

The Relationship between the Mutation rs3741378 of *SIPA1* Gene and Breast Cancer in Vietnamese Women

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ABSTRACT The product of signal-induced proliferation-associated (*Sipa*) gene is a mitogen induced GTPase activating protein that is specific for Rap 1 and Rap 2 GTPase. It is related to the cell proliferation, differentiation and cell adhesion. The *Sipa1* enzyme may hamper mitogen-induced cell cycle progression when abnormally expressed and to be linked to the metastatic potential via Rap1 GTPase-activating protein, which inducing the cell adhesion. The genetic population study was performed to see the association between SNP rs3741378 of *SIPA1* genes with breast cancer disease in Vietnamese. An ARMS-PCR was designed for screening the allele C and T of SNP rs3741378 in women Vietnamese population with 50 breast cancer patients and 50 healthy persons. This preliminary study showed the association between SNP rs3741378 and the breast cancer in Vietnamese population, with the C allele as the risk allele ($P=0.006$, OR= 2.843). However, the correlation analysis did not show the association between the mutant genotypes and the disease. As using the small sample size the power of this study for the association is inadequate (46.18%). The estimated sample size to get power at 90% is 136 cases and 136 controls.

INTRODUCTION

Breast cancer is the most frequently occurring cancer and the cause of cancer related death in women worldwide (Treeck et al. 2009). Mortality rate of breast cancer occupies 23% of all kinds of cancer and increases annually in both developed and developing countries (Liang et al. 2008). Vietnam is one of the developing countries in Asia with a relatively high breast cancer incidence (18 cases per 100.000 females) and it is the most common comparing with 6 other types of cancers like stomach, prostate, lung and bronchus, liver, esophagus, colon and rectum (all of them happen in below 10 cases per 100.000 females) or the second-most common cancer disease comparing with uterine cancer (20 cases per 100.000 females) in Vietnamese women (The Burden of Cancer in Asia 2008). As other cancers, breast cancer treatment is only most effectively at the early stage whereas the last stage is not curable as it relates to metastasis. Modified factors also related to lower risk of breast including breast feeding, moderate or vigorous physical activity, and maintaining a healthy body weight (Cancer Facts and Figures 2012). Furthermore, genetic factors are considered as significantly important factors because they are associated with metastasis which cannot be treated effectively. Many researches involved in detection of the causative gene of breast cancer

have been performed. In the early 1990s, *BRCA1* and *BRCA2* gene were considered as the two susceptibility genes of breast cancer by using the linkage analysis of families (Hall et al. 1990; Miki et al. 1994) followed by many genetic researches demonstrated that other genes can confer susceptibility to this disease (Wooster et al. 1994, 1995). Potential causative genes consist of *SLC4A7* and *NEK10* on chromosome 3q and *COX11* on chromosome 17q (Ahmed et al. 2009). Fletcher and his colleagues found that five SNPs in *ATM* (S49C, S707P, F858L, P1054R, and L1420F) have been related to a small increased risk of 0.03 % breast cancer in family (Fletcher et al. 2010). Other researches indicated that genetic variants in fibroblast growth factor receptor 2 (*FGFR2*) may significantly involve in breast cancer in Chinese women through the estrogen and progesterone pathways (Liang et al. 2008). *CTLA-4* gene may contribute to the breast cancer susceptibility in Chinese Han women (Wang et al. 2007). Many genetic variants (rs2046210, rs3803662, rs7484226, *CASP10* rs13010627 missense SNP, *PGR* rs1042838 missense SNP, *BID* rs8193015 missense SNP, *ESR2* rs2987983 in promoter – China, *SIPA1* SNP rs3741378 missense) have been shown to be at high risk of breast cancer in Chinese population and other populations. Among these gene factors, the novel breast cancer signal-induced proliferation associated 1 (*SIPA1*) gene has recent-

ber: NC_000011) *SIPA1* gene consists of 16 exons in which some parts are untranslated, included exon 1, 91 bp of exon 2 and 205 bp at the 3' end of exon 16. SNP rs3741378 (C/T) is located at position 65408937 within the exon 3 of *SIPA1* gene on chromosome 11. The general structure of *SIPA1* gene and the position of the missense are showed in Figure 1. SNP rs3741378 is located in the exon 3 of *SIPA1* gene, namely in the coding region. The *Sipa1* protein consists of three domains which are Rap GTPase-activating (GAP), PDZ and LZ. *Sipa1* exhibits a GAP that is specific for RAP1 and RAP1. Both these factors are member of Ras family of GTPase and are involve in cell proliferation, cell differentiation and cell adhesion. PDZ is a protein-protein interaction domain of *Sipa1*. LZ likes domain which resembles myosin tail.

SNP rs3741378 in the coding region was identified from NCBI SNP database (accession num-

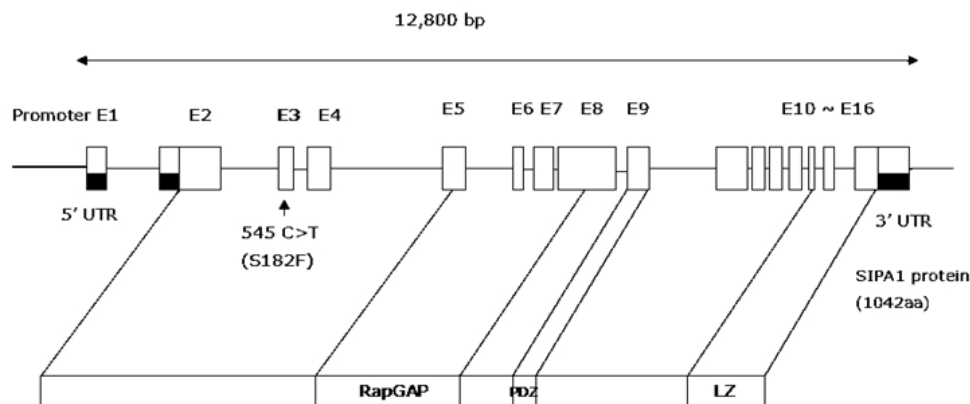


Fig. 1. General structure of *SIPA1* gene and location of SNP rs3741378.

cancer development and heterozygous may help protecting against breast cancer incidence (Hsieh et al. 2009). According to the results, it indicated that this polymorphism in women with breast cancer has a higher C allele frequency. Hsieh's finding is consistent with early researches showed that the C allele is highly associated with the breast cancer incidence (Hsieh et al. 2009). Thus, our study will prove that the polymorphism rs3741378 may play an important role in the breast cancer risk. The aim of this study is to determine the relationship of SNP rs3741378 of *SIPA1* susceptibility gene and the breast cancer in Vietnamese women.

MATERIAL AND METHODS

Samples Collection

This study was performed at the laboratory of International University – Vietnam National University, Ho Chi Minh City. The present analysis used the data from case-control study conducted under the approval of the Ho Chi Minh City Oncology Hospital and International University Committees. Blood samples of breast cancer case were provided by Ho Chi Minh City Oncology Hospital in a randomized manner. Control samples were collected from the volunteer students in International University, and used as the population control.

Bloods plasma was discarded and the pellet was stored in the freezer (-20°C) for later experiments. The population screened consists of 50 female individuals with breast cancer and 50 healthy volunteer individuals. All consent forms were obtained from the individuals admitted into the study.

DNA Extraction

Genomic DNA was extracted by salting-out method (Cardozo et al. 2009). Genomic DNA was extracted from 500µl whole blood by adding 1000µl Cell Lysis Buffer (10mM Tris HCl, 11%, w/v Sucrose, 1%, v/v Triton X-100 and 5mM

MgCl₂, pH 8) followed by centrifugation at 6000rpm in room temperature for 2 minutes, in which supernatant was discarded. This step was carried out two more times. Pellet then was re-suspended in 300 µl Nuclei Lysis Buffer (10mM Tris HCl, 1%, w/v SDS, 10mM EDTA, 10mM Sodium acetate, pH 8). Saturated NaCl (3M) and Chloroform with the volume 100 µl and 600 µl respectively were added to the lysates and inverted gently. After centrifugation at 6000 rpm for 2 min at room temperature, the upper phase was taken out in a new micro-centrifuge tube and precipitated in 600 µl absolute Ethanol. Then the sample was washed by Ethanol 70% which followed by air-dry, then DNA was collected by adding 100µl dH₂O.

ARMS-PCR Assay

ARMS-PCR (amplification refractory mutation system) is a general technique for the analysis of any point mutations. To control the activity of the PCR reactions, in ARMS-PCR beside a specific primer set, one more extra set of primers was used to amplify a control fragment from a house keeping gene (Table 1). In this case, the HGH gene is used. PCR was carried out by reaction containing 1 µl of specific primers 10µM, approximately 50ng DNA, 0.5 µl of pure dimethyl sulfoxide (DMSO), 0.35µl of primer IU7 10µM, 0.35 µl of primer IU8 10µM (amplify the house keeping gene), and 12.5 µl Toptaq Master Mix (1.25 units of Toptaq DNA polymerase, Qiagen, Duesseldorf, Germany) which finally filled up to 25µl by RNase free water (from Toptaq Master Mix kit, Qiagen, Germany). PCR was run by using Mastercycler® ep realplex (Canada). PCR program was set up as 94°C for 3 min followed by 30 cycles of 94°C for 30s, at annealing temperature for 30s and 72°C for 1min; finally a final extension was applied at 72°C for 3 min. PCR products were analyzed by gel electrophoresis with 1.5% concentration of agarose gel containing 1 µg/ml ethidium bromide and screened under ultra-violet illumination. The desired band which represented for allele specific for SNP

Table 1: PCR primers used for *SIPA1* gene SNP analysis

Control (HGH gene)	Forward primer (IU7)	5'-AGGTCGTGTCCCCAGCCACT-3'
	Reverse primer (IU8)	5'-GCACAGACACTGCCCCAGGC-3'
SNP rs3741378	Wild-type Forward primer (F-C)	5'-GCCTGGGTGGACCAGCATC-3'
	Mutation Forward primer (F-T)	5'-GCCTGGGTGGACCAGCATT-3'
	Reverse primer-R	5'-GCGGTAGTAGCCAGCACCC-3'

rs3741378 was at 134bp. Control band is expected at 641bp.

Statistical Analysis

The significant differences between the case and control populations in allele and genotype frequencies were evaluated by Chi-square method (Griffiths 2000). Hardy-Weinberg equilibrium was carried out to determine where the population is in Hardy-Weinberg equilibrium (Salanti et al. 2005). The population was equilibrium if the p value is less than 0.05 (Salanti et al. 2005). Odds ratio was calculated. Statistical test for association (C.I.: 95% confidence interval) and for significant were performed using the SPSS version 16.0. The less frequency allele was considered as the variant allele ($P < 0.05$). To estimate the value of cases-control analysis, the power calculation is estimated based on the sample size (Hong et al. 2012) and the odds ratio (OR) between risk allele and normal allele by using the <http://sampsiz.sourceforge.net/iface/s3.html> software.

RESULTS

PCR Optimization

The effects of annealing temperature on PCR performance are shown in the Figure 2. The amplification reactions were performed with different annealing temperature but all other PCR parameters was the same. With the calculated primer annealing temperature at 70.5°C; the gradient T_m for optimization was ranged from 65°C – 72°C. High yields of specific PCR products were ob-

tained over a wide range of annealing temperature. Figure 3 shows an agarose gel with the sample that was loaded. Clearly, all of the bands in the gel have the relatively same brightness. However, the bands at $T_a = 72^\circ\text{C}$ has no extra bands. Therefore, the best annealing temperature for PCR reaction is 72°C.

The first lane is 100 bp ladder. The upper bands are internal control at 641 bp and the lower bands are desired products at 134bp. For each sample, the left well is for T allele and the right well is for C allele. The gradient PCR with the range of T_a from 65°C to 72°C.

Identifying Heterozygote Control Sample

Screening for 10 patient samples by the optimal PCR condition, the sample appeared desired band in both reaction of ARMS PCR were identified as the heterozygote samples (Fig. 3). One of the heterozygote samples was sequenced to confirm the genotype for mutation rs3741378. The result of sequencing showed that the heterozygote sample contained two alleles C and T of SNP rs3741378 (Fig. 4). This sample was then used as the control sample for further genotyping.

The heterozygote sample with desired band at 134bp in both reactions of ARMS PCR (allele T and allele C are present).

The double peaks at the position 494 showed the presence of two alleles C and T of SNP rs3741378.

Genotyping

The optimal PCR condition and the control samples were used for genotyping 50 cases and

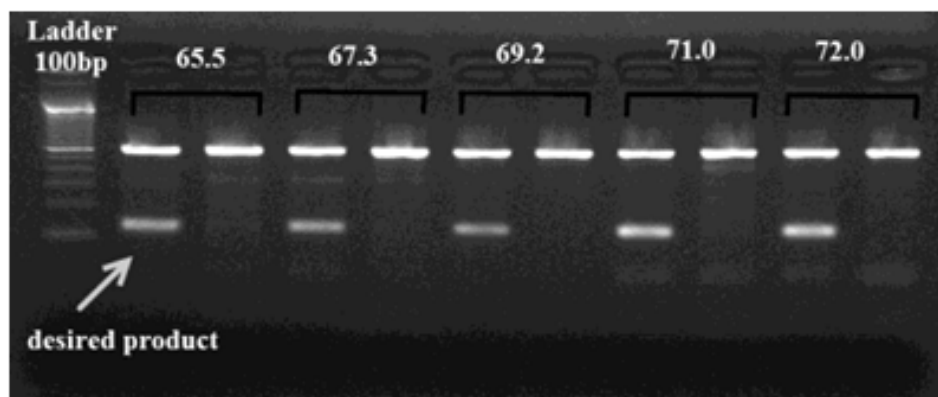


Fig. 2. Optimization of T_a for designed primers

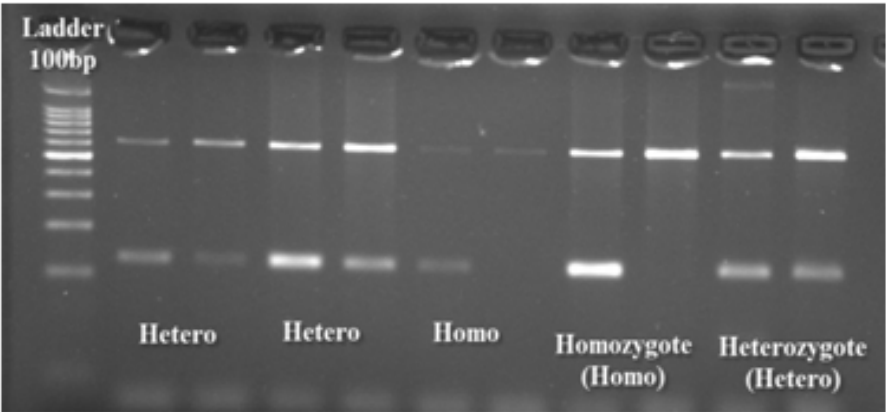


Fig. 3. The primary screening in the patient samples.

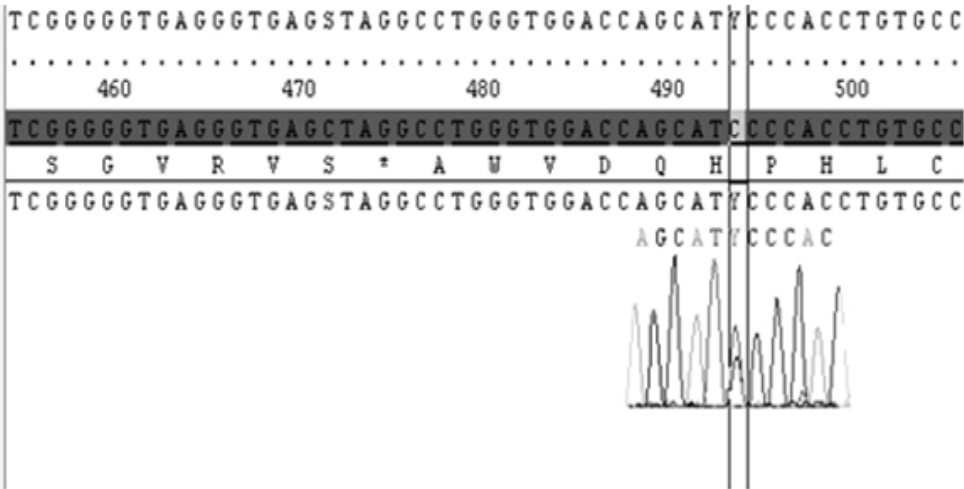


Fig. 4. Confirming the heterozygote sample by sequencing.

50 control samples An agarose gel analysis for genotyping was shown in Figure 5. The presence of normal and mutated allele was recognized by the bands at the size 134 bp. The upper bands at 641bp was always shown in the gel indicated that PCR results is believable.

The upper bands are internal control at 641bp. The lower bands at 134bp are desired bands.

The genotype and allele frequencies of the *SIPA1* gene SNP in breast cancer patients and healthy controls are shown in Table 2. There are three types of genotypes: CC, CT and TT that appear in the results of genotyping. The results

showed that frequency of each genotype is quite different. The frequency of CT and TT genotype in the case group are lower than in the control group. In contrast, the frequency of CC genotype in the case group is higher than in the case group (84% vs. 60%) (Table 2). This table was also presented by the significant difference between the frequencies of individual allele. In more detail, the difference between the frequencies of C and T allele in the case group is much higher than the difference between the frequencies of these two alleles in the control group. This means that the C allele is associated with the disease or C allele is a risk allele.

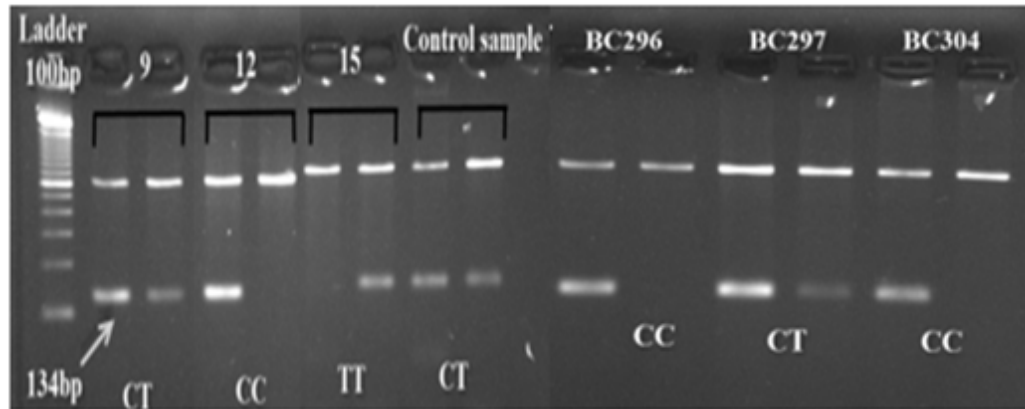


Fig. 5. Genotyping result for mutation rs3741378 of *SIPA1* gene in the Control group (healthy individuals) and in the Breast Cancer group (patients).

Table 2: Genotype/allele frequency for rs3741378

	Cohort	Genotypes		Alleles		
		CC	CT	TT	T	C
Cases	Observed	42 (84%)	5 (10%)	3 (6%)	11%	89%
	Expected	39.605 (79%)	9.79 (19.5%)	0.605 (1.5%)		
Controls	Observed	30 (60%)	14 (28%)	6 (12%)	26%	74%
	Expected	27.38 (55%)	19.24 (38%)	3.38 (7%)		

Testing for the distribution of allele in the population the Hardy Weinberg was performed. This analysis was conducted in both cases and controls where normally only performed in control group as in the control group, the other factors leading disease are consider have no effect. By analyzing in cases and control the result demonstrated the disequilibrium in cases but not in controls. This disequilibrium showed in HW-probability=0.00054 in cases and equilibrium in control with HW-probability= 0.054 as it is higher than 0.05 (not shown).

Association Analysis

The Pearson Chi-square was performed based on the data in Table 2 using SPSS version 16 to test genetic association of case-control study with the hypothesis that the control data is the normal distribution. In the case group the CC genotype is more common than in control group (84% vs 60%). The result also showed

that CT genotype and TT genotype in the case group is lower than in the control group (Table 2). In more detail, the analysis was performed to see the association between alleles or genotypes and the disease. Comparing frequency of allele C and T in the population between cases and control showed that it is significant difference with $p=0.006$ (Table 3). As the frequencies of C allele in cases are higher than in control, C allele is considered as the risk allele. The genotype CC is considered as the risk genotype. However, the comparing between normal genotype and heterozygote CT genotype which contains 1 risk allele, this result showed no significant difference with odds ratio 0.701 in the C.I = [0.128-3.995]. Comparing normal genotype with genotypes containing risk allele (CC and CT), the result showed no significant difference also with odds ratio 2.800 in the C.I = [0.648-12.092] ($p=0.155$) (Table 3). Comparing between the normal genotype (TT) and genotypes containing risk allele (CC+ CT) was showed no significant dif-

ference ($p=0.295$) (Table 3). Thus, in general the C allele showed the association with the disease however genotype CC and genotype CT did not show the association with the disease.

DISCUSSION

The objective of this research is to study the association between rs3741378 mutation and the breast cancer in Vietnamese. Screening frequency of this mutation on the Vietnamese population of breast cancer and comparing this frequency between populations and disease were carried out to confirm the role of genetic mutations in causing breast cancer in the Vietnamese community. The results of this study not only broaden the research and development of diagnostic methods and early diagnosis of breast cancer risk in Vietnamese but also provide the information for the field of cancer research over the world. From that, the development of diagnostic methods and treatment as well as cancer prevention are contributed so as to improve quality of life for people. The *SIPA1* gene was correlated to the metastatic capacity in the tumor cell line in mouse study and metastatic progression in human prostate cancer ($p = 0.001$) (Park et al. 2005). *SIPA1* may have other effect to the cancer characteristic such as the estrogen and progesterone pathways ((Park et al. 2005). Some previous studies have determined the association of *SIPA1* SNPs and breast cancer risk (Hsieh et al., 2009; Gaudet et al. 2009). In recent studies, some selected *SIPA1* SNPs were examined in the Australian population. One of them, SNP rs3741378, has been shown the relationship with the breast cancer incidence. In this study the allele C of SNP rs3741378 was also showed the association with breast cancer in Vietnamese ($p=0.006$). However, a recent study from Gaudet et al, reported a different trend in which an increased risk of breast cancer was associated with TT genotype of SNP rs3741378 (Gaudet et al. 2009). Thus, the question is whether the association between this SNP and the

breast cancer in Polish, British, Vietnamese completely different. It may be happened and this will be very helpful for development of diagnosis which very specific for each population. However, logically when the C allele associated with the disease, the homozygote CC genotype and the heterozygote CT genotype should also associated with the disease (Tsai 2005) in this study, the association between the homozygote CC or the heterozygote CT and the disease was not determined. This may due to the sample size is too small only 50 cases and 50 controls, and they are not represented for the whole Vietnamese population.

In this study, the power for case-control study is performed based on the genotyping result to evaluate the reliability of association result of SNP rs3741378 and disease. The data showed that the estimated power of this study with sample size of 50 cases/50 controls was only about 46.18% (OR=2.8). Thus, the analysis result about the CC and CT genotypes do not associate with the disease is still not true. This result may be affected by the small sample size. The HWE testing for the control group with $p=0.054$ showed that there is a disequilibrium distribution of allele in the control also while this disequilibrium only expected to see in the cases. This result indicated that the small sample size has a big effect on the association analysis. The control group is inadequate for the association analysis or for the study. This again indicated that the result of association analysis is affected by the small sample size. Based on the frequency of alleles in the population and the OR of the previous analysis and if the power of study is required for 90%, the sample size should be increased up to 136 cases/ 136 controls by using Sample size calculation which created by Glaziov (2005). If the association between C allele and the disease is true, logically the CC and CT genotype also associated with the disease. However, in some cases only homozygote CC shows the association and the heterozygote do not need to associate. In this study, neither CC

Table 3: Association between allele and genotypes and the disease.

Association (C= risk allele)		Odds ratio	95% CI	χ^2	p value
Allele	[C] ↔ [T]	2.843	C.I.=[1.317-6.135]	7.46	0.006
Heterozygote	[CT] ↔ [TT]	0.714	C.I.=[0.128-3.995]	0.15	0.701
Homozygote	[CC] ↔ [TT]	2.800	C.I.=[0.648-12.092]	2.02	0.155
Allele positivity	[CC] + [CT] ↔ [TT]	2.136	C.I.=[0.503-9.068]	1.10	0.295

nor CT shows the association. Thus, this needs to be confirmed by a bigger sample size.

CONCLUSION

This study reported the primary frequency of SNP of *SIPA1* gene in Vietnamese women. Our data suggest that the *SIPA1* gene may be related to the susceptibility to and progression of breast cancer in Vietnamese women. The allele C of SNP rs3741378 is the risk allele, which may relate to the development of breast cancer in Vietnamese. The association between genotypes and the disease has not been demonstrated due to the small sample size. To confirm the association between rs3741378 and the breast cancer in Vietnamese the bigger sample size is need to be screened. Additionally, there are many another single polymorphism nucleotide of *SIPA1* may relate to breast cancer. Further study should be carried out to examine the association of these SNPs and breast cancer risk in Vietnamese women and find out which SNP is frequent in breast cancer patients.

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