Predisposing Haplotypes of TGF-ß1 among Malay Women with Breast Density in Kuala Terengganu, Malaysia: A Pilot Study

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ABSTRACT

Introduction and Aim: Breast density has been used as a potential cofounder factor in breast carcinoma risk. The breast density is determined by several genes that involved in cell proliferation, particularly Transforming Growth Factor-Beta 1 (TGF-ß1). A defect in TGF-ß1 molecular pathway may lead to abnormal cell proliferation which is seen in human cancers, particularly breast carcinoma. This preliminary study was aimed to predict the predisposing haplotypes of TGF- ß1 among Malay women in Kuala Terengganu with breast density.

Materials and Methods: The participants were recruited among women who underwent mammography check-up at Department of Radiography, Hospital Sultanah Nur Zahirah of Kuala Terengganu. The case group (n=44) was consisted of individuals with BIRADS 1 and BIRADS 2, while the control group (n=48) was taken among individuals with BIRADS 3 and BIRADS 4. SNP genotyping was conducted for three main TGF-ß1 variants, assigned as rs1800469, rs1800470 and rs4803455 using PCR-RFLP technique.

Results: Two SNPs (rs1800469 and rs180047) did reveal a significant association with breast density with a p value of 0.008 and 0.006, respectively. However, rs4803455 did not yield association in this small cohort. In a further investigation, haplotype GAA conferred a susceptible haplotype (p value=0.027, OR=2.28 [1.09-4.76]) towards breast density whilst haplotype AAC projected a significant association with protective effect (p value=0.018, OR=0.45[0.23-0.88]) in breast density development.

Conclusion: This pilot study might provide baseline information on the role of TGF-ß1 haplotypes in determining the density of breast that projected a genetic determinant as a risk factor for breast carcinoma among Malay women.

Key Words: Breast density, TGF- ß1, Haplotypes, Association.

INTRODUCTION

A mammogram is a screening tool to detect the mass of breast by assessing the ratio of the epithelial and stromal tissue towards fat mass in which can determine the density of the breast. According to the Breast Imaging-Reporting And Data System (BIRADS) classification, there are four main classes, namely BIRADS 1, BIRADS 2, BIRADS 3 and BIRADS 4 that is based on the level of density. Breast density has been recognized as a breast cancer predictor in diagnosis alongside with other factors such as family history, environmental factor and genetic determinants (1). Individuals with higher breast density assigned as BIRADS 3 and BIRADS 4 are prone to develop breast cancer with 1.8- to 6-fold increase risk compared to individuals with lesser breast density (BIRADS 1 and BIRADS 2) (2). The mammographic density is determined by gene regulation in mammary cell development that includes Transforming growth factor-beta (TGF-ß) gene. The gene is a cytokine that involves cell proliferation, differentiation and cell signaling in which consists of three isoforms assigned as TGF-ß1, TGF-ß2, and TGF-ß3.TGF-ß have been shown to promote in ductal branching in mammary gland

which leads to alveolar hyperplasia of breast and premature functional differentiation (3).

Transforming Growth Factor-Beta 1 (TGF- ß1) is the most isoform that was observed in upregulating in tumourogenesis(4) and has been extensively studied in association with its role in cell divisions and proliferation especially in mammary cell development (5). The gene was observed to associate in breast carcinogenesis, and thus, this study was conducted to determine the role of TGF- ß1 variants in breast density development among Malay women in our population in which to the best our knowledge no study is carried out yet in Malaysia for this purpose.

MATERIALS AND METHODS

Subject Recruitment

This case-control study was conducted among Malay women (n=92) who underwent mammography screening either for diagnosis or follow up examination at Hospital SultanahNur Zahirah, Kuala Terengganu. They were given informed consent before participating in this study. The human ethical approval was obtained from UniSZA Human Research Ethical Committee (No:UniSZA.C/1/UHREC/628-1(38)) and National Medical Research Registry (No:NMRR 15-1021-26292 (IIR). For those who developed with other cancer than breast carcinoma was excluded in this study.The mammography parenchymal pattern was determined using BIRADS (breast imaging-reporting and data system) classification. BIRADS 1 and 2 with lesser breast density were identified/categorized as a control group (n=44), whilst BIRADS 3 and 4 were grouped into case cohort (n=48) with denser breast density.BIRADS 1 consists of the mostly fatty breast with 0-24% density and BIRADS 2 is a scattered fibroglandular breast with 25-50% density. Meanwhile, BIRADS 3 is categorized as a heterogeneously dense breast with 51-75% density, and BIRADS 4 is classified as an extremely dense breast with 76-100% density. The diagnosis on BIRADS classification was conducted by radiologists.

SNP Genotyping

DNAs were extracted from 3 ml of peripheral blood using the commercialized extraction kit according to the manufacturer's protocol (Qiagen, Germany). Three SNPs of TGF-ß1 were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, assigned as rs1800469 (G/A), rs1800470 (A/G) and rs4803455 (C/A). The primers and restriction enzymes used in this technique are listed in the table below (refer Table 1).PCR technique was performed in a total of 25µl that consisted of 5X PCR buffer, 2.0mM MgCl2, 200M dNTP, 0.2M of forward and reverse primer and 0.08 U/µl of Taq polymerase (Promega, Madison, WI) before restriction enzyme digestion procedure. The PCR and PCR-RFLP product was preceded with 2% agarose gel electrophoresis and stained with ethidium bromide before viewed with the Alpha Imager Analyzer.

Statistical Analysis

Allelic and genotypic frequencies were calculated according to Hardy Weinberg equilibrium using SHEsis online software providing odd ratios (ORs) and 95% confidence interval (95%CI) and a significant p value at 0.05 (Chi square). The statistical analysis is used for single association and haplotype association analyses in determining the association of TGF- ß1 with breast density.

RESULTS

Single association revealed a significant difference for rs1800469 and rs1800470 with breast density among Malay women who attended for mammography screening with p value at 0.008, OR=2.23, 95%CI [1.22-4.08] and p value at 0.006, respectively. However, this pilot study did not find any association of rs4803455 (p value=0.130, OR=0.62, 95%CI [0.34-1.15]. The odd ratio for rs1800470 was not obtained due to no heterogeneity observed in case group (refer Table 2). In an attempt to provide evidence of predisposing effect with a combination of an allele from each of the TGF-ß1 variants, haplotype analysis was conducted. It is noteworthy that haplotype GAA conferred susceptible effect in breast density development with p value at 0.027, OR=2.28, 95%CI [1.09-4.76]. In a contrarily, haplotype AAC yielded a significant association with breast density in a protective manner, projected p value at 0.018, OR=0.45, 95%CI [0.23-0.88] (refer Table 3).

Table 1: Primer sequences and restriction enzymes used with PCR product sizes

SNP	Primer sequences	Restriction enzyme	Product sizes
rs1800469	Forward:5'-GGGAGGAGGGGGCAACAGGA-CACCTGC Reverse: 5'-TGGGGGAGGATGGCACAGTG	HpyCH4V	289bp, 30 bp, 259 bp
rs1800470	Forward:5'-ACCAGTAGCCACAGCAGCGGTAG-CAGCAGT Reverse: 5'-AGGACCTCAGCTTTCCCTCG	Bst1	191bp, 31 bp, 160 bp
rs4803455	Forward: 5'-TTTGACACCCTGAATTCTCA Reverse: 5'-TTAGTAGAGACGAGGTTTCAC	MluCI	172 bp, 34 bp, 138 bp

Table 2: Single association analysis of TGF beta polymorphisms with breast density

	Genotype data			Case Control analysis		
SNP	Homozygous wild type n (%)	Heterozygous, n (%)	Homozygous mutant type, n (%)	MAF	P value	OR [95% CI]
Rs1800469	25(0.57)	10(0.23)	9(0.21)	28(0.32)	0.008	2.23 [1.22-4.08]
	16(0.33)	15(0.31)	17(0.35)			
Rs1800470	44(1.00)	0(0.00)	0(0.00)	0(0.00)	0.006	-
	43(0.90)	2(0.04)	3(0.07)			
Rs4803455	18(0.41)	17(0.39)	9(0.21)	35(0.40)	0.130	0.62 [0.34-1.15]
	27(0.56)	14(0.30)	7(0.15)			

***Case data is at the top line while control data is at the bottom line. MAF= Minor allele frequency. P value < 0.05 is considered significant in Pearson Chi Square.**

Table 3: Haplotype association analysis of TGF-ß1 polymorphisms with breast density

Allele combination	Lesser breast density group, n (freq)	Denser breast density group, n (freq)	P value	OR [95%CI]
GAC	28.29(0.295)	35.38(0.402)	0.153	1.56[0.85-2.88]
GAA	13.71(0.143)	24.62(0.280)	0.027	2.28[1.09-4.76]
GGC	3.00(0.031)	0.00(0.000)	0.091	-
AAC	33.71(0.351)	17.62(0.200)	0.018	0.45[0.23-0.88]
AAA	12.29(0.128)	10.38(0.118)	0.793	0.89[0.37-2.14]
AGC	3.00(0.031)	0.00(0.000)	0.091	-

***Alleles from left to right are rs1800469, rs1800470 and rs4803455, respectively. P value < 0.05 is considered significant in Pearson Chi Square.**

DISCUSSION

In this preliminary study, the data showed a significant role of TGF-ß1 polymorphisms with breast density development projected by two variants assigned as rs1800469 and rs1800470. The association study may support the function of Transforming Growth Factor (TGF) gene in the mammary parenchymal cell formation, differentiation, and proliferation with the role of single nucleotide polymorphisms within the gene region. The mammographic density is determined by the ratio of the amount of epithelial and stromal tissue to the amount of fat tissue. The importance of breast density determination and classification is useful as a strong predictor of breast cancer risk. Individuals with higher breast density such as BIRADS 3 which contains 51-75% of density and BIRADS 4 comprises of 76-100% of density may prone to predispose them to breast carcinogenesis. Therefore this study presented the minor allele of rs1800469 (a promoter variant with a substitution of guanosine to adenosine at position -800 bp) to in-

crease risk in breast density development by giving the odd ratio of 2.23 in a single association analysis. Even though the odd ratio for rs1800470 (a non-synonymous variant with a substitution of leucine to proline at position -509 bp) could not be calculated due to no heterogeneity in the case group, the variant showed association with breast density. The data is consistent with other studies in different populations (6,7). The minor allele from these two variants was also implicated in higher TGF-ß1 serum level and suggesting the genetic factor controls and regulates the biochemical in which this study did not perform (8). Meanwhile, rs4803455 did not yield a significant association in this study with the minor allele conferred protective effect based on OR=0.61. The data was contradicted by a study conducted among Singapore Chinese women particularly among nulliparous women (9) with a fair association (p= 0.034). The contradictory factor might be due to different ethnicity in which the women in this study was among 98% Malays.

In a further investigation to evaluate predisposing haplotype in breast density, haplotype GAA with a combination of wild alleles from rs1800469 and rs1800470 with the mutant allele from rs4803455 revealed an association in susceptibility to higher breast density as projected by the odd ratio. A change allele from haplotype GAC to GAA provided an evidence of allele A from rs4803455 with a susceptible effect significantly in haplotype GAA.Thus, individuals with this haplotype GAA are prone to have denser breast density as categorized as BIRADS 3 and BIRADS 4. Contradictory, a protective haplotype assigned as haplotype AAC with a combination of a mutant allele from rs1800469 with wild alleles from rs1800470 and rs4803455 might reduce the risk to denser breast density in which less potential to develop breast cancer among Malay women in our population. This evidence can be observed when an allele change from haplotype GAC with a combination of all wild alleles was changed to haplotype AAC.The role of the protective effect was projected from allele A from rs1800469. It is noteworthy that mutant alleles from rs4803455 and rs1800470 play a major role in giving stronger effect to these predisposing haplotypes either a susceptible or protective manners.

Malaysia is a multiracial country that accounts Malays with 54.7%, followed by Chinese 24.3%, Indian 7.3%, other Bumiputera 12.8% and others 0.9%. The incidence of breast cancer in Malaysia was highest among Chinese, followed by Indian and Malays. However, the incidence of breast cancer among Malay women in Malaysia was 27.2% based on Malaysian National Cancer Registry Report 2007-2011(10). Since this study was conducted in Terengganu where the Malays contribute the majority ethnic group with 96.6%, the data may provide evidence of breast density among Malay women with predicting the breast cancer incidence rate in Terengganu, particularly. Therefore, SNP profile in the different ethnic group is worth to be taken into account in a future direction in a well-characterized population-based study.

This current study revealed that the variations of TGF- ß1 provide molecular information on potential diagnostic biomarkers for a majority of patients-based ethnic in breast cancer risk. The data may facilitate the personalized medicine based on targeted SNP profile and may provide baseline information to other researchers in exploring more evidence in the role of TGF-ß1 variants in breast density.

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