RESEARCH ARTICLE

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Down Regulated Expression Levels of miR-27b and miR-451a as a Potential Biomarker for Triple Negative Breast Cancer Patients Undergoing TAC Chemotherapy

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Abstract

Background: Triple negative breast cancer (TNBC) is associated with poor prognosis, aggressive phenotype(s) of tumours, partial chemotherapy response, and lack of clinically proven therapies. MicroRNAs (miRNAs) can target and modulate key genes that are involved in TNBC chemotherapy. Deregulated miRNA expression is highly involved in anti-cancer drug resistance phenotype and thus, miRNAs tend to be promising candidates for prediction of chemotherapy response and recurrence. **Aim:** This study aimed to investigate the expression levels of selected miRNAs (miR-21, miR-27b, miR-34a, miR-182, miR-200c and miR-451a) in cancerous and normal adjacent tissues of TNBC patients and to correlate with the clinicopathological data. **Methods:** Forty-one (41) FFPE tissue block of histopathologically confirmed TNBC patients was collected. Total RNA from the cancerous and adjacent non-cancerous tissues were isolated, transcribed, and pre-amplified. The relative expression level of miRNAs in tumour and normal adjacent tissues of TNBC patients was analysed using qRT-PCR. Results: Out of six miRNAs studied, the relative expression of miR-27b and miR-451a were found to be significantly lower in cancerous as compared to normal adjacent tissues of TNBC patients. In addition, a significant down regulation of miR-451a was also observed in infiltrating ductal carcinoma subtype, stages I and II, in both grade II and III, premenopausal and postmenopausal as well as in those with positive axillary lymph node metastases. **Conclusion:** The results suggest the possible utilization of miR-27b and miR-451a expression levels as potential predictive risk markers for TNBC patients undergoing TAC chemotherapy.

Keywords: miR-27b- miR-451a- miRNA expression- TNBC- TAC chemotherapy

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Introduction

Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype, characterized by negative estrogen (ER), progesterone (PR) expression and no amplification of human epidermal growth factors 2 (HER2) receptors (Lehmann and Pietenpol, 2015). As compared to other breast cancer subtypes, TNBC is correlated with high histological grade, higher cell proliferation, high rate of relapse, invasion, metastases, and low overall survival (Foulkes et al., 2010; Arpino et al., 2015). Due to tumour heterogeneity, high probability of tumour relapse and lack of specific targets for treatment selection, a large number of TNBC patients fail to respond or acquire resistance to the introduced chemotherapeutic agents that typically lead to relapse and worsening of prognosis. The causes for TNBC recurrence remain unknown. To date, there are no specific genetic biomarkers available to predict the chemotherapy response in TNBC patients and there is no standard module for predicting drug resistance and disease relapse in TNBC. Hence, there is the need for identification of predictive molecular biomarkers to predict response to therapy in TNBC patients undergoing chemotherapy with TAC regimen. A few studies have demonstrated that epigenetic factors such as microRNAs (miRNAs) play key regulatory role in modulating drug resistance (Kutanzi et al., 2011; An et al., 2017).

miRNAs are a family of non-coding small RNAs with a length of 18-24 nucleotides. miRNAs play critical role in tumorigenesis and progression by regulating cell proliferation, invasion, differentiation, apoptosis, and migration by targeting the gene expression at the posttranscriptional level (Bartel, 2004; Herranz and Cohen, 2010). Deregulation of miRNAs may affect the function of multiple target mRNA and alter the expression of multiple proteins which are involved in the response to

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chemotherapeutic drugs (Hummel et al., 2010; Kutanzi et al., 2011). As a result, deregulated miRNA expression is highly involved in anti-cancer drug resistance phenotypes and thus miRNAs tend to be promising candidates for response and recurrence prediction. Few earlier studies reported that TNBC patients' reaction to chemotherapy agents were followed by improvements in the expression of particular miRNAs, which clearly suggested that miRNAs could be used as predictive biomarkers (Li and Yang, 2013; Winther et al., 2016).

miR-451 acts as a tumour suppressive miRNA, mapped on chromosome 17q11.2 and plays a critical role in cancer cellular formation, migration and invasion in various cancers (Pan et al., 2013). Deregulation of miR-451 widely occurs in several human malignancies as well as treatment response (Bai and Wu, 2019). miR-27b is located at chromosome 9q22.1 and acts as an oncogene. Few studies on TNBC samples showed that up regulation of miR-27b attenuated chemoresistance and tumour seeding ability and also involved in initiation, invasion, and migration of breast cancer (Wang et al., 2009; Jin et al., 2013; Ding et al., 2017).

miR-34a is a tumour suppressive miRNA, located at chromosome 1p36.23, and plays a role as an antagonist in different oncogenic processes, such as inhibition of tumour cell differentiation, proliferation, migration, and invasion (Cole et al., 2008; Li et al., 2009). Evidence has indicated that the expression of miR-34a was associated with drug resistance in cancer (Kastl et al., 2012; Ghandadi and Sahebkar, 2016). miR-200c acts as tumour suppressive miRNA and located on chromosome 12p13. miRNA 200c regulates breast cancer cell migration, stress fibre formation, migration, invasion, and elongation. Down regulation of miR-200c is associated with drug resistance in breast cancer in humans (Pogribny et al., 2010; Chen et al., 2012).

miR-182 which is located at chromosome 7q32.2, has been abundantly expressed in the retina, central nervous system, and normal human embryonic stem cells (Segura et al., 2009). This miRNA act as an oncomiR and/or tumour suppressive miRNA in various cancer tissues. miR-182 is frequently overexpressed in human breast cancer tumour tissues and cell lines (Zhang et al., 2017). miR-21 is an oncomiR, located at chromosome 17q23.2 and has been reported to be overexpressed in a variety of solid tumours and haematological malignancies (Volinia et al., 2006) and plays a role in breast cancer pathogenesis (Corcoran et al., 2011; Zhang and Ma, 2012). miR-21 plays a role in controlling cell proliferation, G2/M check point, and metastasis diffusion (Anastasov et al., 2012; Dong et al., 2014; Min et al., 2014).

The present study was undertaken to investigate the expression level of six (6) selected miRNAs (miR-21, miR-27b, miR-34a, miR-182, miR-200c and miR-451a) in TNBC tissues patients and associate the expression level with clinico-pathological features as well as treatment response of TNBC patients undergoing TAC chemotherapy.

Materials and Methods

Recruitment of Study subjects

This is part of research university grant (RU), approved by the Human Research Ethics Committee Universiti Sains Malaysia (USM/JEPeM/20060347) with Declaration of Helsinki. Fourty-one (41) formalin-fixed paraffin embedded (FFPE) tissue blocks from TNBC patients who were immunohistochemically (IHC) confirmed with negative staining of ER and PR as well as no amplification of HER-2 receptor by fluorescent in situ hybridization (FISH) were collected from Hospital Universiti Sains Malaysia. The research was conducted at Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia. These 41 patients had undergone primary surgery followed by six cycles of TAC chemotherapy and completed at least 12 months after chemotherapy. All clinical and pathological data of the TNBC patients including the tumour subtype, staging, grading, status of lymph nodes, menopausal status and treatment status were recorded.

Isolation of total RNA, cDNA synthesis and miRNA preamplification

Total RNA was extracted from 41 cancerous and 17 normal adjacent tissues of FFPE blocks using Ambion Recover All™ Total Nucleic Acid Isolation Kit (Ambion, Austin, TX). The concentration and purity of the extracted total RNA was determined by using a NanoQuant Infinite M200 (Tecan Inc., U.S). The concentration and purity of total extracted RNA ranged from 20-200 ng/µl and 1.8 to 1.9 (A260/280) respectively. The quality of extracted total RNA was determined by performing 1.0 % agarose gel electrophoresis. Next, cDNA was synthesized using TaqMan® MicroRNA RT Kit (Applied Biosystem, USA) and transcribed miRNA was then pre-amplified using TaqMan® PreAmp Master Mix Kit (Applied Biosystems, USA).

qRT-PCR for detection of miRNAs expression levels

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed to measure the expression levels of miR-21b-5p (000397), miR-27b-3p (000409), miR-34a-5p (000426), miR-182-5p (002334), miR-200c-3p (002300) and miR-451a (001141) in cancerous and normal adjacent tissues of TNBC patients. RNU48 (568908) was chosen as a house keeping miRNA.

miRNA. qRT-PCR reaction was carried out using Applied Biosystems Step One Real Time PCR thermal cycler (Applied Biosystems, USA). The reaction was performed in 10.0 µl of final volume in triplicates containing consisting of 0.5 µl of 5X TaqMan® MicroRNA Assay, 0.1 µl of preamplified miRNA, 5.5 µl of TaqMan® Advanced PCR Master Mix and 4.4 µl of nuclease free water. The qRT-PCR started with Uracil-N-glycosylase (UNG) incubation at 50°C for 2 minutes and enzyme activation at 95˚C for 20 seconds followed by 40 cycles of denaturation at 95˚C for 1 sec and annealing/extension at 60˚C for 20 seconds. Negative control also was included to detect possible carry-over contamination if any. The relative expression was determined by using Relative

Expression Software Tools (REST) 2009, V2.0.13 to compare expression level of miRNAs in tumour and normal adjacent tissues of TNBC patients.

Treatment response assessment

TNBC patients who completed six (6) cycles of chemotherapy with TAC regimen were assessed after one year. Those TNBC patients with disease progression, local relapse, primary and secondary tumour at various sites were categorized under chemo-resistance group. TNBC patients were categorized into chemo-responder group if they did not show any of the signs above. The disease relapse was assessed based on ultrasound, computed tomography (CT scan) or magnetic resonance imaging (MRI) findings.

Results

The clinicopathological parameters of TNBC patients

The mean age of diagnosis was 50.4 ± 11.45 years. Among these, majority (85.4%) had infiltrating ductal carcinoma while the remaining (14.6%) had medullary and metaplastic subtypes. Histological grading showed 63.4% to be grade III and the remaining 36.6% to be grade II. Stage wise, 7.3% had stage I, 73.2% had stage II and 19.5% had stage III. A total of 34 of patients (82.9%) showed positivity for axillary lymph node involvement and 26 (63.4%) were pre-menopausal. In the present study, on 41 TNBC patients, 14 (34.1%) were categorized as chemo-resistant and 27 (65.9%) as chemo-responders. The clinicopathological features and treatment response status of the TNBC patients are shown in Table 1.

Table 1. Clinicopathological data of TNBC patients

Characteristics	Tissue sample (N=41)
Type	
Infiltrating ductal carcinoma	35 (85.4 %)
Medullary and metaplastic	6 (14.6 %)
Histological grade	
I	0 (0.0%)
II	15 (36.6%)
III	26 (63.4%)
Stage	
I	3 (7.3 %)
II	30 (73.2 %)
III	8 (19.5 %)
Axillary lymph nodes metastasis	
Negative	7 (17.1%)
Positive	34 (82.9 %)
Menopausal status	
Pre-menopausal	26 (63.4%)
Post-menopausal	15 (36.6%)
Treatment response	
Chemo-responder	27 (65.9%)
Chemo-resistant	14 (34.1%)

Relative expression levels of miRNAs in cancerous versus normal adjacent tissues and its association with clinicopathological data and treatment response

Out of six miRNAs studied, the relative expression of miR-27b and miR-451a was found to be significantly downregulated in cancerous tissues as compared to normal adjacent tissues by mean factors of 0.443 (p=0.036) and 0.163 (p=0.001) respectively. The expression levels of miR-21 and miR-182 were found to be up-regulated and miR-34a and miR-200c were found to be down regulated. However, the results were not significant (Figure 1).

When miRNA expressions with clinicopathological data were correlated. miR-451a expression level was found to be significantly downregulated in infiltrating ductal carcinoma subtype, stages I and II, in both grade II and III, premenopausal and postmenopausal as well as in those with positive axillary lymph node metastases

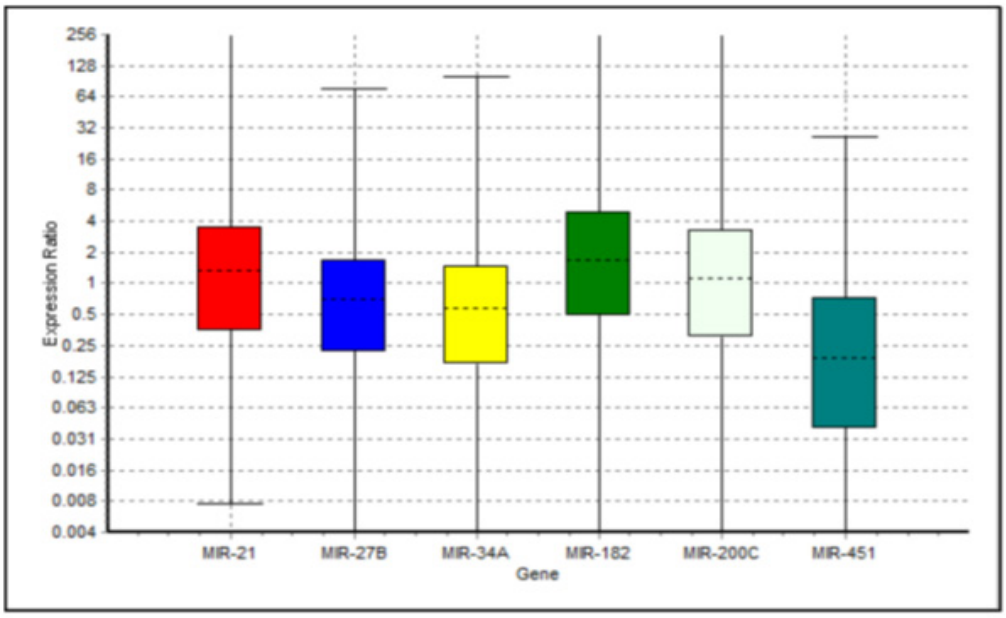

Figure 1. Relative Expression of miRNA Levels in Cancerous versus Normal Adjacent Tissues of TNBC Patients

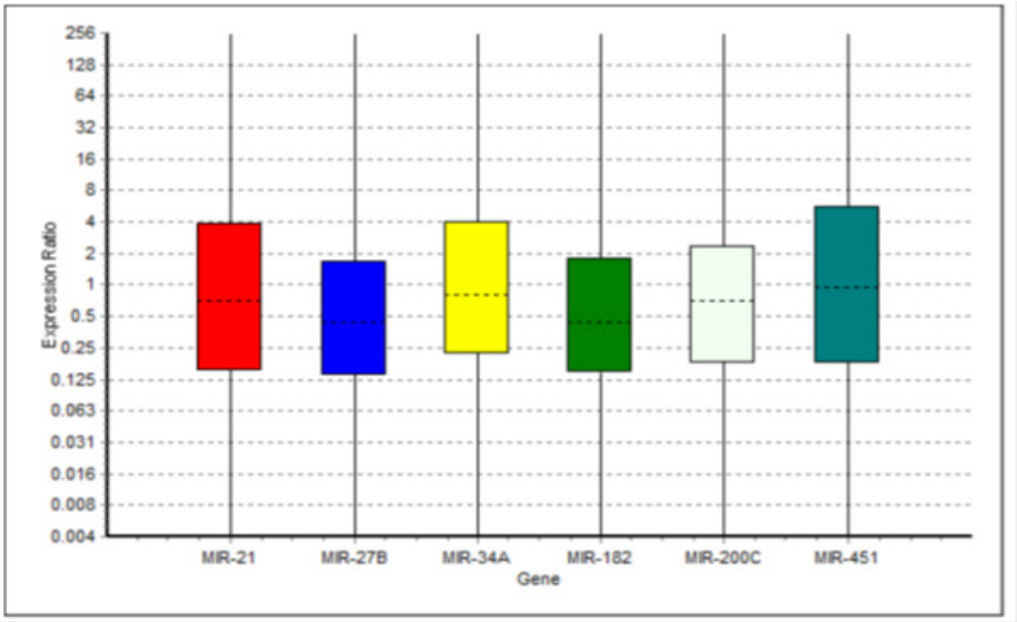

Figure 2. Relative Expression of miRNA Levels in Chemoresistant Versus Chemoresponder of TNBC Patients

(Table 2).

Next, the expression levels of the candidate miRNAs (miR-21, miR-26b, miR-27b, miR-34a, miR-182 and miR-200c and miR-451a) were compared between TNBC patients who were chemoresistant and chempresponders. The results indicated that the relative expression levels of six of the investigated miRNAs were lower in chemoresistant patients as opposed to chemoresponders. However, the difference in the expression level of the investigated miRNAs between chemoresistant versus chemoresponders did not reach any statistically significant level ($p>0.05$) (Figure 2).

Discussion

The present study demonstrated that miR27b and miR451a were found to be significantly downregulated in the tumour as compared to normal adjacent tissues of TNBC patients. A further analysis showed that miR-451a expression level was found to be significantly downregulated in infiltrating ductal carcinoma subtype, stages I and II, in both grade II and III, premenopausal and postmenopausal as well as in those with positive axillary lymph node metastases.

miRNAs represent a class of small non-coding RNAs that control the expression of the protein coding genes through imperfect binding at the 3'-UTR of the target mRNA, thus leading to protein translation inhibition or degradation (Bartel, 2004; Oliveto et al., 2017). Studies have demonstrated that majority of miRNAs are deregulated in most solid tumours and haematological malignancies. miRNAs may act either as oncogenes or tumour suppressor genes or both, depending on their targeted genes and cancer types (Calin et al., 2004). In breast cancer, miRNA deregulation can lead to an upregulated and downregulated pattern in the level of miRNA expression levels, thus affecting the function of multiple target mRNAs, changing the multiple protein expressions involved in the progression of the cell cycle, apoptosis, microenvironment of the tumour, migration, invasion, metastasis, drug resistance, as well as cell differentiation and self-renewal (Li et al., 2012).

miR-27b is located at chromosome 9q22.1 and acts as an oncogene. miR-27b promotes cell proliferation, cell migration and cell invasion. In the present study, the expression level of miR-27b was significantly lower in cancerous as compared to normal adjacent tissues. This finding is in concordance with the study conducted by Zhu et al., (2016) who observed significant down regulation of miR-27b in breast tumour tissues as opposed to the

Table 2. Relative Expression Levels of miR-451a and Its Association with Clinicopathological Features

Clinicopathological feature	Relative expression	Standard Error	p-value
Infiltrating Ductal carcinoma	0.122	0.015-0.996	<0.001*
Stage I and II	0.209	0.029-1.375	0.003*
Grade II	0.067	0.007-0.591	0.014*
Grade III	0.246	0.030-1.448	0.008*
Pre-menopausal	0.24	0.034-1.692	0.005*
Post-menopausal	0.082	0.009-0.583	0.002*
Positive axillary lymph nodes metastasis	0.161	0.020-1.354	0.002

*p<0.05, statistically significant

normal adjacent tissues. Moreover, they also demonstrated that the expression of miR-27b was also lower in both BT549 and MDA-MB-231 TNBC cell lines compared to ER-positive breast cancer cell line. Similarly, a recent study also showed that the expression of miR-27b was down regulated in breast cancer cell lines and tissues (Chen et al., 2018).

A few studies have shown that down regulation of miR-27b is associated with treatment response. Our result clearly demonstrated the down regulation of miR-27b in chemoresistant group of TNBC patients. Earlier studies demonstrated that the low expression of miR-27b subsequently increased *CYP1B1* expression and caused resistance to docetaxel in cancerous cells (Tsuchiya et al., 2006; Martinez et al., 2008). A study by Kovalchuk et al., (2008) observed down regulation of miR-27b expression in MCF-7/doxorubicin as compared to MCF-7 cell lines. Lee et al., (2012) demonstrated that, loss of miR-27b expression was found to be associated with docetaxel resistance and high tumourigenicity in breast cancer stem cells. In a study conducted by Takahashi et al., (2015), the loss of miR-27b was found to inhibit the generation of breast cancer stem cell and attenuate chemo-resistance and tumour seed capacity. In addition, downregulation of miR-27b-3p was reported to enhance tamoxifen and paclitaxel resistance in both breast cancer cell tissues and cell lines (Zhu et al., 2016; Chen et al., 2018).

miR-451a acts as tumour suppressive miRNA and has been shown to be significantly down regulated in cancerous than normal adjacent tissues (Pan et al., 2013). In the present study also, the mean expression level of miR-451a was found to be significantly down regulated in cancerous tissues compared to normal adjacent tissues ($p<0.001$). A study conducted by Ouyang et al., (2014) showed the expression level of miR-451a was down regulated in TNBC patients. Our results are also in agreement with two previous studies which demonstrated that down regulation of miR-451 was correlated with tumour differentiation, advanced pathological stage and positive lymph node metastasis in gastric cancer and non-small-cell lung carcinoma (Wang et al., 2011; Shen et al., 2017). Gu et al., (2015) observed that the expression levels of miR-451 was found to be down regulated in breast cancer patients who showed resistance to neoadjuvant chemotherapy than those who showed good response to neoadjuvant chemotherapy. In addition, the expression level of miR-451 was significantly lower in MCF-7/epirubicin and MCF7/docetacel cell lines than in MCF-7 cell lines. In TNBC patients, miR-451a expression was down regulated and an in-vitro study demonstrated that miR-451 significantly altered the sensitivity to doxorubicin in MDA-MB-231 cell line (Ouyang et al., 2014). Likewise, in the case of other malignancies, such as colorectal cancer (CRC), Bitarte et al., (2011) reported low expression of miR-451 in chemoresistant CRC group compared to CRC patients who responded to the first-line irinotecan therapy. Sun et al., (2017) demonstrated that expression level of miR-451 regulated adriamycin resistance both in-vitro and in-vivo studies in renal cell carcinoma.

In contrast, study by Bian et al., (2011) showed that up regulation of miR-451 increased cisplatin sensitivity to the cell by increasing dichlorodiammine platinum (DDP)-induced apoptosis. Overexpression of miR-451 negatively regulates BCL-2 expression, accelerated apoptosis and influent paclitaxel resistance in breast cancer (Gu et al., 2015). Kovalchuk et al., (2008) demonstrated that 3'-UTR of *ABCB1* was a putative binding site for mature miR-451 at 4742 to 4763 nucleotides. Down regulation of miR-451 increased the *ABCB1* expression. Up regulation of *ABCB1* expression has been shown to increase cancer drug resistance (Hansen et al., 2015). Our previous study demonstrated that the expression of *ABCB1* was significantly lower in tumour as compared to normal adjacent tissues (relative expression: 0.282, SE:0.016–5.291, $p = 0.035$). When compared with chemotherapy response, the mRNA expression of *ABCB1* was 1.834-fold higher in TNBC patients who developed resistance compared to the patients who responded to chemotherapy (Abdul Aziz et al., 2018).

Thus, the present study concluded that down regulation of miR-451 expression could reduce drug sensitivity, slower the apoptosis, increase tumorigenicity and thus resulting in drug resistance.

The mechanism underlying the deregulation of miR-27b and miR-451 is poorly understood. A few reports have demonstrated that the loss of miR-451 expression was associated with epigenetic mechanisms such as DNA methylation and/or histone deacetylation at long distance regions upstream (>2kb) of miR-451 promoter region (Wang et al., 2011; Pan et al., 2013). In case of miR-27b, TCGA data base demonstrated that hypermethylation at miR-27b gene locus in human breast cancer could explain the low expression of miR-27b in malignant as well as in chemoresistant tissues and cells. Moreover, down regulation of miR-27b caused by hypermethylation at promoter region was associated with cell proliferation, evading apoptosis and drug resistance (Chen et al., 2018).

Limitation of the study

The present study has a limitation due to small sample size of TNBC tissues (both cancerous and normal adjacent). Forty-one (41) FFPE tissues samples were collected from TNBC patients. In Malaysia, the incidence of TNBC cases has been estimated to range between 12.3 percent to 17.6 percent of total breast cancer (Tan et al., 2009; Kanapathy Pillai et al., 2012). Therefore, recruiting a sufficient number of patients who have been clinically and histopathologically verified as TNBC has been a challenge for the present study. In addition, it was also a difficult task to select TNBC patients who had undergone surgery and completed six cycles of adjuvant TAC chemotherapy as per the inclusion criteria. Thus, a further study needs to be undertaken in large number of TNBC patients and associate these miRNAs with treatment response that might increase the strength of the study.

In conclusion, the present study demonstrated that down regulation miR-27b and miR-451a expression level in cancerous tissues of TNBC patients could be a candidate biomarker for predicting treatment response for TNBC undergoing TAC chemotherapy regimen.

Abbreviations

TNBC, triple negative breast cancer; miRNA, microRNA; TAC, taxane, adriamycin and cyclophosphamide; ER, estrogen; PR, progesterone; HER2, human epidermal growth factors 2; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; FFPE, formalin-fixed paraffin embedded

Author Contribution Statement

Ahmad Aizat Abdul Aziz carried out the experiment, analysed the data and wrote the manuscript. Md Salzihan Md Salleh was involved in pathological confirmation of the cases, Maya Mazuwin Yahya (Breast cancer surgeon) and Andee Dzulkarnaen Zakaria (Breast cancer surgeon) helped in identification and recruitment of TNBC patients and collection of clinical and pathological data. Ravindran Ankathil conceived the idea, designed the study, reviewed manuscript and supervised the project.

Acknowledgements

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Ethical Statement

Informed written consents were obtained from all participants in this study. The protocol was approved by the Human Research Ethics Committee Universiti Sains Malaysia (USM/JEPeM/20060347) which complies with the Helsinki Declaration of 1975.

Data Availability Statement

Data are provided within the article.

Declaration of Competing Interest

Authors declared no conflict of interest.

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