RESEARCH ARTICLE

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Gene Combination of *CD44 rs187116*, *CD133 rs2240688*, *NF-κB1 rs28362491* and *GSTM1* Deletion as a Potential Biomarker in Risk Prediction of Breast Cancer in Lower Northern Thailand

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Abstract

Background: Biomarkers play an important role in oncology, including risk assessment, treatment prediction, and monitoring the progression of disease. In breast cancer, many genes are used as biomarkers. Since, several SNP variations of hallmark – related genes have been reported to be of value in risk prediction in various cancers and populations, some genetic polymorphism loci were combined and reported as biomarkers for use in the risk assessment of breast cancer in Thai people. **Methods:** Twelve cancer gene hallmarks (15 polymorphic loci) were selected and genotyped in 184 breast cancer patients and 176 healthy individuals in Phitsanulok, Thailand. **Results:** AA genotype of *CD44 rs187116* (c.67+4883G>A), the C allele of *CD133 rs2240688* (c.*667A>C), the *2 allele (4 bp deletion) of *NF-κB1 rs28362491* and the homozygous null allele genotype of *GSTM1* were significantly associated with an increased risk of breast cancer ($p<0.05$). A combination of these 4 significant loci showed that AA-AA-*1*1-homozygous null allele genotype has the greatest correlation with increased risk of breast cancer (OR = 21.00; 95% CI: 1.77 to 248.11; p = 0.015), followed by GA-AA-*2*2- homozygous null allele genotype (p = 0.037) and GG-AC-*1*2- homozygous null allele genotype (p = 0.028). **Conclusion:** These findings suggest that the polymorphisms of *CD44 rs187116* (c.67+4883G>A), *CD133 rs2240688* (c.*667A>C), *NF-κB1 rs28362491* and *GSTM1* homozygous null allele genotype might be associated with an increased risk of breast cancer, and this gene combination could possibly be used as biomarkers for risk prediction, which would be of benefit in planning health surveillance and cancer prevention.

Keywords: Breast cancer- cancer surveillance- genetic biomarker- polymorphism

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Introduction

Breast cancer (BCA) is the most common form of cancer in women (Bray et al., 2018; NCI, 2017). Age, gender, estrogen, family history, gene mutation and unhealthy lifestyles are risk factors for this cancer (Sun et al., 2017). In 2011, Hanahan and Weinberg described the occurrence and progression of cancer, known as the hallmarks of cancer (Hanahan and Weinberg, 2011). These include evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death, deregulating cellular energetics, and sustaining proliferative signaling (Hanahan and Weinberg, 2011). These characteristics result from an abnormality of regulatory genes, such as *VEGF* genes that induce angiogenesis (Hoeben et al., 2004; Carmeliet, 2005), or *GSTM1*, *GSTT1*, and *NF-κB1* genes which induce the inflammation of tumor cells (Kim et al., 2006; Tang et al., 2010; Espın-Palazon and Traver, 2016). Other abnormalities in caspase 8 and caspase 9 genes could affect the death of cells (McIlwain et al., 2013); $TGF\beta_2$ gene can induce cell multiplication via proliferative signaling (Villapol et al., 2013); tumor suppressor gene (*FOXO3*) and proto-oncogene (*MDM2*) could induce cells to evade the growth suppressors (Essaghir et al., 2009; Urso et al., 2016). Cancer stem cells (CSCs) are a factor in cancer occurrence (Al-Hajj et al., 2003; Bozorgi et al., 2015). These cells were recognized as the key drivers of tumor development and progression, including tumor initiation, promotion, and metastasis which regulated cross-talks with tumor microenvironments in breast cancer (Ayob and Ramasamy, 2018; Feng et al., 2018). In order, to identify the CSCs, cell surface phenotypes such as *CD24*, *CD44*, *CD90*, *CD117*, *CD133*, should be checked (Schatton et

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al., 2009).

The molecules that might be used for predicting the occurrence of cancer, known as biomarkers, are DNA, mRNA, enzymes, metabolites, transcription factors, and cell surface receptors (Wu and Qu, 2015). Guidelines from the European Group on Tumor Markers (EGTM) reports that estrogen receptors (ERs), progesterone receptors (PRs), and human epidermal growth factor receptor 2 (HER2) are often used as breast cancer biomarkers (Duffy et al., 2017). The expression levels of *ALDH1*, *CD24*, *CD44*, and *CD133* in breast cancer stem cells can also be used as biomarkers to detect solid tumors. (Jiang et al., 2012; Medema, 2013). The expression of these molecules almost always results from an abnormality in the genes. Mutations or polymorphisms in the gene sequences affected the cancer occurrence, progression, and susceptibility.

Single nucleotide polymorphisms (SNPs) and insertion-deletion polymorphisms (indel) have potential to indicate risk factors and susceptibility of lung, gastric, and breast cancer (Tan et al., 2010; Park et al., 2012; Eskandari-Nasab et al., 2016; Liu et al., 2016; Deng et al., 2017; Jia et al., 2017). For instance, the *rs13347* (c.2392C>T) of *CD44* was reported as a predictor marker for breast cancer risk and prognosis (Jiang et al., 2012; Lin et al., 2018). Indel polymorphism of *GSTM1* was found to be associated with breast cancer risk in Chinese and Mexican people (Soto-Quintana et al., 2015; Xue et al., 2016). However, *GSTM1* genotypes were found to have no association with cancer susceptibility in Thai women (Pongtheerat et al., 2009).

The genetic background of populations play an important role in cancer risk and susceptibility, but there are no reports on the genetic variations in Thais. In this study, we selected 15 polymorphic loci from 12 genes relating to the hallmarks of cancer and cancer stem cell markers. We aimed to find candidate genes which are associated with breast cancer in Thai people from the lower Northern region and that could be used as biomarkers for breast cancer risk prediction, health surveillance, and cancer prevention planning.

Materials and Methods

Blood samples

Blood samples from 184 primary breast cancer patients and 176 healthy individuals, were collected by oncologists from Buddhachinaraj Phitsanulok Hospital, Phitsanulok, Thailand. Genomic DNA was isolated from whole blood by AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea), according to the manufacturer's protocol, and then the DNA concentration was measured by NanoDrop 2000 UV-Vis spectrophotometer (Thermo scientific, US). This project was approved by Naresuan University Research Ethics Committee No. 579/2017.

Genotyping

This study used several methods to analyze the genotypes. Polymerase chain reaction (PCR) was used for analyzing the genotypes of detoxification genes, *GSTM1*, and *GSTT1*. Each reaction contained DNA templates (2 – 5 ng/µl), 2X HS Taq Master Mix (Bioline, Canada), forward and reverse primers (Table 1), sterile water, and used the Albumin gene (*ALB*) as a positive internal control. *MDM2* genotypes were analyzed by amplification refractory mutation system-polymerase chain reaction (ARMS–PCR). The DNA template (2 – 5 ng/µl), 2X HS Taq Master Mix (Bioline, Canada), 5 µM of each forward and reverse primers, and sterile water making up the total volume of 10 µl are contained in the reaction. For amplifying *ALDH1*, *TGFβ2*, *caspase 8*, *caspase* 9, *NF-κB1*, and *VEGF*, PCR with 6-FAM fluorescence dye labeled specific primers were used. The purified PCR products were analyzed by fragment analysis, in a 96-well plate. The reaction contained 1 µl of PCR products, 0.5 µl of GeneScan™ 600 LIZ™ Dye Size Standard (Thermo scientific, US), and 8.5 µl of HiDi formamide (Thermo scientific, US), for a total volume of 10 µl. The genotypes were determined by a Fragment analyzer, ABI 3130 (Thermo scientific, US). The fluorescence of each well was analyzed automatically by Applied Biosystems software v2.2.2 (Thermo scientific, US).

CD44 rs187116, and *CD133 rs3130* were analyzed by polymerase chain reaction -restriction fragment length polymorphism (PCR-RFLP). DNA templates were amplified with primers, as shown in Table 1. Afterwards, the PCR products were cut by restriction enzymes. The enzymatic digestion followed the manufacturer's protocol, and the fragments were analyzed by using 2% agarose gel electrophoresis.

CD44 rs13347, *CD44 rs4756196*, *CD133 rs2240688*, and *FOXO3 rs2802292* were analyzed by TaqMan SNP Genotyping (Thermo scientific, US). Each reaction contained DNA template (2 – 5 ng/µl), 2X HS Taq Master Mix (Bioline, Canada), 40X TaqMan probe and primers, and sterile water. The conditions of the manufacturer's procedure were carefully observed.

Statistical Analysis

The association between the genetic variations and the risk of breast cancer, was calculated by using MedCalC's odds ratio calculator online software (https://www.medcalc.org/calc/oddsratio.php) giving an odds ratio (OR), 95% confidence interval (95% CI), and P – value at the significant level $p < 0.05$. Hardy-Weinberg equilibrium (HWE) analysis of 15 polymorphic loci was performed by using online calculator (http://www.oege.org/software/hwe-mr-calc.shtml) (Rodriguez et al., 2009) and simple calculator of Hardy-Weinberg equilibrium from Laboratory of Immunogenomics and Immunoproteomics, Department of Pathological Physiology, Faculty of Medicine and Dentistry, Palacky University, Czech Republic (http://www.dr-petrek.eu/documents/HWE.xls).

Results

Among the 12 genes studied, 15 polymorphic loci were genotyped to attempt to establish breast cancer biomarkers in the Thai population. The results showed that *CD44 rs187116* (c.67+4883G>A), *CD133 rs2240688* (c.*667A>C), *NF-κB1 rs28362491*, and *GSTM1* were associated with the risk of breast cancer (Table 2).

Table 1. Genotyping Methods, Primer Sequences and Product Size of the Genes

Genes	Genotyping methods	Primer sequence (5' -> 3')	PCR product (bp)	Reference
ALDH1A1	Fragment analysis	F: 5′ 6 FAM - GCACTGAAAATACACAAGACTGAT 3′ R: 5′ AGAATTTGAGGATTGAAAAGAGTC 3′	HWT 213 HDL 196 HET 213, 196	Spence et al., 2003
Caspase 8 (rs3834129)	Fragment analysis	F: 5' 6 FAM - AACTTGCCCAAGGTCACGC 3' R: 5' TGAGGTCCCCGCTGTTAA 3'	HDL 96 HIS 103 HET 103, 96	Kuhlmann et al., 2016
Caspase 9 (rs4645982)	Fragment analysis	F: 5' 6 FAM - CGTTGGAGATGCGTCCTGCG 3' R: 5' CGCCCTCAGGACGCACCTCT 3'	HDL 237 HIS 257 HET 257, 237	Park et al., 2006
CD44 rs187116 G>A	PCR - RFLP (MspI*)	F: 5' CTTTCGCAAGAACCACTTCC 3' R: 5' AGGTGGTTGGAGATCACCTG 3'	HWT 93, 60 HVA 153 HET 153, 93, 60	Winder et al., 2011
CD44 rs13347 C>T	TaqMan probe	Commercial kit	-	Thermo scientific, US
CD44 rs4756196 A>G	TaqMan probe	Commercial kit	-	Thermo scientific, US
CD133 rs3130 T>C	PCR - RFLP (EcoRI*)	F: 5' GTCGCTGGATCTACTCAAGGA 3' R: 5' ACCTGCGTAACTCCATCTGA 3'	HWT 527 HVA 404, 120 HET 524, 404, 120	this study
CD133 rs2240688 A>C	TaqMan probe	Commercial kit	-	Thermo scientific, US
FOXO3 rs2802292 T>G	TaqMan probe	Commercial kit	-	Thermo scientific, US
GSTM1	PCR	F: 5' GTTGGGCTCAAATATACGGTGG 3' R: 5' GAACTCCCTGAAAAGCTAAAGC 3'	Present 215 Absent Null	Hezova et al., 2012
GSTT1	PCR	F: 5' TTCCTTACTGGTCCTCACATCTC 3' R: 5' TCACCGGATCATGGCCAGCA 3'	Present 480 Absent Null	
NF-κB1 rs28362491	Fragment analysis	F: 5' 6 FAM - TGGGCACAAGTCGTTTATGA 3' R: 5' CTGGAGCCGGTAGGGAAG 3'	HWT 281 HDL 277 HET 281, 277	Gautam et al., 2017
MDM2 SNP309 rs2279744 T>G	ARMS - PCR	F1: 5' GGGGGCCGGGGGCTGCGGGGCCGTTT 3' R1: 5' TGCCCACTGAACCGGCCCAATCCCGCCCAG 3' F2: 5' GGCAGTCGCCGCCAGGGAGGAGGGCGG 3' R2: 5' ACCTGCGATCATCCGGACCTCCCGCGCTGC 3'	HWT 224, 122 HVA 224, 158 HET 224, 158, 122	Zhang et al., 2006
TGFB2	Fragment analysis	F: 5' 6 FAM - GAAGCCTTCCCTTCTAGAGCA 3' R: 5' CGCCCTGACAACAGTGATTTA 3'	HWT 146 HDL 142 HET 146, 142	Beisner et al., 2006
VEGF rs35569394	Fragment analysis	F: 5' 6 FAM - AAGATCTGGGTGGATAATCAGACT 3' R: 5' AACTCTCCACATCTTCCCTAAGTG 3'	HWT 185 HDL 168 HET 185, 168	Rezaei et al., 2016

*Restriction enzyme; HWT, Homozygous wildtype; HVA, Homozygous variant; HET, Heterozygous; HDL, Homozygous deletion; HIS, Homozygous insertion

CD44 rs187116, the homozygous variant (AA) was significantly associated with an increased risk (OR = 2.03; 95% CI: 1.02 to 4.02; p = 0.041) when compared to the homozygous wildtype (GG). Significant association of *CD133 rs2240688* was found not only in C allele (OR = 1.46; 95% CI: 1.03 to 2.07; p = 0.032), but also in the recessive model (CC + AC) (OR = 1.57; 95% CI: 1.03 to 2.41: p = 0.034). The 4 - base pair deletion of *NF-κB1 rs28362491* and the homozygous null allele of *GSTM1* were associated with increasing risk of breast cancer. According to *NF-κB1*, the odds ratio of the homozygous deletion (*2*2 genotype) was 1.95 (95% CI: 1.02 to 3.72; p = 0.04) and *2 allele was 1.36 (95% CI: 1.00 to 1.84; p = 0.046). For *GSTM1*, the odds ratio was 1.83 (95% CI: 1.20 to 2.79; p = 0.005). Chi-square (χ^2) was used to analyze HWE. The results showed that the observed genotype frequencies of each locus did not significantly deviate from their expected frequencies, indicating that the population of this study is of infinitely large size and in accordance with an ideal population in Hardy-Weinberg. The HWE of the detoxification genes could not be calculated (Table 2).

Four significant associated loci, *CD44 rs187116G*, *CD133 rs2240688A*, *NF-κB1 rs28362491*, and *GSTM1*, were combined to obtain the candidate genotypes that tended to be associated with a risk for breast cancer. As shown in Table 3, the AA-AA-*1*1- homozygous null allele combination showed the most significant association with an increased risk of breast cancer (OR = 21.00; 95% CI: 1.77 to 248.11; p = 0.015), followed by GA-AA-*2*2-homozygous null allele (OR = 9.00; 95% CI: 1.14 to 71.04; p = 0.037) and GG-AC-*1*2-homozygous null allele (OR = 8.00; 95% CI: 1.24 to 51.50; p = 0.028). The variant genotype, AA-CC-*2*2-homozygous null allele, was not found in this combination.

Discussion

Breast cancer biomarkers play an important role in predicting the progression of tumors, effective treatments, and risk assessments. Mutation of some genes, including *BRCA1*, *BRCA2*, *CD44* and *CD133* are said to be associated with an increased risk of breast cancer (Tulsyan et al., 2013; Mehrgou and Akouchekian., 2016), but the association of these genes with disease has not been comprehensively investigated in a Thai population.

Twelve genes, 15 loci were divided into 7 groups of genes that relate to hallmarks of cancer. The group of detoxification genes (*GSTM1* and *GSTT1*) and an inflammatory gene (*NF-κB1*) are related to tumor-promoting inflammation, while the growth factor genes (*TGFβ2* and *VEGF*) are related to inducing angiogenesis and sustaining proliferative signaling. The

Table 2. Associations between the Groups of Interested Genes and the Risk of Breast Cancer

Genes	Total (n = 360) n, (%)	Patients (n = 184) n, (%)	Controls (n = 176) n, (%)	Odds ratio (95% CI)	P - value (<0.05)
Cancer stem cell marker genes					
CD44					
rs187116 G>A					
Genotypes					
GG	141 (39.17)	71 (38.59)	70 (39.77)	1.00 (reference)	
GA	170 (47.22)	80 (43.48)	90 (51.14)	0.87 (0.56 to 1.37)	0.562
AA	49 (13.61)	33 (17.93)	16 (9.09)	2.03 (1.02 to 4.02)	0.041#
AA + GA	219 (60.83)	113 (61.41)	106 (60.23)	1.05 (0.68 to 1.60)	0.817
Alleles					
G	452 (62.78)	222 (60.33)	230 (65.34)	1.00 (reference)	
A	268 (37.22)	146 (39.67)	122 (34.66)	1.23 (0.91 to 1.67)	0.164
HWE $\chi^2 = 0.04$, p = 0.84					
rs13347 C>T					
Genotypes					
CC	157 (43.61)	82 (44.56)	75 (42.61)	1.00 (reference)	
CT	162 (45.00)	83 (45.11)	79 (44.89)	0.96 (0.61 to 1.49)	0.858
TT	41 (11.39)	19 (10.33)	22 (12.50)	0.78 (0.39 to 1.57)	0.502
TT + CT	203 (56.39)	102 (55.34)	101 (57.39)	0.92 (0.60 to 1.40)	0.709
Alleles					
C	476 (66.11)	247 (67.12)	229 (65.06)	1.00 (reference)	
T	244 (33.89)	121 (32.88)	123 (34.94)	0.91 (0.66 to 1.24)	0.558
HWE $\chi^2 = 0.01$, p = 0.94					
rs4756196 A>G					
Genotypes					
AA	179 (49.72)	84 (45.65)	95 (53.98)	1.00 (reference)	
AG	150 (41.67)	81 (44.02)	69 (39.20)	1.32 (0.85 to 2.05)	0.201
GG	31 (8.61)	19 (10.33)	12 (6.82)	1.79 (0.82 to 3.90)	0.143
GG + AG	181 (50.28)	100 (54.35)	81 (46.02)	1.39 (0.92 to 2.11)	0.114
Alleles					
A	508 (70.56)	249 (67.66)	259 (73.58)	1.00 (reference)	
G	212 (29.44)	119 (32.34)	93 (26.42)	1.33 (0.96 to 1.83)	0.082
HWE $\chi^2 = 0.00$, p = 0.96					
CD133					
rs3130 T>C					
Genotypes					
TT	35 (9.72)	13 (7.07)	22 (12.50)	1.00 (reference)	
TC	143 (39.72)	71 (38.58)	72 (40.91)	1.66 (0.78 to 3.56)	0.186
CC	182 (50.56)	100 (54.35)	82 (46.59)	2.06 (0.97 to 4.34)	0.056
CC + TC	325 (90.28)	171 (92.93)	154 (87.50)	1.87 (0.91 to 3.85)	0.085
Alleles					
T	213 (29.58)	97 (26.36)	116 (32.95)	1.00 (reference)	
C	507 (70.42)	271 (73.64)	236 (67.05)	1.37 (0.99 to 1.89)	0.052
HWE $\chi^2 = 0.78$, p = 0.38					
rs2240688 A>C					
Genotypes					
AA	213 (59.17)	99 (53.80)	114 (64.77)	1.00 (reference)	
AC	126 (35.00)	72 (39.13)	54 (30.68)	1.53 (0.98 to 2.39)	0.058
CC	21 (5.83)	13 (7.07)	8 (4.55)	1.87 (0.74 to 4.70)	0.182
CC + AC	147 (40.83)	85 (46.20)	62 (35.23)	1.57 (1.03 to 2.41)	0.034#
Alleles					
A	552 (76.67)	270 (73.37)	282 (80.11)	1.00 (reference)	
C	168 (23.33)	98 (26.63)	70 (19.89)	1.46 (1.03 to 2.07)	0.032#

Table 2. Continued

Genes	Total (n = 360) n, (%)	Patients (n = 184) n, (%)	Controls (n = 176) n, (%)	Odds ratio (95% CI)	P - value (<0.05)
HWE χ^2 = 0.17, p = 0.68					
ALDH1A1 (17 bp Del)					
Genotypes					
*1*1	329 (91.39)	173 (94.03)	156 (88.64)	1.00 (reference)	
*1*2	29 (8.06)	10 (5.43)	19 (10.80)	0.47 (0.21 to 1.05)	0.066
*2*2	2 (0.55)	1 (0.54)	1 (0.56)	0.90 (0.05 to 14.53)	0.941
*2*2 + *1*2	31 (8.61)	11 (5.97)	20 (11.36)	0.49 (0.23 to 1.06)	0.073
Alleles					
*1	687 (95.42)	356 (96.74)	331 (94.04)	1.00 (reference)	
*2	33 (4.58)	12 (3.26)	21 (5.96)	0.53 (0.25 to 1.09)	0.087
HWE χ^2 = 2.25, p = 0.13					
Detoxification genes					
GSTM1					
Present allele	151 (41.94)	64 (34.78)	87 (49.43)	1.00 (reference)	
Null allele	209 (58.06)	120 (65.22)	89 (50.57)	1.83 (1.20 to 2.79)	0.005#
HWE (ND)					
GSTT1					
Present allele	230 (63.89)	120 (65.22)	110 (62.50)	1.00 (reference)	
Null allele	130 (36.11)	64 (34.78)	66 (37.50)	0.88 (0.57 to 1.36)	0.591
HWE (ND)					
Apoptotic genes					
Caspase 8					
rs3834129 (6 bp InsDel)					
Genotypes					
DelDel	18 (5.00)	10 (5.43)	8 (4.55)	1.00 (reference)	
InsDel	118 (32.78)	67 (36.42)	51 (28.98)	1.05 (0.38 to 2.85)	0.922
InsIns	224 (62.22)	107 (58.15)	117 (66.57)	0.73 (0.27 to 1.92)	0.526
InsIns + InsDel	342 (95.00)	174 (94.57)	168 (95.45)	0.82 (0.31 to 2.15)	0.699
Alleles					
Del	154 (21.39)	87 (23.64)	67 (19.03)	1.00 (reference)	
Ins	566 (78.61)	281 (76.36)	285 (80.97)	0.75 (0.53 to 1.08)	0.132
HWE χ^2 = 0.23, p = 0.63					
Caspase 9					
rs4645982 (20 bp InsDel)					
Genotypes					
DelDel	37 (10.28)	20 (10.87)	17 (9.66)	1.00 (reference)	
InsDel	155 (43.06)	80 (43.48)	75 (42.62)	0.90 (0.44 to 1.86)	0.789
InsIns	168 (46.66)	84 (45.65)	84 (47.72)	0.85 (0.41 to 1.73)	0.655
InsIns + InsDel	323 (89.72)	164 (89.13)	159 (90.34)	0.87 (0.44 to 1.73)	0.705
Alleles					
Del	229 (31.81)	120 (32.61)	109 (30.97)	1.00 (reference)	
Ins	491 (68.19)	248 (67.39)	243 (69.03)	0.92 (0.67 to 1.26)	0.636
HWE χ^2 = 0.02, p = 0.89					
Inflammatory genes					
NF-κB1					
rs28362491 (4 bp Del)					
Genotypes					
*1*1	142 (39.44)	66 (35.87)	76 (43.18)	1.00 (reference)	
*1*2	164 (45.56)	84 (45.65)	80 (45.46)	1.20 (0.77 to 1.89)	0.408
*2*2	54 (15.00)	34 (18.48)	20 (11.36)	1.95 (1.02 to 3.72)	0.04#
*2*2 + *1*2	218 (60.56)	118 (64.13)	100 (56.82)	1.35 (0.88 to 2.07)	0.156

Table 2. Continued

Genes	Total (n = 360) n, (%)	Patients (n = 184) n, (%)	Controls (n = 176) n, (%)	Odds ratio (95% CI)	P - value (<0.05)
Alleles					
*1	448 (62.22)	216 (58.69)	232 (65.91)	1.00 (reference)	
*2	272 (37.78)	152 (41.31)	120 (34.09)	1.36 (1.00 to 1.84)	0.046#
HWE χ^2 = 0.35, p = 0.56					
Growth factor genes					
TGFβ2 (4 bp Del)					
Genotypes					
*1*1	11 (3.06)	3 (1.63)	8 (4.55)	1.00 (reference)	
*1*2	99 (27.50)	50 (27.17)	49 (27.84)	2.72 (0.68 to 10.86)	0.156
*2*2	250 (69.44)	131 (71.20)	119 (67.61)	2.93 (0.76 to 11.32)	0.117
*2*2 + *1*2	349 (96.94)	181 (98.37)	168 (95.45)	2.87 (0.74 to 11.01)	0.123
Alleles					
*1	121 (16.81)	56 (15.22)	65 (18.47)	1.00 (reference)	
*2	599 (83.19)	312 (84.78)	287 (81.53)	1.26 (0.85 to 1.86)	0.244
HWE χ^2 = 0.10, p = 0.75					
VEGF					
rs35569394 (18 bp Del)					
Genotypes					
*1*1	29 (8.06)	15 (8.15)	14 (7.95)	1.00 (reference)	
*1*2	145 (40.28)	77 (41.85)	68 (38.64)	1.05 (0.47 to 2.34)	0.892
*2*2	186 (51.67)	92 (50.00)	94 (53.41)	0.91 (0.41 to 1.99)	0.82
*2*2 + *1*2	331 (91.94)	169 (91.85)	162 (92.05)	0.97 (0.45 to 2.08)	0.945
Alleles					
*1	203 (28.19)	107 (29.08)	96 (27.27)	1.00 (reference)	
*2	517 (71.81)	261 (70.92)	256 (72.73)	0.91 (0.66 to 1.26)	0.59
HWE χ^2 = 0.01, p = 0.0.92					
Proto-oncogene					
MDM2 SNP309					
rs2279744 T>G					
Genotypes					
TT	81 (22.50)	46 (25.00)	35 (19.89)	1.00 (reference)	
GT	193 (53.61)	95 (51.63)	98 (55.68)	0.73 (0.43 to 1.24)	0.253
GG	86 (23.89)	43 (23.37)	43 (24.43)	0.76 (0.41 to 1.40)	0.379
GG + GT	279 (77.50)	138 (75.00)	141 (80.11)	0.74 (0.45 to 1.22)	0.246
Alleles					
T	355 (49.31)	187 (50.82)	168 (47.73)	1.00 (reference)	
G	365 (50.69)	181 (49.18)	184 (52.27)	0.88 (0.65 to 1.18)	0.407
HWE χ^2 = 1.89, p = 0.17					
Tumor suppressor genes					
FOXO3					
rs2802292 T>G					
Genotypes					
TT	167 (46.39)	90 (48.92)	77 (43.75)	1.00 (reference)	
GT	154 (42.78)	79 (42.93)	75 (42.61)	0.90 (0.58 to 1.39)	0.642
GG	39 (10.83)	15 (8.15)	24 (13.64)	0.53 (0.26 to 1.09)	0.085
GG + GT	193 (53.61)	94 (51.08)	99 (56.25)	0.81 (0.53 to 1.23)	0.326
Alleles					
T	488 (67.78)	259 (70.38)	229 (65.06)	1.00 (reference)	
G	232 (32.22)	109 (29.62)	123 (34.94)	0.78 (0.57 to 1.07)	0.126
HWE χ^2 = 0.15, p = 0.70					

#, significant level at $p < 0.05$; ND, no data

Table 3. The Combined Genotypes of Genes that were Significant in Increasing the Risk of Breast Cancer

Genotypes combination				Patients (n = 184) n, (%)	Controls (n = 176) n, (%)	OR (95% CI)	P - value (p < 0.05)
CD44 rs187116	*CD133* rs2240688	*NF-KB1*	*GSTM1*				
GG	AA	*1*1	present allele	3 (1.63)	9 (5.11)	1.00 (reference)	
GG	AC	*1*2	null allele	8 (4.34)	3 (1.70)	8.00 (1.24 to 51.50)	0.028#
GA	AA	*2*2	null allele	6 (3.26)	2 (1.13)	9.00 (1.14 to 71.04)	0.037#
AA	AA	*1*1	null allele	7 (3.80)	1 (0.56)	21.00 (1.77 to 248.11)	0.015#
AA	CC	*2*2	null allele	ND	ND	ND	ND

#, Significant value at p < 0.05; *1 wildtype, *2 deletion

tumor suppressor gene (*FOXO3*) and proto-oncogene (*MDM2*) are involved in evading growth suppressors, and the apoptotic genes (*caspase 8* and *caspase 9*) relate to resisting cell death. Moreover, the variation of cancer stem cell marker genes (*CD44 rs187116*, *CD44 rs13347*, *CD44 rs4756196*, *CD133 rs3130*, *CD133 rs2240688*, and *ALDH1A1*), were reported to be associated with an increasing cancer risk (Winder et al., 2011; Jiang et al., 2012; Liu et al., 2016; 2017; Lin et al., 2018). The results of this study showed that *CD44 rs187116* and *CD133 rs2240688* of cancer stem cell marker genes, the inflammatory gene, *NF-κB1*, and the detoxification gene, *GSTM1* were significantly associated with an increased risk of breast cancer (p = 0.041, p = 0.033, p = 0.046, and p = 0.005, respectively).

Cancer stem cells are important in tumor progression, spreading, and in resistance to conventional therapy for breast cancer (Sin and Lim, 2017). Biomarkers, such as *CD24*, *CD44*, *CD133*, and *ALDH1*, are mostly used to identify CSC in the tumors (Medema, 2013). Previous reports showed that the expression of these biomolecules might increase in CSC (Sheridan et al., 2006; Glumac and LeBeau, 2018). The variation of these biomarker genes was also associated with the risk of cancer (Jiang et al., 2012; Jia et al., 2017). Hence, the variations of *CSC* biomarker genes might relate to risk of cancer. Our study found that the AA genotype of *CD44 rs187116* (c.67+4883G>A) increased the risk of breast cancer compared with wildtype genotype (OR: 2.03; 95% CI: 1.02–4.02; p = 0.041). In contrast, previous studies of *rs187116* variation reported that patients with at least one G allele, had an increased risk and recurrence of cancer after gastric surgery in Iran, Japan, North America, and Northeast Thailand (Winder et al., 2011; Bitaraf et al., 2015; Suenaga et al., 2015; Tongtawee et al., 2017). Our study of *CD133*, *rs22406882* (c.*667A>C) shows that the C allele tended to increase the risk of cancer (OR: 1.46; 95% CI: 1.03–2.07; p = 0.033), which is consistent with previous reports that the AC or CC genotypes were associated with increased risk and reduced overall survival rate in lung cancer patients in China (Liu et al., 2016; 2017). However, the variant AC/CC genotypes were associated with decreased risk of gastric cancer (OR: 0.81; 95% CI: 0.67–0.97; p = 0.023) (Jia et al., 2017). Many studies report that *ALDH1* correlated with cell migration, tumor metastasis, and poor prognosis of breast cancer (Ginestier et al., 2007; Tan et al., 2013; Li et al., 2017), but our study found no such association.

The NF-κB is a main regulator of inflammation, cancer development, immune response, and apoptosis (Chen et al., 2018b; Zhou et al., 2009), and several genetic variations are associated with the risk of oral, esophageal, gastric, and colorectal cancers (Lo et al., 2009; Umar et al., 2013; Song et al., 2013; Chen et al., 2018b). The 4-bp ATTG deletion in the promoter of *NF-κB1 rs28362491* resulted in the loss of binding to nuclear proteins that reduced promotor activity, hence decreased *NF-κB1* transcription, and protein production (Karban et al., 2004; Zhou et al., 2009). This study showed that the *2*2 homozygous genotype (del/del) was associated with a 2 – fold increased risk (p = 0.041) and with the *2 allele was 1.36 increased risk (p = 0.046). This finding is consistent with a previous report that this polymorphism was not only associated with the risk of oral cancer (Chen et al., 2018b), but also with the development of gastric cancer and colorectal cancer (Cavalcante et al., 2017).

GSTM1, one of the glutathione-S-transferase gene family, produces the *GSTM1* enzyme involved in the detoxification of polycyclic aromatic hydrocarbons and other carcinogens (Strange and Fryer, 1999). The homozygous null allele genotype increase damage to DNA caused by these agents and this genotype is a risk factor for breast cancer (Strange and Fryer, 1999; de Aguiar et al., 2012; Chirilă et al., 2014). In this study, the homozygous null allele genotype was associated with an increased risk of breast cancer (OR: 1.83; 95% CI: 1.20-2.79; p = 0.005). It was found in 65.22% in patients, similarly to other studies that found it in over 50% (Possuelo et al., 2013; Chirilă et al., 2014).

The other polymorphisms including detoxification genes (*GSTT1*), cancer stem cell marker genes (*CD44 rs13347*, *CD44 rs4756196*, *CD133 rs3130*, and *ALDH1A1*), apoptotic genes (*caspase 8* and *caspase 9*), growth factor genes (*TGFβ2* and *VEGF*), tumor suppressor gene (*FOXO3*), and proto-oncogene (*MDM2*) did not show an association with breast cancer in this study, indicating that these polymorphisms do not necessarily increase the risk of breast cancer in our population. However, these genes were reported to be associated with other cancers, such as nasopharyngeal, gastric, lung, and colorectal cancer (Son et al., 2006; Xiao et al., 2013; Aravantinos et al., 2015; Jia et al., 2017). The genetic background of the population might be the cause of this discrepancy.

We combined the four significant associated polymorphic loci, including *CD44 rs187116*, *CD133 rs2240688*, *NF-κB1 rs28362491* and *GSTM1*, and we enquired as to which marker combinations might increase the risk for breast cancer. The results showed that the

AA-AA-*1*1-homozygous null allele combination was significantly the highest association (OR = 21.00; 95% CI: 1.77 to 248.11; p = 0.015), followed by GA-AA-*2*2-homozygous null allele (OR = 9.00; 95% CI: 1.14 to 71.04; p = 0.037) and GG-AC-*1*2-homozygous null allele (OR = 8.00; 95% CI: 1.24 to 51.50; p = 0.028). A report in 2013 by Sharma and colleagues reported that there is no association of *CD44* haplotypes in gallbladder cancer, but the combined haplotype was significantly associated with a decreased risk of gallbladder cancer in a North Indian population (Sharma et al., 2014).

The biological functions of the 4 selected genes have been previously described. The *CD44 rs187116* associated with a higher expression of *CD44* protein in carcinogenesis, is involved in cancer progression and cancer cell metabolism (Chen et al., 2018a). The functions of *CD133 rs2240688* are not fully understood; it has been identified as the transcription factor binding site relating to the tumor initiation, maintenance and metastasis (Cheng et al., 2013). For *NF-κB1*, 4 bp deletion in the promoter affected to reduce the response of cells to inflammation (Karban et al., 2004; Zhou et al., 2009). The deletion of *GSTM1* affected the detoxification of the cells by reducing the function of glutathione S transferase, leading to accumulation of the carcinogens within the cells (Strange and Fryer, 1999).

This study shows that the gene combination of *CD44 rs187116*, *CD133 rs2240688*, *GSTM1* and *NF-κB1 rs28362491* could act as a new genetic biomarker to predict the risk of breast cancer in a Thai population and it could benefit cancer surveillance. However, among 360 samples in this study, the demographic and clinical characteristics of breast cancer patients and controls were not available due to the limitations in the data retrieval from medical records and histopathologic reports. For the further study, authors suggest the larger sample sizes with more information on demographic and clinical characteristics of participants must be obtained to provide more comprehensive and accurately representative results.

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