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Research Article

miR-21 Expression and its Correlation with Demographics, Subtypes, and Tumour Suppressor Genes; PTEN and PDCD4 in Breast Cancer Tissues in Malaysia

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Abstract

Objectives: Despite extensive research in breast cancer (BC) genomics, most studies are from Western countries, which do not reflect the multi-ethnic make-up of Malaysia. Hence, microribonucleic acid-21 (*miRNA-21*), which is known to be an oncogenic stimulator of BC will be investigated by comparing its expression between breast tumour tissues and normal adjacent tissues excised from 67 BC patients, ethnic groups, age groups distribution, neo-adjuvant chemotherapy (NAC) treated and untreated patients, as well as BC subtypes.

Methods: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to measure the distribution of *miR-21* expression in the paired BC tissues. The expression of the tumour suppressors; PTEN and PDCD4 was also investigated via RT-qPCR and Western Blot for its gene and protein expressions.

Results: The results only showed the significance of *miR-21* and *PTEN* expression between the normal adjacent tissues and BC tissues ($p<0.05$). Additionally, there was a lack of correlation between gene expression of *miR-21* against *PTEN* and PDCD4. Protein expression analysis did not show a significant difference in tumour suppressor proteins; *PTEN* and *PDCD4* expression in both tissue types.

Conclusion: *miR-21* has a notable presence in BC and is a suitable biomarker to be evaluated further in patients of all ethnicities and age groups.

Keywords: Breast cancer, Malaysia, *miR-21*, PTEN, PDCD4.

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Breast cancer (BC) is a complex disease that poses a significant challenge to human health, quality of life, and financial burden, both in Malaysia, and worldwide.[1] The International Agency for Research on Cancer's GLOBOCAN statistical analysis reveals that the BC mortality rate has reached 8,418 new cases among Malaysia's female popula-

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tion of all ages, with a total of 29,453 cases within a 5-year prevalence span.[2] Additionally, a joint study between two tertiary academic hospitals in Malaysia and Singapore concluded that 50% of women in their sample group were diagnosed before the age of 50 years. Meanwhile in Western countries, 20% are diagnosed before age 50. This is supported by data that reveals the mean age of BC presentation in Malaysia is 26.1 years, compared to 39.8 years of age in the United Kingdom.[3] Ethnicity is a key risk factor for one's lifetime risk of developing BC. In Malaysia, BC risk is highest among the Chinese population, followed by Indians, then Malays.[4] Thus, the genetic underpinning differences of BC risk in different ethnicities and age groups are poorly understood.

Breast tissue is highly heterogeneous, and is composed of breast stem cells, myoepithelial cells, epithelial cells, and glandular cells. BC is similarly heterogeneous, as neoplastic changes may occur in any of these cell types.[5] BC can be classified into subtypes depending on the presence or absence of receptors expressed by cancerous cells. These receptors include oestrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor 2 (HER2).[6] BC subtypes include Luminal A (ER-positive, PR-positive, HER2-negative), which accounts for 50-60% of BCs, and is associated with good prognosis.[6, 7] Luminal B (ER-positive, PR-negative, HER2-positive or negative)[8, 9] accounts for 15-20% of BCs, characterised as a more aggressive phenotype, and is associated with poorer prognosis.[10] Triple negative BCs (TNBC) (negative for ER, PR, and HER2),[6] accounts for 12-17% of BCs, have high recurrence rates, and poorer prognosis.[11] TNBC's behaviour is relatively aggressive compared to other subtypes and have characteristic metastatic patterns.[12] Lastly, HER2-positive BC (ER & PR-negative, HER2-positive) accounts for 15-20% of BC cases.[13] HER2 positivity is associated with more aggressive and invasive cellular behaviour but has a remarkably better prognosis due to the availability of effective targeted treatment.[14, 15] There are, however, no known biomarkers that provide prognostic information in any of the above BC subtypes. The identification of BC biomarkers that provide insights into disease progression and outcome may have important clinical value to medical practitioners and patients alike.

The primary methods of treating BC include surgical excision, radiotherapy, and chemotherapy.[16-19] All three modalities may be used in a number of different combinations depending on tumour grade (aggressiveness) and staging (degree of spread). A general approach typically utilises a course of neoadjuvant (or pre-treatment) chemotherapy to reduce tumour size, followed by surgical excision of the tumour, and then adjuvant (post-treatment) chemotherapy or radiotherapy to minimise remaining microscopic, undetectable cancer cells.[19] The usage of neoadjuvant therapy is associated with lower rates of BC recurrence and mortality.[20-24] Despite the promising outcomes of NAC in BC,[25, 26] the effect of its administration against oncogenic miRNAs is unknown.

The main post-transcriptional regulators of gene expression in different tissues and developmental stages are miRNAs. They accomplish this through highly specific interactions and complex regulatory networks.[27] miRNAs can be divided into oncogenic miRNAs (oncomiRs) and tumour suppressor miRNAs (tsmiRs). OncomiRs are typically upregulated in BC,[28] while tsmiRs prevent cancer initiation through modulating oncoproteins that code for gene expression.[29] The oncomiR of interest in this study is *miR-21*, a key oncomiR in many cancer subtypes, and whose expression is dramatically up-regulated in BC. *miR-21* targets and inhibits the activity of programmed cell death 4 (*PDCD4*) and phosphatase and tensin homolog (*PTEN*), both of which are tumour suppressor genes.[30-32] This is supported by evidence that associates the downregulation of *PTEN* and *PDCD4* with poor prognosis in BC.[33, 34] However, studies that aim to establish a link between *miR-21* overexpression with *PTEN* and *PDCD4* expression have yielded mixed results with some studies exhibiting an expected negative correlation while others exhibited negligible correlation.[35-38]

In BC, the overexpression of *miR-21* is significantly correlated with advanced clinical staging, lymph node metastasis, and poor prognosis.[39] The over-expression of *miR-21* in BC has potential clinical implications that require further investigation. Additionally, its association with more advanced disease may make it an important biomarker to monitor for disease progression and inform prognostication. To validate its use, further investigation should attempt to quantify its expression among different ethnic populations, given that BC incidence and prevalence is known to vary between ethnicities. Therefore, the question persists if *miR-21* gene expression levels differ in different ethnic groups. That being said, the vast majority of the existing evidence regarding *miR-21* expression has been performed with populations of homogenous ethnicities, e.g. Caucasian North Americans, or Han Chinese, or Indians.[3, 40-42] Further, attempts to compare *miR-21* expression between these studies may be confounded by the variable access to cancer therapies and quality of the therapies between these countries. Malaysia offers a multi-ethnic population that is exposed to a standardised approach to cancer care, and comparable quality of said care between patients, thus making it a promising location for a comparative multi-ethnic study comparing *miR-21* expression.[3] Additional investigations must also be conducted to further elucidate the proposed correlation between *miR-21* overexpression, and *PTEN* and *PDCD4*

downregulation. This study aims to investigate differential expression of *miR-21* in BC cases among Chinese, Indian, and ethnic Malays in Malaysia, across different age groups, subtypes, treatment status, while concurrently investigating the association between *miR-21* overexpression with *PTEN* and *PDCD4* downregulation.

Methods

Ethical Considerations

This present study involves the use of human subjects which include tissue specimens. Therefore, human ethics approval has been applied and obtained for this project to collect the patients derived tissue specimens via the University Putra Malaysia institutional ethics review board and Ministry of Health Medical Research Ethics Committee (MREC) prior to the commencement of the study. The ethics approval code is NMRR-21-246-58614 (IIR). Clinical information was obtained from archived medical records. Informed patient consent were obtained.

Specimen Collection and Storage

Surgical mastectomies were excised by surgeons in Putrajaya Hospital. The nature and characterisation of both tissue specimens (breast tumour and normal adjacent tissue) were confirmed by the surgeon who has conducted mammograms and ultrasounds to locate the tumour and confirm the normal tissue. A segment of the excised tissues were then sent to the hospital's histology specialists to confirm the subtypes via immunohistochemistry through the expression of biomarkers such as ER, PR and HER2 receptors along with Ki67 and E-cadherin. The remainder of the excised breast tissues were then submerged in transport media containing either phosphate buffered saline (PBS) with 10% antibiotics for same-day collection or Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) for next-day collection.[43, 44] Once the specimens have arrived at Taylor's University Research Lab, each tissue specimen (breast tumour and normal adjacent tissue) were dissected for different experimental purposes such as; 1) RNA extraction for *miR-21*, *PTEN* and *PDCD4* detection, and 2) protein extraction for *PTEN* and *PDCD4* detection. The breast tumour tissues were labelled as hBC001 (human breast cancer 001) while the normal adjacent tissue is labelled as hBN001 (human breast normal 001). Collectively, this set of specimens refers to patient number 1. The tissues were snapped frozen in liquid nitrogen in cryovials and stored in the cryotank until RNA and protein extractions are performed (Loken and Demetrick, 2005, Zhang et al., 2019b, Zheng et al., 2019). Patient demographical data such as ethnicity, treatment prescription and clinical history was also collected from Hospital Putrajaya's patient medical records to conduct demographical analysis based on this study's results.

Inclusion and Exclusion Criteria

The inclusion criteria for this study consisted of Malaysian BC patients with informed consent, of any age and ethnicity, diagnosed with BC of any subtype, tumour size received has to be larger than 2 cm, patients who have undergone NAC treatment and treatment naïve patients. The exclusion criteria consisted of non-Malaysian BC patients and patients diagnosed with other chronic disease(s).

RNA Extraction

Tissue RNA extractions were carried out with a TRIzol® RNA extraction kit, following its user manual (Zymo Research, USA. Cat No: R2052). The eluted RNA was then measured for its concentration and quality using the LVIisplate (BMG Labtech, Germany) and quantified using the NanoDrop quantification software. An acceptable reading of the 260/280 purity test should equate to a value above 1.8. After that, the extracted RNA samples were stored in -80°C until further use.

RT-qPCR Assay

The gene expression levels of *miR-21*, *PTEN* and *PDCD4* were measured using RT-qPCR with their relative fold change expression calculated with the $2^{-\Delta\Delta CT}$ method. [45] Primers used were: *miR-21* forward primer: 5'-GCCC-GCTAGCTTATCAGACTGATG-3', *miR-21* reverse primer: 5'-CAGTGCAGGGTCC GAGGT-3',[46] *U6 snRNA* forward primer: 5'-CTCGCTTCGGCAGCACA-3', *U6 snRNA* reverse primer: 5'-AACGCTTCACGAATTTGCGT-3',[47] *PTEN* forward primer: 5'-GACGAACTGGTGTAATGATATG-3', *PTEN* reverse primer: 5'- GTGCCACTGGTCTATAATCC-3',[48] *PDCD4* forward primer: 5'- TCTGGGAAAGGAAGGGGACTAC-3', *PDCD4* reverse primer: 5'-TTCATAACACAGTTCTCCTGGTCAT-3'[49], *β-actin* forward primer: 5' -CTTCCTTCCTGGGCATG-3' and *β-actin* reverse primer: 5'-GTCTTTGCGGATGTCCAC-3'.[48] *U6 snRNA* and *β-actin* were used as housekeeping genes for microRNA and mRNA detection, respectively. cDNA synthesis and RT-qPCR master mix for *miR-21* detection purposes were prepared with GeneCopoeia's All-in-One miRNA RT-qPCR Detection Kit 2.0 (GeneCopoeia Inc, USA. Cat No: QP115) for quantitative detection of mature miRNA while *PTEN* and *PDCD4* cDNA synthesis was prepared with HiScript II 1st Strand cDNA Synthesis Kit (Vazyme Biotech, China. Cat No: R211) and the RT-qPCR master mix were prepared with ChamQ Universal SYBR® qPCR Master Mix (Vazyme Biotech, China. Cat No: Q711). RNA concentration of 10ng/μL was used to synthesise the respective cDNA. The reaction mix

was prepared according to the manufacturer's protocol. Once cDNA synthesis was completed, cDNA concentration of 500ng/µL was used for RT-qPCR master mix preparation. The RT-qPCR master mix for *miR-21*, *PTEN* and *PDCD4* gene expression analysis was performed following the manufacturer's protocol. The prepared samples were placed into the real-time machine; CFX Opus 96 Real-Time PCR System (Bio Rad, USA). The RT-qPCR run setting for *miR-21* gene expression consisted of a 3-step method; initial denaturation at 95°C for 10 minutes, then denaturation step at 95°C for 10 seconds, annealing step at 56°C for 20 seconds and finally the extension step at 72°C for 10 seconds. Meanwhile, the RT-qPCR run setting for *PTEN* and *PDCD4* gene expression consisted of a 2-step method; initial denaturation at 95°C for 30 seconds, then denaturation step at 95°C for 10 seconds, annealing and extension step at 63°C for 30 seconds. The melting curve analysis for both sets of gene expression was performed based on the default conditions set by the instrument. In this study, the epithelial human breast cancer cell line, metastatic mammary adenocarcinoma1 (MDA-MB-231) is used as the reference sample for *miR-21* RT-qPCR analysis due to its aggressive, invasive and poorly differentiated nature.[50, 51] Meanwhile, the green African monkey kidney cell line (Vero), which resembles as a normal cell line is used as the reference sample for *PTEN* and *PDCD4* RT-qPCR analysis.[52, 53]

Protein Extraction

Tissue protein lysates were prepared based on the manufacturer's protocol using Radioimmunoprecipitation Assay (RIPA) lysis buffer (Elabscience Biotehcnology Inc, USA. Cat No: E-BC-R327). The protein concentration of samples was then measured with the bicinchoninic acid (BCA) assay (Elabscience Biotechnology Inc, USA. Cat No: E-BC-K318-M), adhering to manufacturer's protocol. The desired protein concentration used for all samples were standardised to 10µg. Following protein lysate standardisation, 10µL of 10µg samples were then mixed with an equal amount of SDS loading buffer (200mM, pH6.8 Tris-HCl, 8% (w/v) SDS, 0.4% bromophenol blue, 40% glycerol, add 100µL of β-mercaptoethanol per 900µL of whole volume), hence, producing a final volume of 20µL. The mixture was heated at 95°C for 5 minutes before proceeding with gel electrophoresis.

Western Blot Protocol

Reagents used for Western Blot protocols were prepared in house and methodology used was adapted from Zhang et al., (2021) and Liu et al., (2014). Sample proteins were resolved on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% resolving gel and 4% stacking gel) in 1x electrolysis running buffer (1L: 250mM tris base, 192mM glycine and 10mL of 10% SDS) and the gel was run at 125V for 1 hour and 15 minutes. Filter papers and gel were soaked in 1x transfer buffer (1L: 25mM tris base, 192mM glycine, 20% (v/v) methanol) before proteins were semi-dry transferred onto polyvinylidene difluoride membrane (PVDF) (Millipore, USA. Cat No: R1JB27545) and was set at 20V for 1 hour to allow complete protein transfer. Once the transfer was done, the membrane was rinsed briefly in 1x tris buffered saline with tween-20 (TBST) (1L: 20mM tris, 150mM NaCl and 0.1% (w/v) Tween 20 detergent, pH adjusted to 7.6) and blocked with 5% Bovine Serum Albumin (BSA). Membranes were then incubated at 4˚C overnight with primary antibodies: *PTEN* (SC-7974, Santa Cruz, USA), *PDCD4* (SC-376430, Santa Cruz, USA) or *β-actin* (SC-47778, Santa Cruz, USA) which were diluted to working concentration of 1:1000 in 1x TBST. After washing steps with 1x TBST, the membrane was then incubated with diluted horse radish peroxidase (HRP) secondary antibody (sc-516102, Santa Cruz, USA) of a working solution of 1:10,000 for 1 hour in room temperature. The membrane was washed again with 1x TBST. The protein signals were then visualised with enhanced chemiluminescence (ECL) substrate (Elabscience Biotechnology, USA. Cat No: E-IR-R307). The membrane was incubated in the substrate for 1 minute and then exposed to autoradiography film in a dark setting and imaged with a chemiluminescent imaging system (Azure Biosystems A600, USA) (54, 55). The relative fold change of each protein expression were analysed by using Image J analysis software (National Institutes of Health, USA).

Data Analysis

The fold change ($2^{-\Delta\Delta CT}$) of gene expression for *miR-21*, *PTEN* and *PDCD4* were converted into the Log2 formula. The protein bands for western blot imaging was quantified with ImageJ software. After confirming the data obtained in this study is not normally distributed, non-parametric tests such as the Mann Whitney-U test was used to compare values across two groups such as *PTEN* and *PDCD4* protein expression between the two tissue types and *miR-21*'s expression in NAC treated and untreated patients. The Kruskal-Wallis Test was also utilised to assess a relationship between *miR-21* across different ethnicities, different subtypes and age groups of the patient cohort. Correlative statistics to deduce a correlation between gene expression of *miR-21* against *PTEN* and *PDCD4* was determined using the Spearman's rho of correlation coefficient. All statistical tests were conducted using the statistical software; SPSS Statistics 27.0 (IBM, USA).

Results

Breast Cancer Cases in Hospital Putrajaya from Year 2020-2021

Demographic data collection for BC patients was conducted in Hospital Putrajaya, including age and ethnic groups, as per Table 2 to stratify BC incidence. The accumulated data was tabulated and presented (Fig. 1) to visualise the distribution of each demographic factor with BC incidence in years 2020-2021. Table 1, which represents the frequency of BC incidence, showed that the year 2020 had a total of 315 BC cases in Hospital Putrajaya. Meanwhile, the percentage of BC patients' distribution among the age groups 25-40, 41-50, 51-64 and 65-86 were 15.6%, 23.5%, 38.1% and 22.9% cases, respectively, with a median age of 55 years old. Additionally, the distribution of ethnicity included 68.6% of Malay patients, 17.8% of Chinese ethnic, 12.1% Indian patients and 1.6% of patients categorised as 'Others'. In the following year of 2021, a total number of 147 BC cases was recorded. The age groups distribution consisted 20.4% of patients in the 25-40 age group, 24.5% of patients in the 41-50 age group, 35.4% of patients in the 51-64 age group, and finally 19.7% patients in the 65-86 age group with a median age of 53 years old. Based on the ethnic groups, there were 79.6%

Table 1. Frequency of BC incidence based on demographic data in the years 2020-2021

Category	Year 2020 (n=315)	Percent (%)	Year 2021 (n=147)	Percent (%)
Age				
25-40	49	15.6	30	20.4
41-50	74	23.5	36	24.5
51-64	120	38.1	52	35.4
65-86	72	22.9	29	19.7
Total	315	100	147	100
Median Age	55		53	
Ethnicity				
Malay	216	68.6	117	79.6
Chinese	56	17.8	19	12.9
Indian	38	12.1	11	7.5
Others	5	1.6	0	0
Total	315	100	147	100

Table 2. Summary table of miR-21 expression and demographic variables of 67 breast cancer patients in Hospital Putrajaya

Category	n=67	miR-21 Expression (mean±SD)	p
Age			
25-40	15	9.38±0.81	0.515
41-50	25	4.21±6.81	
51-64	13	5.77±8.93	
65-85	14	6.09±7.50	
Ethnicity			
Malay	53	6.03±7.95	0.233
Chinese	7	9.25±9.92	
Indian	7	3.09±8.17	
Prescription			
Neo-Adjuvant Chemotherapy	27	4.17±7.50	0.167
Untreated	40	7.34±8.45	
Subtype			
Luminal A	39	5.65±7.33	0.939
Luminal B	7	5.33±8.01	
HER 2 Enriched	9	4.98±7.73	
Triple Negative	12	8.66±11.30	

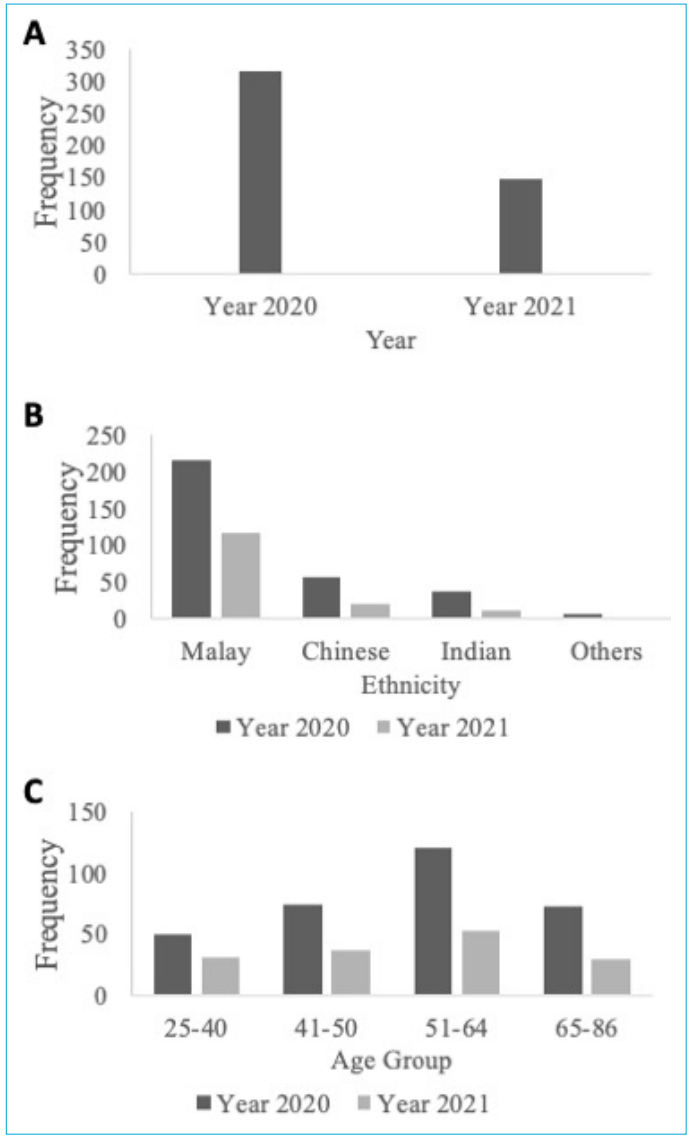

Figure 1. Bar chart of breast cancer cases in Hospital Putrajaya from **(a)** year 2020-2021, further segregated based on **(b)** ethnicity and **(c)** age group.

Malay patients, 12.9% Chinese patients and 7.5% Indian patients. The drastic decrease of BC patients admitted to Hospital Putrajaya between year 2020 to 2021 was because of a national lockdown during the novel COVID-19 outbreak. Not only that, but there was also a reduction of the operating theatre, therefore, only a selected number of cases can be performed on a weekly basis. Consequentially, a large quantity of BC patients were transferred to private hospitals to accommodate the influx of COVID-19 patients in Hospital Putrajaya.[56] The total cases of BC in Hospital Putrajaya in years 2020 and 2021 were shown in Figure 1A to visualise the difference in frequency. Additionally, Figure 1B and Figure 1C are clustered bar graphs that represented the frequency of BC cases in Hospital Putrajaya based on ethnicity and age group in 2020 and 2021, respectively.

miR-21 Expression in BC Patients among Malaysian Ethnic Groups

The analysis illustrated in Table 2 showed that the Chinese ethnicity exhibited the highest *miR-21* expression despite having only 7 (10%) Chinese patients out of the total of 67 patients. This is followed by the Malay ethnicity with 53 (80%) patients, and finally the Indian ethnicity having the lowest expression among 7 (10%) patients. After conducting the Kruskal-Wallis test, no significance was found among the ethnic groups (p=0.233), based on *miR-21* ex-

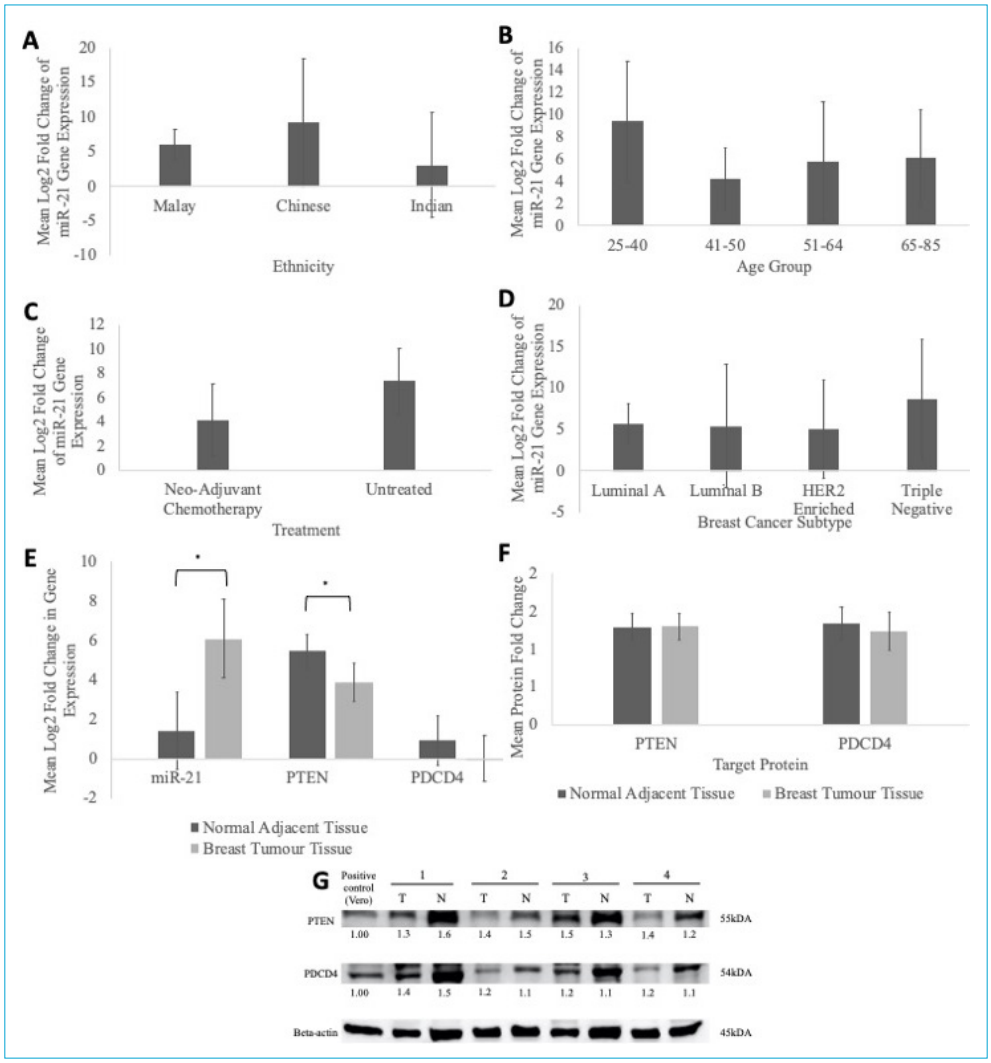

Figure 2. Log2 bar charts of miR-21 expression among 67 breast cancer patients against **(a)** ethnicity, **(b)** age group, **(c)** neoadjuvant chemotherapy treated and non-neoadjuvant chemotherapy treated patients and **(d)** subtype, **(e)** miR-21, PTEN and PDCD4 against specimen type (breast tumour and normal adjacent tissue), **(f)** bar chart of protein expression of PTEN and PDCD4 of 67 breast cancer patients, **(g)** blot images of PTEN and PDCD4 protein expression for paired breast tumour tissues (T) and normal adjacent tissues (N) with protein fold expressions normalised with housekeeping protein: B-actin. The symbol '*' represents significant difference between variables (p<0.05).

pression. The bar chart in Figure 2A showed the expressional levels of *miR-21* for each ethnic group.

miR-21 Expression in BC Patients and its Association with Age Groups

The expression levels of *miR-21* were also measured among age groups. Based on Table 2, the patients were divided into four age groups to represent young, middle-age and senior patients, namely 25-40 (younger age group) that consisted of 15 patients (22%), 41-50 (early middle-age group) that consisted of 25 patients (38%), 51-64 (late middle-age group) that consisted of 13 patients (19%) and 65-85 (senior age group) consisted of 14 patients (21%) of the sample size. The results denoted no significance of *miR-21* expression between the different age groups (p=0.515). However, *miR-21* expression was found to be the highest among those in the 25-40 age group, followed by 65-85 age group, 41-50 and finally 51-64 age group. Figure 2B visualises the difference in *miR-21* expression among all age groups.

miR-21 Expression among Neo-Adjuvant Treated Patients and Untreated Patient Groups

miR-21's expressional difference was also measured among BC patients who were treated with neo-adjuvant chemotherapy (27 patients, 40% of sample population) and patients who did not receive treatment (40 patients, 60% of sample population). Comparing *miR-21* expression between patients who have undergone neoadjuvant treatment versus patients without prescribed treatment, showed no significant difference (p=0.167), as presented in Table 2. Figure 2C showed that the untreated patient group had a higher expression of *miR-21* compared to the neo-adjuvant treated group.

miR-21 Gene Expression and Breast Cancer Subtypes

In this study, the expression of *miR-21* was investigated among the four main breast cancer subtypes. Among the 67 patients, luminal A consisted of 39 patients (58%), 7 (10%) luminal B patients, 9 (14%) HER-2 enriched patients, and 12 (18%) triple negative patients. The results in this section are presented in Table 2 that distinguished the different *miR-21* expression among the four BC subtype groups with the respective number of patients in each group. Figure 2D aided in visualizing the different *miR-21* expression for each patient group. However, no significant difference of *miR-21*'s expression among the four subtypes (p=0.939) was observed. Generally, triple negative subtype showed the highest *miR-21* expression, followed by luminal B, luminal A and finally HER-2 enriched.

Gene Expression of *miR-21*, *PTEN* and *PDCD4* in Specimen Types

Based on 67 paired tissue samples from breast cancer patients, Table 3 showed a significant difference between *miR-21* (p<0.001) and *PTEN* (p=0.010) gene expression in both tissue types. The expressional difference between the paired tissues were tabulated as mean and standard deviation (STDEV). It showed that *miR-21* expression is higher in the tumour specimen compared to its normal adjacent counterpart. Meanwhile *PTEN* showed a higher gene expression in the normal tissues compared to the tumour specimen. Finally, *PDCD4* showed no expressional significance between the tissue pair, p=0.451. However, *PDCD4* was expressed higher in the normal tissues compared to the tumour specimens. These differential expressions can be observed in Figure 2E.

Protein Expression of *PTEN* and *PDCD4* in Tissues

After conducting western blot procedure for 67 pairs of tissues for protein detection and quantification of *PTEN* and *PDCD4* (representative blots shown in Figure 2G), no significant difference was found between the two tissue types and among both proteins (Table 3). When comparing the proteins expression however, Figure 2F, there was a slight increase of *PTEN* protein observed in the normal tissue

Table 3. Genes and Proteins Expression Between Normal Adjacent Breast Tissues and Breast Cancer Tissues

Gene Expression	n=67	Gene Expression of Normal Adjacent Tissue (mean±SD)	Gene Expression of Cancer Tissue (mean±SD)	p
Gene of Interest				
miR-21	67	1.42±8.09	6.06±8.18	0.000
PTEN	67	5.43±3.59	3.86±3.93	0.010
PDCD4	67	0.92±5.10	0.02±4.72	0.451
Protein Expression	**n=67**	**Protein Expression of Normal Adjacent Tissue (mean±SD)**	**Protein Expression of Cancer Tissue (mean±SD)**	**p**
PTEN	67	1.293±0.722	1.297±0.738	0.938
PDCD4	67	1.331±0.889	1.235±1.004	0.296

when compared to the cancer tissue (p=0.938). For *PDCD4* protein expression on the other hand, the normal tissue specimen showed a relatively higher expression compared to the tumour counterpart (p=0.296). This suggested that in the cancer tissues, there was slight downregulation of the protein's expression of both *PTEN* and *PDCD4* compared to the normal tissues, a result that was consistent with that of the mRNA expression level.

The Correlational Expression of *miR-21*, *PTEN* and *PDCD4* Among BC Patients

To investigate the correlational expression of this study's target genes; *miR-21*, *PTEN* and *PDCD4* among 67 BC patients, Spearman's rho was utilised to deduce if the gene expression of *miR-21* with *PTEN* and *miR-21* with *PDCD4* were correlated. This correlation between the two variables is illustrated in Table 4. The relationship (or correlation) between the two variables is denoted by the letter r (Spearman's rho value) and quantified with a number, which varies between -1 and +1.[57] If the value is close to zero, it is said to have a negligible or a lack of correlation. Meanwhile if the value is close to one, the variables is said to have a strong correlation. Additionally, the sign of the r shows the direction of the correlation. A positive correlation is identified by a positive value while a negative correlation is indicated by a negative value.[57, 58] To visualise the trend of correlation between gene expression, a scatterplot graph was plotted between *PTEN* against *miR-21* (Fig. 3A) and *PDCD4* against *miR-21* (Fig. 3B).

Based on Spearman's rho analysis for the level of correlation between *PTEN* against *miR-21* as well as *PDCD4* against *miR-21*, there was a lack of correlation between the two pairs (Table 4). The r_s values were a mere r_s=-0.020 and r_s=0.037, respectively. As the Spearman's rho coefficient value is close to zero, this value is too low to indicate the presence of a correlation, which consequently did not reach significance (p=0.875 and p=0.767, respectively).

Discussion

Significant progress has been made in the 21st century when it comes to diagnosis and treatment of human malignancies. In this report, we've shown that *miR-21* expression was significantly higher in BC tissues compared to its normal tissue counterpart (p<0.001). These results are further reinforced with recent studies which also reflected the same outcome between these two tissue types when utilising the relative quantification method.[59-64]

Table 4. PTEN and PDCD4 Genes Expression Correlation in Breast Cancer Tissues

Gene Expression	n=67	Spearman's rho Correlation Coefficient	p
PTEN against miR-21	67	-0.020	0.875
PDCD4 against miR-21	67	-0.037	0.767

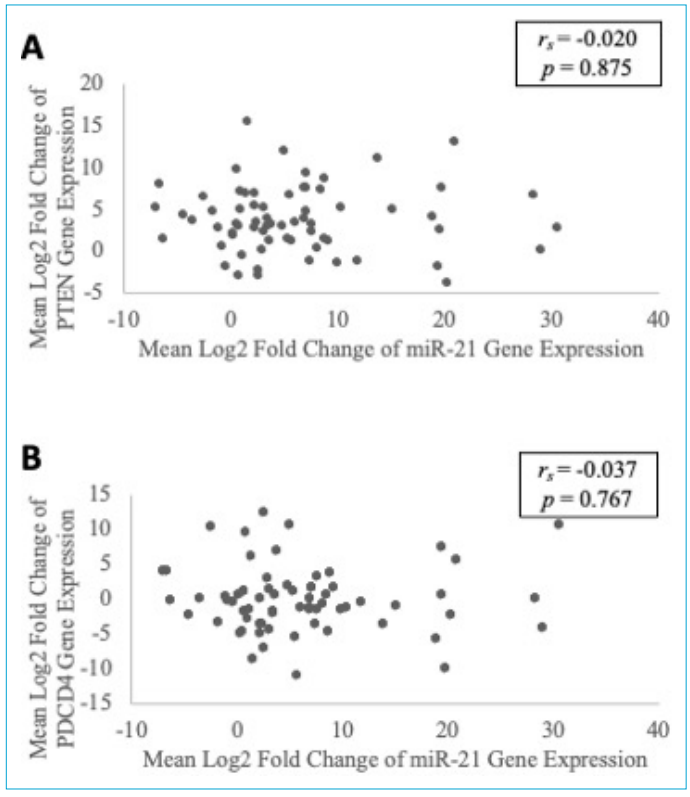

Figure 3. Spearman's rho scatterplot of **(a)** PTEN against miR-21 gene expression and **(b)** PDCD4 against miR-21 gene expression.

The regulation of *miR-21* expression and its targeting of *PTEN* is complex and involves many factors, including transcription factors, epigenetic modifications, and signalling pathways.[65-67] As of recent, studies that analysed the expressional patterns of *PTEN* among breast specimens and its gene expression correlation with *miR-21* have been conflicting.[36] In this study, *PTEN* was significantly expressed in the normal adjacent tissue compared to the breast tumour tissue. Five other studies compiled in a meta-analysis also compared *PTEN* expression in breast tumour tissues and its matched normal tissues and unanimously showed a significantly higher expression of *PTEN* in the matched normal tissues compared to the tumour counterpart.[68-72] In this study, *PTEN* and *miR-21* was also investigated for its correlation in gene expression. Based on the Spearman's rho, *PTEN* and *miR-21* showed a lack of correlation, with no significant difference in expression. Such results could be due to this study's low sample population along with the inclusion of NAC treated patients that could have plausible treatment-induced changes to gene expression profiles. This assumption could be justified based on similar previ-

ous studies that showed a correlation in gene expression between *miR-21* and *PTEN*, which excluded BC patients that had undergone treatment.[35] Moreover, *miR-21* expression was observed to be highly expressed in the breast tumour tissues despite the patients have undergone several cycles of chemotherapy. This trend can also be observed in a study that analysed the correlative expression of *miR-21* and *PTEN* among 120 BC patients did not reach a significant correlation coefficient. The nature of the collected breast specimens, whether the patients have undergone BC treatment or not was not disclosed.[38]

Despite the novelty of *PDCD4* and its regulatory effects in preventing carcinogenesis, many open questions persist regarding the molecular basis of its functionality when there is an overexpression of *miR-21* in BC.[73] This study's results showed no significance in expression of both tissue types, although upregulated in the normal adjacent tissue. Meanwhile, there was also negligible correlation of *miR-21* and *PDCD4* gene expression. Abdulhussain et al. (2019) had a significant inverse correlation between *miR-21* and *PDCD4* expression in 60 matched BC tissue specimens ($p<0.001$, r=0.-59).[74] The aforementioned study, however derived their BC specimens from patients who have not undergone any BC treatment while this current study included BC specimens from treated BC patients, which could potentially be the cause of the absence of significance of *PDCD4*'s expression in tissue and correlational expression. A similar BC study also had a significant inverse correlation between *miR-21* and *PDCD4* expression in breast tumour and normal adjacent tissue among 20 BC patients who have not undergone BC treatment.[75] Conversely, the aforementioned study also investigated the genetic profiles of treatment resistant BC cell lines and discovered the overexpression of *miR-21* while *PDCD4* mRNA expression remain unchanged.[75]

At present, there is very limited information that is available regarding the effect of chemotherapy on *miR-21* expression in BC and its correlation with clinical improvement. Sukhija et al. (2023) collected blood samples from BC patients before and after receiving chemotherapy and compared the respective expression of *miR-21*. After NAC, the expression of *miR-21* was significantly increased by 5.65-fold. They have deduced that NAC causes clinical improvement in BC patients but is not correlated with *miR-21* expression despite being significantly increased after chemotherapy.[76] Additionally, this study's findings were congruent to a previous study that investigated the same variables of gene expression correlation and protein expression of the tumour suppressors in matched BC tissues. There was a significant expressional difference of *miR-21* where it was upregulated in the tumour tissues compared to its normal counterpart. However, there was no significance in gene expression correlation between *miR-21* and *PDCD4*, while *PTEN* and *PDCD4* proteins had no significant difference of its expression in matched BC tissues.[77]

After conducting western blot procedure for 67 pairs of tissues for protein detection and quantification of *PTEN* and *PDCD4*, no significance was found between the two tissue types and among both proteins although the normal adjacent tissues showed a slight increase of both tumour suppressor proteins compared to the cancer tissue counterpart. Both studies conducted by Kechagioglou et al. (2014) and Qi et al. (2009) have failed to detect loss of *PTEN* expression in invasive and in situ ductal breast carcinoma.[78, 79] The depletion of *PTEN* functionality has been attributed to inactivation of *PTEN* protein via post-translational alterations.[80, 81] The phosphorylation of *PTEN* in specific residues transposes the molecule from an open into a closed conformation thus inactivating the molecule.[82] Therefore, a plausible explanation for this study's *PTEN* protein expression could be related to Kechagioglou et al. (2014), where the expression of phosphorylated *PTEN* is more pronounced among the patient cohort with breast cancer compared to their healthy controls. This suggests that despite the presence of gene expression, phosphorylation can be a pathway of protein inactivation in breast cancer.[78] Not only that, while such phosphorylated modifications occur, this can also affect *PTEN* localisation to the plasma membrane which limits its interaction with PIP3.[83]

Other than the possibility of phosphorylated *PTEN* that conceived such results in this study, recent studies have proven that *PTEN* has three alternative translation isoforms; *PTEN*α, *PTEN*β and *PTEN*ε, that are originated from the same mRNA as canonical *PTEN*.[84-86] Interestingly, in contrast with canonical *PTEN* and its tumour suppressive role, *PTEN*α and *PTEN*β expression promote tumourigenesis.[87] In a 2019 study that investigated the expression of canonical *PTEN* and its isoforms in liver cancer tissues in comparison with the normal tissues, it was discovered that the expressional trend of *PTEN*α and *PTEN*β were not always consistent as canonical *PTEN* since the levels of *PTEN*α and *PTEN*β proteins remain unchanged, or an increase was observed in tumour tissues with decreased canonical *PTEN* as compared to the normal adjacent tissues. The same variables were also tested on xenograft models which had consistent results with the liver patient cohort.[87] Frankel et al. (2008) also investigated *PTEN-miR-21* interaction in breast cancer cells by transfecting MCF-7 cells with a *miR-21* precursor, a *miR-21* inhibitor, and appropriate controls. Interestingly, these treatments caused only subtle changes in *PTEN* protein levels which suggests that cell and tissue type specific differences may result in different functional *miR-21* targets.[88]

Typically *PDCD4* protein are down-regulated in cancer cells, however there has been an increasing amount of cancer patients with upregulated *PDCD4* protein originated from tumours that showed poor survival.[89] A study was done with MCF-7 breast cancer cell line which demonstrated that the methylation of *PDCD4* had caused inactivation and upregulation of *PDCD4* protein which was associated with tumour cell growth and viability.[89] When it comes to *PDCD4* with its mRNA and protein expression, this disparity was identified in a lung cancer study by comparing its expression in both lung tumour and normal adjacent tissue.[90] Based on the results derived from the lung tissues, *PDCD4* protein was observed to increase in expression in the lung tumour tissues compared to the non-tumour counterpart. Additionally, *PDCD4* mRNA and protein changes in expression was not in parallel in most of the tissue pairs and this also meant a significant increase of protein levels was observed in tumours where no changes in *PDCD4* mRNA level were detected or suppressed.[90]

The multi-ethnic composition of Malaysian society offers a population in which comparative studies of genetics may be performed. Our results revealed that there was no significant difference in the expression of *miR-21* between BC patients from each of Malaysia's three main ethnic groups (Malay, Indian and Chinese). This consistency among ethnicities is similarly seen among other cancer types, such as oral squamous cell carcinomas[91] and lung adenocarcinoma.[92] Furthermore, this consistency is not limited to Asian ethnicities, as one meta-analysis of *miR-21* expression in multiple cancer types demonstrated no correlational significance among Asian, Caucasian and African American populations.[93] This evidence supports our results. Moreover, another rationale of this study's results is the ethnic distribution in the patient cohort, which consisted of 53 Malay patients, 7 Chinese and 7 Indian patients, which could have skewed the expression of *miR-21* due to the evident clustering of the Malay ethnicity in the patient cohort.

It was deduced in this study that *miR-21* expression had no significant correlation with the 4 patient age groups. Despite this study consisting of only 67 patients, other studies conducted that carried out similar correlational studies discovered that there was no significance between age groups and *miR-21* expression.[62, 94, 95] Even with a larger patient cohort of 252 participating BC patients, it did not demonstrate a correlation between age groups with the oncogenic *miR-21*.[96] Therefore, based on this study's results and previous literature which consisted of larger patient cohort, it can be presumed that *miR-21*'s clinical significance can be suitable to be evaluated further in patients of all ethnicities and age groups.

In this study, *miR-21* was upregulated in patients with BC, regardless of previous exposure to chemotherapy, which consequently, showed no significant difference. However, chemotherapy naïve patients still presented higher relative fold change values than the chemotherapy treated patients. This finding is aligned with the report of Sales et al. (2022) who assesed the expression of *miR-21* and reported that *miR-21* expression did not show any significant difference between the chemotherapy-treated and chemotherapy naïve patients.[97] These findings reinforce a possible downregulation of *miR-21* following an optimal response to chemotherapy. The prognostic potential of circulating *miR-21* had also been previously investigated where a significant proportion of BC patients who had undergone neoadjuvant chemotherapy had an increased expression of exosomal *miR-21* that eventually develop metastatic disease.[98]

The majority of current studies of the role of microRNA have been coordinated regardless of the tumour's molecular subtype. This study's results revealed that the expression of *miR-21* was upregulated in all subtypes but did not reach a significant difference, which could be attributed by the final sample size (67 patients). However, other reports with higher number of BC patients confirmed that *miR-21* expression was similar in different BC subtypes which did not exhibit a clear discrimination between the subtypes, thus reinforcing our results.[97, 99, 100] Additionally, luminal breast cancers are known to be a result of somatic mutations[101] while, triple negative breast cancers are due to germline mutations with 80% of cases that arise from the BRCA1 or BRCA2 mutation.[102] As this study's total luminal A and B BC subtype (46 patients) is evidently more than the total TNBC subtype (12 patients), this could also be the source for the absence of discrimination between *miR-21* expression and the subtypes as the distribution of the subtypes are heavily skewed.

Evidently, BC staging and subtype determination is dependent on the expression of hormone receptors, including oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) in tumours, which also defines prognosis and aid in deciphering treatment options.[103] Thus, it is clear that gene expression defines BC and therefore, the possibility of miRNA expression to display subtype specificity is presumed to exist.[104, 105] A meta-analysis published in 2022 compiled a list of distinct miRNAs that correspond to every BC subtype.[104] Despite the extensive list of biomarkers proven to be molecularly specific for each subtype, the common denominator in this metanalysis depicts too many miRNAs to allow testing for specific subtypes. Therefore, having a sole miRNA such as *miR-21* that is constantly proven in its upregulation in BC would be more convenient. To aid the issue of specificity of *miR-21* in BC, the development of an enzyme-powered

miRNA discriminator (T7 Exonuclease powered digestion) was designed in a 2023 study to distinguish BC cells from normal cells. Moreover, this enzyme could further identify subtype features using *miR-21* as a universal biomarker and *miR-21*0 to identify triple negative subtype features. Interestingly, this method was successful in distinguishing BC cell lines respective to the subtypes; MCF-7 (ER positive), BT-474 (HER-2 positive), MDA-MB-231 (triple negative) with MCF-10A as a control. *miR-21* levels were found to have a constant increase in the BC cell lines according to the increasing severity of each subtype using the T7 Exo powered miRNA in relative to the normal cell line.[106] Ultimately, future works of defining oncomiR expression with the use of innovative techniques could bring us a step closer to a reliable universal biomarker.

When it comes to the Malaysian demographic and its influence on *miR-21* expression, it is evident that there is no clear correlation between age groups ethnicities, subtypes and treatment received. Therefore, we can say that based on this study's patient cohort, *miR-21*'s gene expression is independent, as it is upregulated when a patient has BC. Nevertheless, significant differences were found between *miR-21* expression and the specimen type (normal adjacent tissue and breast cancer tissue), which reflects other similar studies with the same results obtained when utilising the $2^{-\Delta\Delta CT}$ method where patients diagnosed with BC had a higher *miR-21* fold change compared to the normal tissues ($p<0.001$). Moreover, significance was also found in *PTEN*'s expression within the specimen types, where *PTEN* was significantly upregulated in the normal adjacent tissues compared to the breast tumours ($p=0.010$). *PDCD4*'s expression was also upregulated in the normal adjacent tissues and downregulated in breast tumours, although no significant difference was found ($p=0.451$). However, there was a lack in correlation, based on the Spearman's rho analysis between gene expression of *miR-21* against *PTEN* and *miR-21* against *PDCD4* which showed: $rs=-0.020$, $p=0.875$ and $rs=-0.037$, $p=0.767$, respectively. *PTEN* and *PDCD4* protein expression in this study showed little difference of the tumour suppressor proteins in both tissue types. This could be theorised to occur due to post-translational modifications such as phosphorylation of the proteins or the existence of isomers from a single mRNA which allowed the proteins to be expressed but is instead non-functional and therefore prompted tumourigenesis. Despite the absence of significance in most demographical factors and groups in this study, therefore, we can agree that *miR-21* has a notable presence in BC and is a suitable biomarker to be evaluated further in patients of all ethnicity and age groups. Additionally, *miR-21* should further be studied for its possibility as a circulating biomarker.

Disclosures

Ethics Committee Approval: This present study involves the use of human subjects which include tissue specimens. The human ethics approval obtained for this project to collect the patients derived tissue specimens via the University Putra Malaysia institutional ethics review board and Ministry of Health Medical Research Ethics Committee (MREC) prior to the commencement of the study. The ethics approval code is NMRR-21-246-58614 (IIR).

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Conflict of Interest: None declared.

Authorship Contributions: SRW carried out all experiments, specimen and patient information collection, participated in all the statistical analysis and drafted the manuscript. SHL supervised, conceived, reviewed, revised, drafted and edited the manuscript. AB assisted in the tissue specimen collection. CPP, TMIM, and GLT co-supervised and reviewed the manuscript. SN assisted with the statistical analysis. All authors read and approved the final manuscript.

Availability of data and materials: The datasets used and/or analysed in the current study are available from the corresponding author upon reasonable request.

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