Association of single nucleotide polymorphisms in miR-101 and Pre-miR618 with breast cancer susceptibility in a Vietnamese population

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Abstract

Breast cancer is the most common cancer diagnosed in women worldwide. Among BC gene regulation factors, microRNAs play as oncogenes or tumor suppressors in the cancer development. Functional polymorphisms of microRNA gene may alter their expression and affect target binding specificity. The risk of cancer can be modified by Single Nucleotide Polymorphisms (SNPs) and thus, SNPs may be considered as potential markers of carcinogenesis. Among SNPs of mir-618 and miR-101 gene, rs2682818 and rs1053872 had been found related to BC risk in different populations. This study aims to investigate the genetic susceptibility of these two SNPs in the risk of BC in Vietnamese women. SNP genotyping was analyzed by PCR- High Resolution Melting (PCR-HRM) and Tetra-Arms-PCR Melting Analysis (TAPMA) in 100 cases and 100 controls.

The logistic regression analysis revealed that the A allele of rs2682818 and G allele of rs1053872 tend to confer protection against BC (A vs. C: OR = 0.94, 95% CI = 0.63 – 1.42 and G vs. C: OR = 0.82, 95% CI = 0.56 – 1.21 respectively) but still not statistically significant (P value = 0.78 and 0.32 respectively). Further studies are suggested to confirm these findings.

Keywords: Breast cancer, Single Nucleotide Polymorphisms (SNPs), rs2682818, rs1053872, mir-618, miR-101.

Introduction

Breast cancer (BC) is the most common cancer among women worldwide. Several low-frequency, high-penetrance BC predisposition genes have been identified including *BRCA1* and *BRCA2*. However, most of BC cannot be explained by the above genes[1]. As a common complex disease, there are some indications of a potential contribution of moderate and low-penetrance BC predisposition genes to genetic susceptibility[2]. A large proportion of the genetic factor of hereditary cases, however, remains undisclosed[3].

Studies on acknowledged genes continue with a growing interest in epigenetics and gene regulation to further explain BC formation. The discovery of microRNA (miRNA) became one of the most remarkable advances in understanding the mechanisms of gene regulation[4]. MiRNAs are single-stranded RNAs of approximately 22 nucleotides that can regulate gene expression by either degrading or blocking translation of mRNA, mainly by binding to their 3'-UTR[5]. One miRNA may target to various mRNAs and, therefore, miRNA regulates approximately 30 % of all human genes[6]. MiRNAs and their important and diverse roles has been found in many molecular pathways and biological processes[7]. The function of miRNAs has been widely found in many types of cancer. Depending on regulated-gene, miRNAs can act as oncogenes or tumor suppressors[8]. A variety of miRNAs expression in the growth of several human cancers has been observed[9].

Single nucleotide polymorphisms (SNPs) are the common form of variations of genes within the human genome. The appearance of SNPs in the DNA sequence encoding pre-miRNAs and miRNAs can contribute to aberrant miRNA maturation and affect binding affinity and specificity of target[10]. The association of SNPs in miRNA genes with cancer susceptibility has been examined in many epidemiological studies[11]. In BC, the associations between polymorphisms in miRNA gene and BC risk have been evaluated in different populations including Asians[12], Caucasians[13], Brazilian[14], Iranian[15], Pakistani[16], Chinese[17], and Vietnamese populations[18].

Recent studies have shown the connection between the abnormal miR-618 expression and tumorigenesis[19,20]. Rs2682818 polymorphism was found on the miR-618 stem-loop precursor sequence which could influence both the pre-miR-618 secondary structure and the mature miR-618 release[21]. The contribution of the rs2682818 miR-618 gene to BC in various populations had been explored. In South American women, the finding showed that the CA genotype of rs2682818 increases BC risk in non-familial early-onset BC[22]. A novel meta-analysis had found that the dominant genotype of miR-618 rs2682818 polymorphism is related to BC[23]. On the other hand, there was no substantial interaction within the Chinese population between miR-618 rs2682818 and BC risk[24].

A few variations are present in the mature microRNA sequence. These polymorphisms might have a direct effect on microRNAs binding to hundreds of target mRNAs. One is rs1053872, which is situated in the mature miR-101. The predicted targets of miR-101 include EZH2 and STMN1[25,26]. MiR-101 family was involved in several cellular events including cell proliferation, invasion and apoptosis[25].

Among several malignancies, particularly BC, miR-101 is often presented at low levels[26,27]. In BC, miR-101 over-expression has a tumor-suppressing effect[25,26]. Nevertheless, little is known about the effect of miR101-associated SNPs in the risk of BC. So far, only one case-control study found an association for the miR101 rs1053872 with BC risk[28].

These SNPs have not yet been adequately evaluated in the Vietnamese population. In this study, we initially analyzed the association of rs2682818 in pre-mir-618 gene and rs1053872 in miR-101 gene with BC susceptibility in the Vietnamese population.

Material and Methods

Study population: This population-based study is part of an ongoing study, the goal of which is to determine potential SNPs associated with BC susceptibility in Vietnamese Kinh Women. The full sample set in this study included about 100 BC female patients and 100 healthy female controls. All cases were diagnosed with malignant breast tumors. The control group consisted of individuals attending for a screening check-up in a hospital or were healthy blood donors without BC. The age of participants was a range from 40-65 years and belonged to Kinh ethnic, Viet Nam. The study was conducted with the approval of the ethical committee in the Oncology Hospital of Ho Chi Minh City under the decision number 177/HĐĐĐ-CĐT. All patients signed informed consent forms.

Genotyping and mutation screening: Genomic DNA was extracted from whole blood using a salting-out method following Hue et al's[29] protocol with some modifications. DNA samples were evaluated by spectrophotometry using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) to determine DNA concentration and purity. The purity of the DNA samples was validated by the value of the 260 nm/280 nm absorbance ratio which should be from 1.7 to 2.0.

The genotyping for two candidate SNPs was performed by Polymerase chain reaction – High Resolution Melting (PCR-HRM) method for rs2682818 and Tetra-Arms-PCR Melting Analysis (TAPMA) for rs1053872. Information about sequences of miR-618 gene (containing SNP rs2682818) and miR-101 gene (containing SNP rs1053872) obtained from the NCBI databases was used as input data for designing primers with specialized software (NCBI Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Most potential primers for each SNP with in-silico product's sequences from UCSC were used to predict the melting curves and melting peaks corresponding with three genotypes of each SNP through uMelt-HETS software (https://www.dna.utah.edu/hets/umh.php).

Besides, the secondary structure formation was rechecked with Oligo Analyzer online's tool (https://sg.idtdna.com/calc/analyzer). The designed sets of primers for two SNPs genotyping were shown in table 1.

One negative control and three positive controls (genotypes confirmed by Sanger sequencing) were included in each run. In other to identify the genotype of a sample, four criteria based on the melting curve (Normalized melting curves, Normalized melting peaks and Different plots) and the amplification value (Ct value) obtained from the DNA amplification (Amplification curves) were taken into consideration.

Pre-mir-618 rs2682818 genotyping: The SNP genotyping assays using optimal HRM analysis were executed by LightCycler 480 High-Resolution Melting Master (Roche Diagnostics, Germany) and a LightCycler 96 Instrument with a 96-well thermal block (Roche Diagnostics). The PCR-HRM analysis was performed in a reaction containing 1X LightCycler 480 High-Resolution Melting Dye, 0.2 μM forward and reversed primers, 3.5 mM $MgCl_2$, 10–20 ng of genomic DNA and PCR-grade water.

The PCR thermal cycling was as follows: initial denaturation at 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, an annealing step at 64 °C for 30 s and an elongation step at 72 °C for 30 s. HRM analysis was then carried out as the default. Typical genotyping results for rs2682818 is demonstrated in figure 1. All the Ct value was in range from 22 to 30.

miR-101 rs1053872 genotyping: SNP rs1053872 was genotyped using TAPMA. The SNP genotyping assays were executed by HotStartTaq DNA Polymerase (Qiagen) and a LightCycler®480 ResoLight Dye (Roche).

Table 1
Primer sequences for selected SNPs genotyped using PCR - HRM and TAPMA

SNP (Genotyping method)	Primer	Sequence (5'-3')
rs2682818 (PCR-HRM)	Forward	GAATTTTTTCAGACTCATCCACAGGG
	Reverse	GTACATGCAGTAGCTCAGGAGAC
rs1053872 (TAPMA)	Forward outer	CCTACTATCATAGGCTTCCTCAGC
	Reverse outer	AATCCCTACAACAAAAACCAAGGCTC
	Forward inner (Allele G)	GCCCTCTGTCATATGGCTGTTTTG
	Reverse inner (Allele C)	CAGTAACAGACTCCACAGGTTTGG

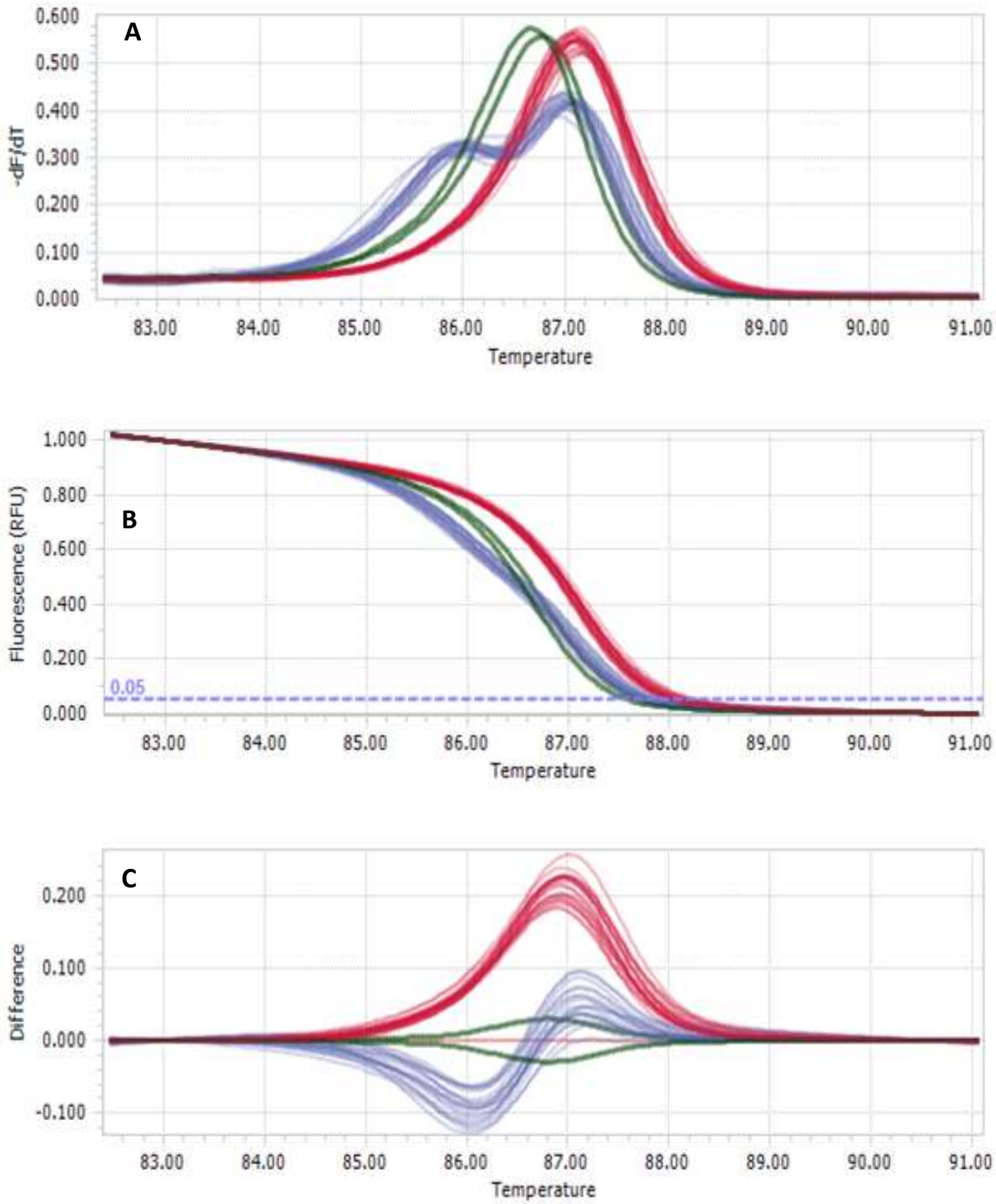

Figure 1: Example of HRM curve genotyping for rs2682818 (A/C). (A) Normalized melting peaks. The ΔT of the two homozygotes must be higher than 0.05. (B) Normalized melting curves. The heterozygote curve cuts the homozygote with the lower Tm in the middle and does not cut the other homozygote curve. (C) Difference plots. The lower-Tm homozygote is set as the baseline and the heterozygote curve must divide into two sides of the baseline. The homozygote with the higher Tm curve must be on the upper side. Red, blue and green represent CC, CA and AA genotypes respectively

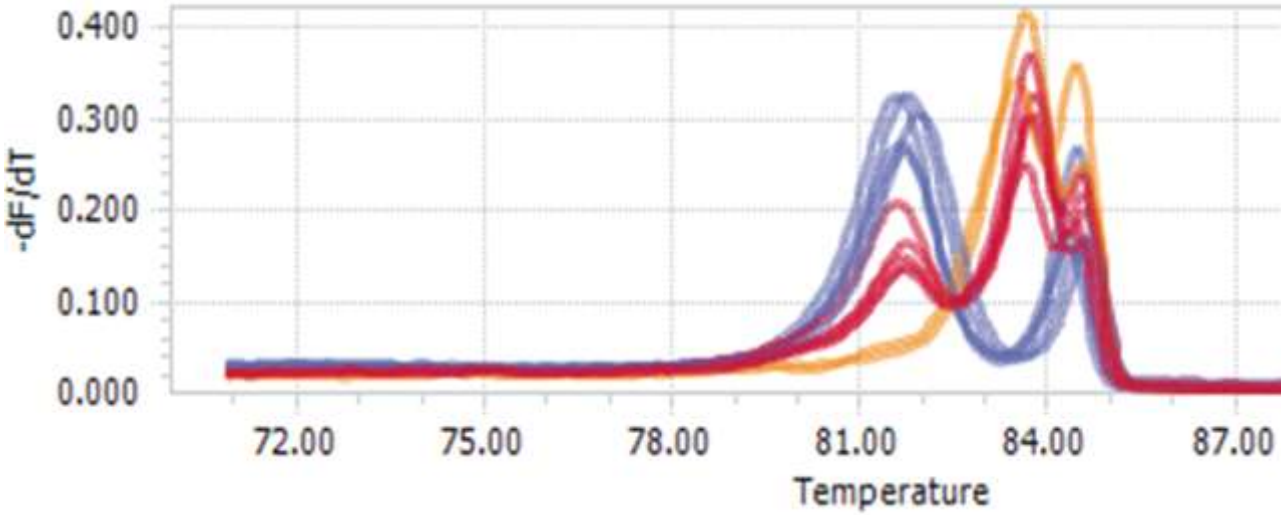

Figure 2: Example of HRM curve genotyping for rs1053872 (C/G). The heterozygote CG genotype (red line) was identified based on three distinct peaks comprising an internal control (Tm = 84.08 ± 0.22°C), G allele amplicon (Tm = 83.34 ± 0.18°C) and C allele amplicon (Tm = 80.76 ± 0.23°C). The homozygote CC genotype (blue line) had only two peaks consists of an internal control and C allele amplicon. The homozygote GG genotype (yellow line) was recognized by two separated peaks including an internal control and G allele amplicon

TAPMA reactions were carried out in a reaction containing 1X HotStarTaq buffer (Qiagen), 200µM each dNTP (Promega), 0.3 µM forward outer primer (Sigma), 0.5 µM reverse outer primer (Sigma), 0.05 µM forward inner primer (G allele) (Sigma), 0.4 µM Reverse inner primer (C allele) (Sigma), 1.5 mM $MgCl_2$ (Qiagen), 2.5 units HotStarTaq (Qiagen), 0.5µL LightCycler®480 ResoLight Dye (Roche), 10-20 ng of genomic DNA and PCR-grade water. Thermal cycles for PCR amplification consisted of an initial pre-incubation at 95°C for 15 minutes followed by 40 cycles of a denaturation step at 95°C for 30 seconds, an annealing step at 62 °C for 30 seconds and an elongation step at 72°C for 30 seconds.

HRM analysis was then carried out as the default. One negative control and three positive controls of each SNP (genotypes confirmed by Sanger sequencing) were included in each run to ensure the identified genotype accuracy. The typical HRM curves for rs1053872 were demonstrated in Figure 2. All the Ct value ranged from 22 to 30.

Statistical analysis: All statistical analysis was undertaken using RStudio Version 1.2.1335. Genotype and allele frequencies of each SNP in the population were calculated as percentages. The genotype frequencies of each SNP were tested for deviation from the Hardy-Weinberg equilibrium (HWE) in both cases and controls [30]. Further analysis, including the genetic model association, was carried out using the 'SNPassoc' package. The ORs and 95% CIs were calculated to assess BC risk. The threshold to determine a statistically significant association was set at P = 0.05.

Results and Discussion

In this study, two SNPs in pre-miRNA and miRNA genes were investigated by the relationships with BC risk.

The genotype and allele frequency are summarized in table 2. All SNPs were highly variable polymorphisms, with the minor allele frequencies higher than 32.5%. The genotype frequencies of two polymorphisms were all in agreement with the HWE in cases and controls with all *P* values being greater than 0.05 indicating that there was a normal distribution between genotypes inside each SNP in the tested population.

This means the research sample represented the Vietnamese population and the genotyping for these SNPs was reliable. As also shown in table 2, the Chi-squared test indicated that there was no significant difference between BC cases and healthy controls for both allelic and genotypic frequency of rs2682818 and rs1053872 ($P > 0.05$). Further association analysis of genetic models of rs2682818 and rs1053872 was carried out (table 3).

Our findings showed the A allele of rs2682818 tend to confer protection against BC (OR = 0.94) but still not statistically significant ($P = 0.78$) (table 3). A case-control study of 252 women with BC and 248 controls from Chinese population also reported no significant association between the miR-618 rs2682818 and BC risk[24]. On the other hand, the AC and AA genotypes of miR-618 rs2682818 were shown to enhance BC risk (AC vs. CC: OR = 1.291, 95%CI = 1.012–1.648, P = 0.040; AA + AC vs. CC: OR = 1.280, 95%CI = 1.009–1.623, P = 0.042) in a meta-analysis of 10 independent studies along with 4099 cancer cases and 5057 healthy controls[23].

The miR-618 rs2682818 AC and AA genotype were also associated with the increased BC susceptibility in 440 cases and 807 controls from South American population CA vs CC: OR = 1.4, 95% CI = 1.0 – 2.0, P = 0.01; CA + AA vs.

CC: OR = 1.4, 95% CI = 1.0 – 2.0, P= 0.02; A vs. C: OR =1.3, 95% CI = 1.0 – 1.8, P = 0.03[22]. For the mechanism investigation, pre-mir-618 containing rs2682818 showed the effect on BC. The expression of miR-618 could regulate CBX8 expression via targeting the 3'UTR of CBX8, resulting in enhanced CBX8 protein[31]. CBX8 knockdown results in more severe DNA damage indicating that CBX8 participates in DNA repair to promote carcinogenesis[32].

Furthermore, Song and colleagues[33] reported that miR-618 directly targets to the 3'UTR of FOXP2. The downregulation of MiR-618 leads to FOXP2 upregulation followed by the increased TGF-β expression and then inducing EMT. In other words, the effect of miR-618 knockdown could lead to the inhibition of cell migration and invasion through the high level of FOXP2.

In the meanwhile, Ivanovic and colleagues[34] found out that the overexpression of miR-618 could suppress the function of MMP-9 to prevent the progress of tumorigenesis. Since rs2682818 is part of the stem-loop sequence of the miR-618 precursor, this can influence the levels of miR-618. This SNP might change the secondary stem-loop structure which in turn affects the mature process of pre-miR-618[21]. The presence of the A allele of rs2682818 was suggested to negatively affect the production of mature miR-618. The above evidences indicated that the A allele of rs2682818 may decrease the level of mature miR-618 which could downregulate CDX8, FOXP2 and MMP-9 and finally resulting in increased cancer risk.

In our study, the logistic regression analysis also revealed that the G allele of rs1053872 seems likely to decrease the risk of BC (OR = 0.82) but still not statistically significant (P = 0.32) (table 3). Chen and colleagues[28] found that two genotypes carrying G allele of rs1053872 were significantly associated with increased risk of BC (CG + GG vs CC: OR = 1.179, 95% CI = 1.040 – 1.337, P = 0.010) in a study of 1064 BC cases and 1073 cancer-free controls from Chinese.

Several reports have recently been published supporting miR-101's significant role in the development of BC. One of the miR-101-3p target is Med19 by directly binding to the 3'-UTR regions. The increased level of miR-101-3p might mainly cause Med19 downregulation in BC.

Table 2
Allele and genotype frequency distribution of the SNPs

MicroRNA SNP		**Genotype No. (%)**			**Allele No. (%)**		**P_{HWE}**
		CC	CA	AA	C	A	
Pre-mir-618 rs2682818	Case (n = 97)	46 (47.4)	39 (40.2)	12 (12.4)	127 (66.2)	65 (33.8)	0.366
	Control (n = 96)	44 (45.8)	39 (40.6)	13 (13.5)	131 (67.5)	63 (32.5)	0.486
	$P_{Chi\text{-}square}$	0.96			0.77		
		CC	CG	GG	C	G	
miR-101 rs1053872	Case (n = 99)	38 (38.4)	45 (45.5)	16 (16.1)	121 (61.1)	77 (38.9)	0.675
	Control (n = 99)	33 (33.3)	45 (45.5)	21 (21.2)	111 (56.1)	87 (43.9)	0.422
	$P_{Chi\text{-}square}$	0.60			0.31		

Table 3
Logistic regression analysis of the associations of the SNPs with BC

MicroRNA SNP	**Genetic model**	**OR**	**95% CI**	**P**
Pre-mir-618 rs2682818	A vs. C	0.94	0.63 – 1.42	0.78
	AA vs. CC	0.88	0.36 – 2.14	0.78
	CA vs. CC	0.96	0.52 – 1.75	0.89
	(CA+AA) vs. CC	0.94	0.53 – 1.65	0.83
	AA vs. (CC+CA)	0.90	0.39 – 2.09	0.81
miR-101 rs1053872	G vs. C	0.82	0.56 – 1.21	0.32
	GG vs. CC	0.66	0.29 – 1.47	0.31
	CG vs. CC	0.87	0.46 – 1.62	0.66
	(GG+CG) vs. CC	0.80	0.45 – 1.44	0.46
	GG vs. (CG+CC)	0.72	0.35 – 1.47	0.36

The knockdown of Med19 levels decreased EGFR expression followed by the decreased expression of molecules in the MEK/EK pathway and finally suppressed tumor growth and metastasis[35].

Another pathway was reported that miR-101 suppresses Janus kinase 2 (Jak2) expression by directly interacting with the 3'-UTR of Jak2. The Jak2 activation was reported to be required for the growth of breast tumor cells[36]. In other mechanism, miR-101 has been shown to negatively regulate oncogenes including EZH2 and STMN1[25,26]. To sum up, the miR-101-3p down-regulation can enhance the incidence of cancer through the increase of Med19, Jak2, EZH2 and STMN1 level.

In contrast, the miR-101 up-regulation induces BC cell survival through the decrease of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) level. The increased level of Nrf2 can upregulate the expressions of several antioxidants, ultimately protecting cells from carcinogen-induced DNA injury and inhibiting the occurrence of cancer.[37]

The increased level of miR-101 can reduce the expression level of Nrf2, and, therefore, cancer arises. Meanwhile, the SNP rs1053872 is in the pre-miR-101-2 flank region. In the biogenesis of miRNA, after transcription from miRNA gene, the long pri-miRNA is cleaved into a nearly 70-nucleotide-long and hairpin-shaped pre-miRNA with the help of Drosha and its cofactor, DGCR8[38]. Protein DGCR8 recognizes the position on pri-miRNAs and serves as a ruler for Drosha to cut off. The additional flanking sequences can initially be needed to attach the Drosha-DGCR8 complex to pri-miRNA[39].

Previous studies have demonstrated that genetic variants in the flanking sequences extensions may affect the Drosha recognition and cleavage of pri-miRNA into pre-miRNA[40,41]. Therefore, the rs1053872 at 10 kb downstream of pre-miR-101-2 may influence the processing of mature miRNA by affecting cleavage of Drosha.

Although miR-101, miR-618 and their belong variants might play key roles in breast carcinogenesis, neither of the association of rs2682818 and rs1053872 with BC susceptibility showed significance in Vietnamese population. The differences in ethnicity and other genetic and environmental factors could partially explain observed heterogeneities between studies. Additionally, the non-association results may be due to a small sample size, thus a larger sample size will be required to confirm this finding.

Conclusion

To investigate the association between polymorphisms in miR-101 and miR-618 gene and the risk of BC in a Vietnamese population, this study demonstrated that miR-618 rs2682818 and miR-101 rs1053872 are not significant association with BC risk. Nevertheless, further studies are needed to validate the current findings.

Acknowledgement

The study was supported by The Youth Incubator for Science and Technology Programe managed by Youth Development Science and Technology Center - Ho Chi Minh Communist Youth Union and Department of Science and Technology of Ho Chi Minh City, the contract number is "28/2019/HĐ-KHCN-VƯ". The authors would like to thank the Oncology Hospital HCMC for their contribution in collecting samples.

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(Received 11th March 2020, accepted 10th May 2020)