC tissue and it was related to the high expression level of miR-10b compared with FA. This study cannot investigate and compare the normal tissue with FA in this study because it's constraint with the ethical clearance. No normal fresh frozen tissue is available in Biobank. The result in this study is similar with previous studies that miR-10b expression level in BC was lower than FA[34]. This result due to the tissue was originated from primary BC tissue and metastasis has not yet occurred. Primary BC tissue is a condition where cells are only found in breast and has not expanded to other organs.

Typically, primary BC is an early stage of BC tumourigenesis. According to grading values, lower expression level of miR-10b related to Grade II was possibly occurred. This condition showed that BC in west Sumatra has a dominant character; premenopausal patients with level of miR-10b expression were lower than in FA. But based on qPCR result, three samples of BC tissue with positive metastasis showed lower Cq value than FA. Low level of Cq tends to result high expression of miR10b, so that sample with positive metastases showed high expression of miR-10b.

Biagioni *et al.*[35] found that miR-10b expression was lower in BC than controls. The decrease of miR-10b expression level was found in triple-negative subtypes, luminal and HER-2. Presumably, the decrease in expression level of miR-10b was preceded by proliferation which contributing to BC. Similar result was described by Baffa *et al.*[36]. They found that the expression of miR-10b was linearly associated with several types of advanced cancer. High expression level in miR-10b was obtained in metastatic tissues compared to primary tumours. The expression level of miR-10b showed no effect in

primary BC/primary tumour growth and so the expression level of miR-10b remains lower than benign tumours. Conversely, when cells of BC have already metastasis, over expression of miR-10b will occur[37].

Expression level of miR-10b does not change in primary BC tissue or tissue that has not metastasized. There are certain mechanisms involved when BC cells strongly urge to have metastasis and a positive metastasis has an increase in expression miR-10b dramatically. This mechanism remains unclear. TWIST mechanism is presumably involved to induce miR-10b[38].

RhoC is a pro-metastasis gene[39]. The beginning of metastasis followed by the occurrence of a molecular mechanism in the extracellular matrix is marked by TIMP2 and MMP2 genes. These genes are important to investigate the correlation with BC, especially with the character of the BC samples in West Sumatra.

In this study the expression level of the RhoC gene was lower in BC tissue compared with benign tumour ($p<0.05$). It's due to BC tissue was primary BC tissue/primary tumour and in early to middle stage, thus metastasis has not been yet occurred. Moreover the expression level of RhoC gene in BC is significantly lower than in FA. In metastatic BC tissue, there was an increased expression level of RhoC compared with normal tissue. These results may explained the underlying mechanism of BC and provide new therapeutic targets to inhibit the invasion and metastasis of cancer cells[40]. In primary BC tissue, miR-10b expression level is low. This condition occurs because BC cells are still undergoing proliferation but remains in primary tissue and have not invaded the adjacent tissue.

Low expression level of TIMP2 and significantly different with FA tissue indicated that BC tissue was not metastasized or primary BC tissue that strongly urged to invade. Cells in invasion and metastasis phase showed the connective tissue of stroma degrades together with basal membrane as an important element of invasion and metastasis. Several components of extracellular matrix, particularly interstitial collagen, are highly resistant to proteolytic attacks, degraded only by metalloproteinase matrices (MMPs)[41].

In this study, TIMP2 expression level was significantly different between in BC tissue and FA tissue. Unfortunately, this study did not describe the distinction between positive metastasis and non metastasis tissue. Tissue inhibitor metalloproteinase-2 may act as a defence mechanism against metastasis by inhibiting MMP-2 activity.

Expression level of MMP2 was lower in BC than in FA indicated that extra cellular matrix component of laminin-5 and type 4 collagen have not degraded yet, cancer cells have been inactivated and no metastasis have occurred by degrading extra cellular matrix. Over expression in MMP2 emerges destruction of extra cellular matrix membrane. Matrix metalloproteinase (MMP)-2 is very active in degrading of extracellular matrix, affecting by an activator, type 14 MMP (MMP-14) and Tissue Inhibitor of Metalloproteases (TIMP)-2.
By investigating the expression of miRNA, treatment of BC can be improved. Based on previous and current studies data, the administration of anti-miR-10b (miR-10b antagomiR) may be a neoadjuvant to treat BC in early stage or in non-metastatic cancers. miR-10b has a potential gene as a biomarker by distinguishing between primary tumour and positive metastatic tissue, as a step to prevent BC toward advanced stage. Prevention and early detection is crucial in comprehensive management of BC because of the impact of BC.

This study concluded that BC in West Sumatra mostly occurs in premenopausal age, Grade II and commonly in primary BC. Correlation between BC and clinical status of patients and identification of positive metastasis BC and primary BC tissue in balancing sample are important to investigate for further study.

By determining the expression of miRNA, the treatment of BC may be improved. Based on previous and current data obtained, the administration of anti-miR-10b (miR-10b antagomiR) potent to be a neoadjuvant to treat BC in early stage or in non-metastatic. The antimiR-10b is expected to inhibit genes inducing metastases. Previous study showed that modified synthetic miRNA as a target of pro metastases (RhoC and TIMP2) regulates the equivalence MMPs and TIMP2, so degradation of extracellular matrix may decline and prevent advanced metastasis[42].

It is suggested that miR-10b is potential to be a biomarker for distinguishing primary tumour tissue and positive metastatic tissue as an early step to prevent BC toward advanced stage. Because early prevention and detection is crucial in comprehensive management of BC, due to the given impact of BC.

CONCLUSION

This study concluded that miRNA-10b was expressed in BC tissue, this result may be used as a differentiator between tissue with BC and FA. Lower expression of miRNA-10b, RhoC, TIMP2 and MMP2 in BC than FA showed that the result can be a marker in BC tissue which has not yet metastasis and still in primary BC tissue. It gives a chance as an indication in

determining of diagnosis and prognosis. Besides, this study will be useful to develop miRNA-10b therapy of BC in the future.

SIGNIFICANCE STATEMENT

This study identified the correlation of miR-10b expression and metastases-induced genes; RhoC, TIMP2 and MMP2. There is a positive correlation between BC with lymph node metastasis with MMP2 and TIMP2 ratios, it's caused by an alteration in the expression of TIMP2 and MMP2. The changes in miRNA-level are expected to becomes a biomarker to detect disease earlier and the intervention of treatment may be performed at the stage of disease progression, because each miRNA has different target genes and works on different phases.

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