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ASSOCIATION BETWEEN SINGLE NUCLEOTIDE POLYMORPHISM RS4919510 (C>G) IN MIRNA-608 AND BREAST CANCER IN VIETNAMESE POPULATION

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ABSTRACT

Breast cancer (BC) is a complex disease throughout the world and it is one of the most common cancer among women both in developed and developing countries. To enhance the survival of BC patients, genetic factors are used for early diagnosis because they are non-changed factors and present ability of cells to proliferate and metastasize. MiR-608 targets many genes which are vital for development, differentiation, motility, apoptosis and angiogenesis. The SNP rs4919510 (C/G) affects the processing of the pre-miRNA to its mature form and the ability to regulate target genes. This SNP has been demonstrated to relate to breast cancer in Chinese and Iranian population. Our study aimed to investigate the association between the SNP rs4919510 and BC in Vietnamese population. 106 cases and 101 controls were genotyped using optimized tetra-ARMs-PCR method, then statistical analysis was applied to examine the correlation of the SNP. The results show that this SNP is high polymorphism with the frequencies of minor allele C is 24.8% and major allele G is 75.2% in Vietnamese population. Statistic result revealed an obvious increased risk of BC among Vietnamese women when compared of heterozygote model and dominant model (CG vs. GG: OR=1.93, 95% CI: 1.09-3.45, $p=0.02$; CC+CG vs. GG: OR=1.83, 95%CI: 1.05-3.17, $p=0.03$). Our study suggested that the polymorphism of rs4919510 may be associated with BC risk in Vietnamese individuals.

Keywords: rs4919510, miR-608, breast cancer, polymorphism, tetra-ARMs-PCR method.

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INTRODUCTION

Breast cancer is a major health problem throughout the world. It is one of the most common cancer among women both in developed and developing countries. It is also the leading cause of cancer death in female, with over 2 million new cases diagnosed and more than 625,000 breast cancer deaths in 2018[3]. In Vietnam, the breast cancer incidence has increased steadily with an approximate 15,229 new cases in 2018; however, the mortality rate for this disease accounts for 40%[3].

Reducing the burden of disease mortality and morbidity can be done by early detection of individual having breast cancer risk. Breast tumorigenesis proceeds through the accumulation of genetic and epigenetic alteration. Some of these changes are expressed during early stage of the tumor development and thus provide a chance for diagnosis and treatment strategies. Among them, microRNAs have recently emerged as novel biomarkers that can help detect tumor at early stage. MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs with sizes of 17-25 nucleotides. A single miRNA might bind to as many as 200 gene targets and these targets can be diverse in their function[2]. Tumor formation may arise from reduction of a tumor suppressive miRNA and amplification of an oncogenic miRNA.

Single nucleotide polymorphisms (SNPs) in pre-miRNAs or mature miRNAs can modify the biological processes of miRNA and thus are highly potential targets for study of cancer, including breast cancer. It has been proposed that SNP rs4919510 C>G can alter binding of miR-608 to target genes[8]. Therefore, it may affect the signaling path of insulin receptor (INSR), interleukin-1 alpha (IL1A), growth hormone receptor (GHR), and TP53[8]. They are vital genes for development, differentiation, motility, apoptosis and angiogenesis. Several studies investigated the impact of miR-608 rs4919510 C>G on the risk of various cancers,

especially breast cancer [6, 7, 9-13]. Although SNP rs4919510 in miR-608 is reported to be associated with breast cancer in Chinese and Iranian, conclusions of the relevant studies remain inconsistent[4, 5]. Even other study also investigated in the Chinese population, but there was no significant association between this SNP and breast cancer[1]. So, this case-control study was designed to assess the possible association between rs4919510 and susceptibility to BC in an Vietnamese population.

MATERIALS-METHOD

Study population

Blood samples of 109 breast cancer women and 103 healthy women without breast cancer were collected from Oncology Hospital, Ho Chi Minh city, Vietnam. All of the participants had received and signed the consent form which was approved by the Ethical Committee of Oncology Hospital – HCMC Vietnam under the decision number 177/HĐĐĐ-CĐT, 18th November 2014. The collected whole blood was stored in EDTA containing tubes at -20°C for further use.

DNA extraction

Genomic DNA from blood samples were extracted by salting-out method followed protocol by Hue et al. First, white blood cells are isolated from the whole blood. After treatment with various reagents. The DNA will be collected by ethanol precipitation before ethanol is also removed. Finally, the DNA pellet is resuspended in molecular or RNase-free water. Then, stored at -20°C until performing PCR reaction.

Genotyping methods

Tetra-ARMs-PCR (Tetra-primer Amplification Refractory Mutation system PCR) method was used to identify the genotypes of DNA samples in this study. It contains two primer pairs: two outer primers and two allele-specific inner primers. One inner primer is specific to only one allele of the SNP and refractory to the other allele. Consequently, the DNA polymerase will

only be able to extend a primer when its 3' end is perfectly complementary to the template. When this condition happens, a PCR amplicon is produced. The result of tetra-ARMS-PCR is analyzed by using gel electrophoresis. The DNA sequence around the SNP rs4919510 was first obtained from NCBI SNP database and used as input data to design PCR primers which amplify a fragment including interested SNP by Primer3Plus (<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>). The specificity of designed primers was checked by NCBI Primer-BLAST tool ([https://www.ncbi.nlm.nih.gov/tools/primer-](https://www.ncbi.nlm.nih.gov/tools/primer-blast)

blast) to limit any PCR undesired product. The secondary structures of primers were tested by OligoAnalyzer 3.1 software (<https://sg.idtdna.com/calc/analyzer>). The best primer set for rs4919510 is described in Table 1. In PCR, three primer pairs F1-R1, F1-R2, and F2-R1 resulted in the creation of internal control (696bp), C allele-specific product (322bp), and G allele-specific product (417bp), respectively. Therefore, heterozygote CG will give three products of 696bp, 417bp and 322bp; homozygote GG will give two products of 696bp and 417bp; while homozygote CC will give two products of 696bp and 322bp.

Table 1. Primers for tetra-ARMS-PCR analysis of SNP rs4919510.

Primer	Sequence (5'-3')	Tm (°C)
Outer F1	ATGAAGCATCAGCCCTTTCCAG	68.4
Outer R1	TTTGACATTTAAACCCCTCCTTTGA	67.0
Inner F2	GGGTGGTGTTGGGACAGCTG	70.2
Inner R2	TCTTGGAGATGCCTTTTAAACGG	67.7

The PCR reaction was performed in T100™ Thermal Cycler (Bio-Rad Laboratories, Inc.) and used HotstarTaq DNA polymerase kit (Qiagen). For all stages, the amplification protocol was as describes: pre-incubation at 95°C for 15mins, followed by 40 cycles of 95°C for 30s, 63°C for 30s, 72°C for 30s, final elongation at 72°C for 10mins and hold at 4°C. The best conditions for tetra-ARMS-PCR analysis was optimized with the heterozygous control and used for genotyping samples. The components for a 10µl reaction including 1X PCR buffer, 0.2mM dNTP, 3.0mM MgCl₂, 0.5u HotStarTaq DNA Polymerase, 0.5µM outer forward primer, 0.5µM outer reverse primer, 0.1µM inner forward primer, 0.1µM inner reverse primer, 60ng DNA and molecular water. PCR products mixed with dye were then loaded in the gel. Gel electrophoresis were then run at 90V for 30 minutes. Finally by using a Gel

imaging system (Biorad), the gel was viewed under the ultraviolet (UV) light so that bands can be visualized.

Association analysis

Hardy-Weinberg equilibrium (HWE) of the control subjects had been analyzed. HWE $P_{\text{value}} > 0.05$ was considered the HWE in sample sets. The associations between miRNA SNP and breast cancer were determined by computing the odds ratios (ORs) and 95% confidence intervals (CIs) from logistic regression analyses. All statistical tests were 2-sided, $P_{\text{value}} < 0.05$ were considered statistically significant. Finally, the power of the case-control study was evaluated. Association analysis performed using R version 3.3.4.

RESULTS

Genotyping

To determine the genotype SNP rs4919510 of total 220 samples, the optimized tetra-ARMS-PCR method was developed by finding out the suitable T_a (temperature annealing), the concentration of $MgCl_2$, primers and identifying three controls corresponding three genotypes. Figure 1 shows 3 samples predicted as controls that were analyzed on gel electrophoresis. Lane A has 3 bands indicating heterozygote CG; lane B and C have 2 bands indicating

homozygote GG and CC, respectively. These controls were also confirmed by sequencing (Figure 2). The optimal condition for genotyping reaction which was mentioned in the Method part was applied to identify the genotype of samples. 106 of case set and 101 of control set were successfully genotyped. There were 5 samples that could not identify genotypes because they had not amplified products or lack of internal control band.

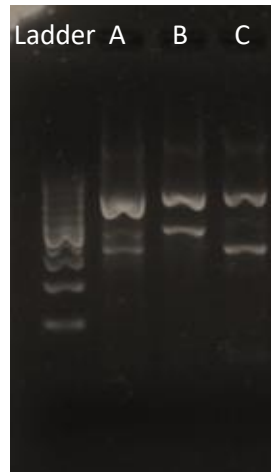


Figure 1. Gel electrophoresis result for controls

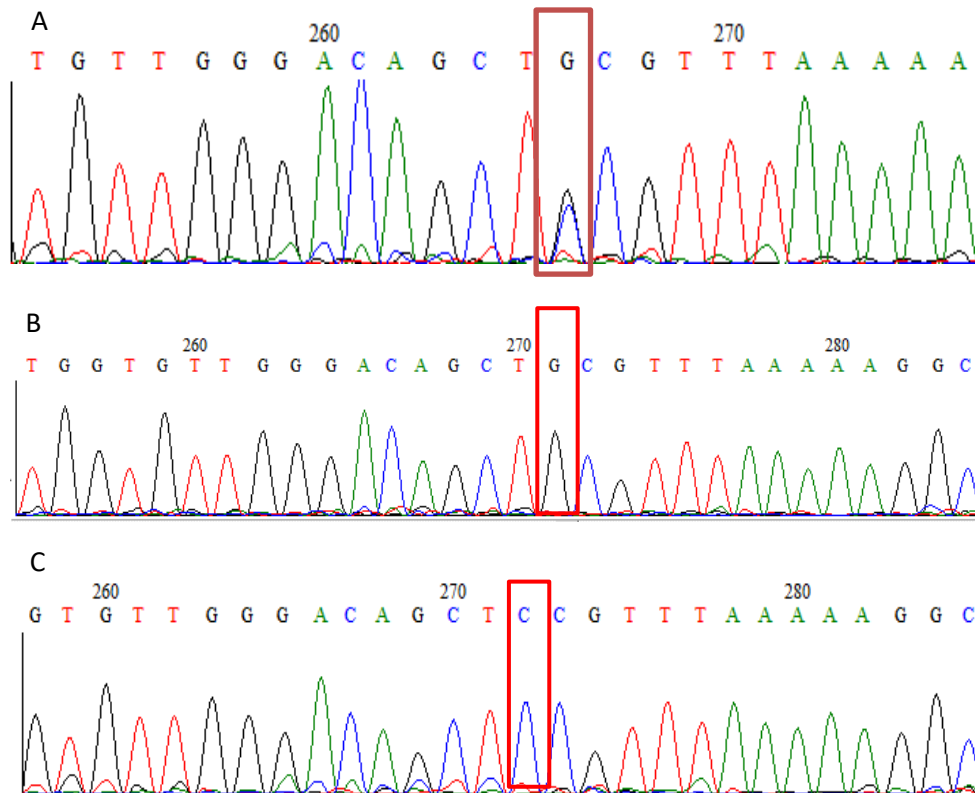


Figure 2. Sequencing results showed the exact alleles.

Alleles's frequency and Hardy-Weinberg equilibrium (HWE)

Allele and genotype frequencies were calculated based on the number of each allele and genotype that were identified by tetra-ARMS-PCR analysis (Table 2). The GG genotype and G allele were more common in the control population. The frequency of minor allele (C allele) counted for 32.1% in case set and 24.8% in control set. It is likely that the C

allele in case set appears more frequently than in control set.

HWE p-value was 0.6 in control set and 0.1 in case set, indicating that both groups were in Hardy Weinberg equilibrium (>0.05). Thus, the selected sample sets can represent the population. The association analysis of this SNP can be a reliable reflection of the relationship of this SNP and breast cancer in Vietnamese.

Table 2. Allele and genotype frequency of SNP rs419510 and HWE of sample sets

	Genotype			Allele		HWE p_value
	CC	GC	GG	C	G	
Case (106)	7 (6.6%)	54 (51%)	45 (42.4%)	68 (32.1%)	144 (67.9%)	0.1
Control (101)	7 (6.9%)	36 (35.7%)	58 (57.4%)	50 (24.8%)	152 (75.2%)	0.6

Association analysis

Table 3 shows an association of SNP rs4919510 with breast cancer. Analysis on the allelic model, revealing that the C allele may increase the risk of breast cancer 1.48-fold. However, the Chi-squared test showed 0.08 of p_value for allele difference between case and control population ($p_value > 0.05$); therefore, it suggested that SNP rs4919510 was not

associated with breast cancer risk in the Vietnamese population. In addition, genotypic association analysis showed that the number of the minor allele in genotypic model also have an effect on increasing breast cancer risk. Especially, there were significantly increased risk in heterozygote model (GC vs. GG: OR=1.93, $p=0.02$) and dominant model (CC+GC vs GG: OR=1.83, $p=0.03$).

Table 3. Association analysis of SNP rs4919510

Association analysis	Analysis model		OR	95%CI	P_value
Allelic analysis	C vs. G		1.48	0.94 – 2.32	0.08
Genotypic analysis	Additive	CC vs. GG	1.29	0.41 – 4.02	0.65
		GC vs. GG	1.93	1.09 – 3.45	0.02
	Recessive	CC vs. (GC+GG)	0.95	0.32 – 2.81	0.92
	Dominant	(CC+GC) vs. GG	1.83	1.05 – 3.17	0.03

The power analysis

The estimated values of association between SNP rs4919510 and breast cancer in our study were confirmed through computing the statistical power. With 207 samples including 106 cases and 101 controls, the power of this study was just 23.9%, which much lower than expectation (75%). One reason for the low power of the study is small sample size. To raise the power up to 50% or more and have

enough confidence to conclude the association between SNP rs4919510 with breast cancer in Vietnamese, a larger sample size was needed to be investigated. The table shows some predicted sample sizes that could be investigated to increase the power of the study. An estimated sample size of 827 cases/controls will be investigated to reach 95% of power for this study.

Table 4. Predicting power, sample size for this study

Power (%)	23.9	50	75	95
Case/Control	106/101	246/246	442/442	827/827

DISCUSSION

In this study, C allele of SNP rs4919510 was identified as a minor allele with a frequency of 24.8%. Distribution of this allele in Vietnamese population is roughly similar to its distribution in Chinese women with a frequency of 32.3%[5]. However, in a study on Iranian populations, C has a higher frequency than the G allele, therefore, C is the common allele (88.8%)[4]. The opposite result shows that the frequency of SNP rs4919510 is not the same between populations. Moreover, the previous studies investigated the association of this SNP and breast cancer, but the results were different between populations[1, 4, 5]. It suggests that association of SNP rs4919510 with breast cancer depends on the ethnic group.

Dai et al. identified that rs4919510 located in mature miR-608 was not associated with breast cancer in Chinese population[1]. Also in this cohort, other study showed that the specifically association between rs4919510 and HER2+ breast cancer was observed in the stratified population[5]. The individuals carrying G allele were able to increase the risk of breast cancer with OR of 1.62 (95% CI 1.23–22.15, $p=3.4 \times 10^{-4}$). They found that patients with GG-genotype also had larger HER2-positive tumors. In contrast, a study on Iranian population showed

that the G allele decreases the risk of breast cancer (OR=0.53, 95%CI 0.30–0.92, $p = 0.024$)[4]. Furthermore, GC genotype significantly reduced the risk of BC with OR of 0.49 (95% CI 0.28–0.88, $p=0.018$) compared to CC genotype. Our findings identified that no correlation was found between rs4919510 allelic model and BC risk. But in the analysis of additive and dominant models, we proved that SNP rs4919510 contribute to breast cancer susceptibility among Vietnamese population. The individuals who carried the GC genotype had an increased risk of BC as compared with the individuals who had GG (OR=1.93, 95%CI=1.09–3.45, $p=0.02$). Moreover, the result from dominant (OR=1.83, 95% CI=1.05–3.17, $p=0.03$) also showed that the minor allele C was associated with a increased risk of BC.

There are still some complications in our study. Although both the heterozygous and dominant models proved that C allele increased the disease risk, the CC genotype had no effect on breast cancer susceptibility ($p = 0.65$). Furthermore, when analyzing on the allelic model to determine whether C allele may contribute to breast cancer risk, the p-value was just nearly borderline ($p = 0.08$). This could be due to some limitations of the study. Because of small sample size, the BC and

control groups were not large enough, therefore, the power was not high (Power = 23.9%). If sample size was larger, when comparing T and G allele, the association might be seen more clearly. Thus, a future study needs to be conducted with a larger sample size to confirm this finding and power can be up to 95% with the 827/827 case/control set.

CONCLUSION

In conclusion, our study found significant associations between rs4919510 and the risk of breast cancer in a Vietnamese population. However, with small patient and control cohorts, the present study is the primary research screening the association of this SNP. Therefore, the future studies with larger sample size are needed to confirm these findings.

ABBREVIATIONS

95% CI: 95% confidence interval

BC: breast cancer

GWAS: Genome Wide Association Study

Tetra-ARMs-PCR: Tetra-primer Amplification Refractory Mutation system PCR

HWE: Hardy-Weinberg equilibrium

NCBI: National Center for Biotechnology Information

OR: Odd ratio

SNP: Single nucleotide polymorphism

WHO: World Health Organization

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AUTHOR CONTRIBUTIONS

HHL contributed to study design, performed the experiment, analysis, interpretation of data and wrote the manuscript. NTNT reviewed and edited the manuscript for intellectual content. Nguyen Thi Hue oriented, gave important idea and revised the manuscript of this review. All

authors gave final approval of the version to be published.

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