Med & Health Dec 2017; 12(2): 312-320 https://doi.org/10.17576/MH.2017.1202.15

ORIGINAL ARTICLE

Study of Association of TGF-ß1 Polymorphism with Breast Density in a Tertiary Medical Center of Malaysia

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ABSTRAK

Pengesanan awal risiko kanser payudara menggunakan mammografi dapat mengurangkan kos pengurusan rawatan dan prognosis penyakit ini. Paras ketumpatan payudara yang dikesan menggunakan mammogram memberi potensi faktor penyebab kepada risiko kanser payudara. Penghasilan sel epitelial pada mamari dapat ditentukan oleh faktor genetik. Gen transforming growth factor-beta (TGF-ß) telah dikenalpasti terlibat di dalam regulasi proliferasi sel dan pembahagian sel. Kajian ini bertujuan untuk mengukur hubung kait polimorfisme TGF-ß1 dengan ketumpatan payudara di kalangan wanita yang menjalani saringan mamografi. Pengesanan genotip bagi tiga polimorfisme TGF-ß1 yang dikenali sebagai rs1800469, rs1800470 dan rs4803455 dilakukan dengan teknik PCR-RFLP. Frekuensi alel dan genotip dikira bagi kumpulan kawalan yang terdiri dari kalangan wanita yang telah dikelaskan BIRADS 1 dan BIRADS 2, manakala kumpulan kes terdiri dari kalangan wanita yang telah dikelaskan BIRADS 3 dan BIRADS 4.Dua polimorfisme (rs1800469 dan rs1800470) telah menunjukkan hubung kait yang signifikan dengan ketumpatan payudara dengan nilai P= 0.004 dan 0.003. Namun begitu polimorfisme yang ketiga iaitu rs4803455 tidak menunjukkan hubung kait (nilai P=0.090). Analisa hubung kait haplotaip mencadangkan haplotaip GAA berupaya memberi kecenderungan ketumpatan payudara (nilai P=0.02, OR=2.21 [1.07-4.55]) berbanding haplotaip AAC yang berupaya untuk memberi kesan perlindungan dari penghasilan ketumpatan payudara (nilai P=0.004, OR=0.40

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[0.21-0.77]). Data asas hubung kait dan haplotaip ini memberi penjelasan kaitan polimorfisme TGF-ß1 dengan ketumpatan payudara dan memberi maklumat fungsi polimorfisme di dalam mempengaruhi pembentukan ketumpatan payudara.

Kata kunci: asosiasi, ketumpatan payudara, polimorfisme, gen TGF-ß1

ABSTRACT

Early detection of breast cancer risk by screening tools such as mammography can reduces the cost of treatment management and the prognosis of the disease. The level of breast density of in mammogram is one of the potential cofounder factor for breast cancer risk. The development of mammary epithelial cells is determined by the genetic factor. Transforming growth factor-beta (TGF-ß) gene involves in the regulation of cell proliferation and cell division. This study aimed to assess the association of TGF-ß1 polymorphisms with breast density among women who underwent breast screening using mammogram. The detection of genotypes for three polymorphisms of TGF-ß1 assigned as rs1800469, rs1800470 and rs4803455 were performed using PCR-RFLP technique. The allele and genotype frequencies were calculated for control group that consists of BIRADS 1 and BIRADS 2 class whilst case group consisted of BIRADS 3 and BIRADS 4 class. Two polymorphisms (rs1800469 and rs1800470) yielded a significant association with breast density with p value of 0.004 and 0.003, respectively. However, the third polymorphism, rs4803455 did not yield a significant association (p value=0.090). Haplotype association analysis might suggest the haplotype GAA conferred susceptibility (p value= 0.02, OR=2.21[1.07-4.55]) rather than haplotype AAC predispose a protective effect (p value=0.004, OR=0.40[0.21-0.77]) to breast density development. This preliminary data on single and haplotype association might reveal the association of polymorphisms of TGF-ß1 with breast density and give an insight on the role of polymorphism in predisposing to breast density development.

Keywords: association, breast density, polymorphism, TGF-ß1 gene

INTRODUCTION

Breast carcinoma ranks second in all death due to cancer, worldwide and the first mortality factor in Malaysia. There are many cofounding factors that can lead to breast carcinoma. These include strong family history of breast carcinoma, patient with history of radiation to chest area, dense breast detected on mammogram and genetic factor such as BRCA1 and BRCA2 gene mutation (Armstrong et al. 2000). Mutations in BRCA1 and BRCA2 are relatively uncommon that predispose a susceptibility to breast cancer development. Therefore, other genes might play a pivotal

role in gene-gene networking and modify the risk of breast cancer (Ziv et al. 2001). The genetic entities that stimulate the mammary cell growth and proliferation determine the density of breast. Women with dense breasts showed a 1.8- to 6-fold increased risk of developing breast cancer (Boyd et al. 2002). Mammogram can assess density of the breast by relative amount of fat, connective tissue and epithelial cells determines the radiographic features of the breast on mammogram. Association of density percentage, dense areas with risk of breast cancer might reflect the potential associated genes in understanding the etiology and pathogenesis of breast cancer (Pettersson et al. 2014).

In relation to the genetic factor contribution, the genetic entities are believed to determine the density of the breast that include TGF-ß gene. Transforming growth factor-beta (TGF-ß) is a cytokine that regulates the proliferation, cellular differentiation and other functions in the cell. The gene consists of three isoforms namely TGF-ß1, TGF-ß2 and TGF-ß3. The most isoform that upregulated in tumourgenesis is TGF-ß1 (Derynck et al. 2001). Defect in TGF-ß1 pathway have been implicated in oncogenesis, particularly in breast cancer development. Transforming growth factors-ß (TGF-ßs) regulates mammary epithelial cell division. Loss of expression of TGF-ß receptor II (TGF-ß-RII) is related to cell proliferation and tumor progression in breast cancer (Gobbi et al. 1999). Joseph and colleagues have shown that TGF-ß promotes increased in ductal branching and resulted in alveolar hyperplasia of breast and premature functional differentiation (Joseph et al. 1999). Alterations in the TGF-ß pathway maybe due to mutation causing abnormal proliferation of the cells which is seen in human cancers (Elliot & Blobe 2005). TGF-ß has been reported to be involved in oncogenesis in transgenic mice where the deletion of one copy of the gene leads to increased cell turnover. However, the risk of mammary carcinoma was reduced when the expression of TGF-ß was increased (Pierce et al. 1995). There were a consistent reports that link to a functional TGF-ß1 polymorphism with increased serum TGF-ß level to decreased breast cancer incidence in women in marked with decreased breast density (Ziv et al. 2001). Thereby, the associated TGF-ß can be used to determine the risk of breast cancer in women. Association of TGF-ß is varies between different population group. There is paucity of study in Malaysia for determining the association of TGF-ß with the mammographic density. Thus, this study was carried out.

MATERIALS AND METHODS

SUBJECTS RECRUITMENT

A total of 100 patients aged between 40-65 years old who underwent the screening procedure of the mammogram at Hospital Sultanah Nur Zahirah were recruited and given informed consent in this study. An ethical clerance was obtained

from Universiti Sultan Zainal Abidin (UniSZA) Human Research Ethical Committee (No:UniSZA.C/1/ UHREC/628-1(38)) and National Medical Research Registry (No:NMRR 15-1021-26292 (IIR). According to the BIRADS (breast imaging-reporting and data system) Classification. BIRADS 1 (mostly fatty breast with 0-24% dense) and BIRADS 2 (scattered fibroglandular breast with 25-50% dense) with less breast dense were considered as control group (n=50) whilst BIRADS 3 (heterogeneously dense breast with 51-75% dense) and BIRADS 4 (extremely dense breast with 76-100% dense) with higher breast dense were considered as case group (n=50). The diagnosis on BIRADS classification was conducted by radiologists. Overall, SNPs give MAF of 0.35 with OR of 3 require 50 samples for each case and control sample sets project a power of 97% (Johnson et al. 2001). This priori power calculation was based on the data generated by Hap Map database and Lee et al. (2013) in Singapore Chinese women.

SNP GENOTYPING

An amount of 3 ml of peripheral blood was extracted for DNA according to the manufacturer's protocol (Qiagen, Germany) and analyzed for TGF-ß polymorphisms using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for three variants that were assigned as rs1800470 (A/G), rs1800469 (G/A) and rs4803455 (C/A).The PCR pimers used for rs1800470 were5′-AGCCACAGCAGCGGTAGCAGCAGT in the forward direction and 5′-AGGACCTCAGCTTTCCCTCG in the reverse direction that results in cleavage of the 191 bp product into 31/160 bp fragments using Bst1; PCR pimers for rs1800469 were5′-GGAGGGGGCAACAGGACACCTGC in the forward direction and 5′-TGGGGGAGGATGGCACAGTG in the reverse direction that results in cleavage of the 289 bp product into 30/259 bp fragments using HpyCH4V; and PCR primers used for rs4803455 were5′-TTTGACACCCTGAATTCTCA in the forward direction and 5′-TTAGTAGAGACGAGGTTTCAC in the reverse direction that results in cleavage of the 172 bp products into 34/138 bp fragments using MluCI restriction enzymes. PCR technique was performed in a total of 25 l that consisted of 5X PCR buffer, 2.0mM MgCl2, 200mM dNTP, 0.2 mM of forward and reverse primer and 0.08 U/ l 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

Hardy-Weinberg equilibrium was calculated using SHEsis online software for cases and controls providing odd ratios (ORs) and 95% confidence interval (95%CI). Allelic and genotypic frequencies were compared between case and control sample sets. SHEsis online software

Table 1: Single association analysis of TGFbeta polymorphisms with breast density.

	Genotype data			Case-control analysis		
SNP	Homozygouswild type	Heterozygous	Homozygous mutant type	MAF	P value	OR [95% CI]
Rs1800469	29(58.0)	10(20.0)	11(22.0)	32(32.0)	0.004	2.30 [1.29-4.09]
	16(32.0)	16(32.0)	18(36.0)	52(52.0)		
Rs1800470	50(100.0)	0(0.0)	0(0.0)	0(0.0)	0.003	-
	45(90.0)	2(4.0)	3(6.0)	8(8.0)		
Rs48033455	21(42.0)	19(38.0)	10(20.0)	39(39.0)	0.090	0.61 [0.33-1.10]
	29(58.0)	14(28.0)	7(14.0)	28(28.0)		

*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.

was also used to determine haplotype frequencies and to test for association of haplotypes with disease. Logistic regression analysis was used to correlate the breast density with TGF-ß polymorphisms.

RESULTS

The single association analysis yielded a significant association of rs1800469 and rs1800470 with breast density with p value of 0.004 and 0.003, respectively. Odd ratio for rs1800470 could not be calculated due to no variation in case group. Eventhough rs48033455 projected a fairly association with p value of 0.090 with breast density, the trend of association was towards protective manner as interpreted by odd ratio with 0.61 [0.33-1.10]. Table 1 showed the allelic and genotype frequencies in the single association analysis.

While performing further investigation, haplotype analysis was conducted to test the predisposing affect of haplotypes with breast density. A sequential SNP that was tested in this analysis is rs1800469, rs1800470 and rs4803455. Haplotype GAA and AAC yieled a significant p value with 0.020 and 0.004, respectively in a opposing manner of effect. Haplotype GAA conferred a susceptible effect as projected by odd ratio with 2.21 [1.07-4.55] whilts protective haplotype was shown in allele combination with AAC (OR=0.40 [0.21-0.77]) as shown in Table 2.

DISCUSSION

Mammographic density measures the ratio of the amount of epithelial and stroma tissue to the amount of fat tissue in the breast in which the criteria has been used as strong predictor of breast cancer risk (McCormack & dos Santos Silva 2006). TGF-ß polymorphisms were shown to determine mammographic density in which the breast dense is considered as risk factor for breast cancer. The contribution from TGF-ß arise from its main function as cell proliferation,

Table 2: Haplotype association analysis of TGFbeta polymorphisms with breast density

Allele combination	Control group	Case group	P value	OR [95%CI]
GGC	3(3.0)	0(0.0)	0.07	-
GGA	2(2.0)	0(41.5)	-	-
GAC	29.2(29.2)	41.5 (41.5)	0.08	1.67[0.93-3.00]
GAA	13.8(13.8)	26.5(26.5)	0.02	2.21[1.07-4.55]
AGC	3(3.0)	0(0.0)	0.07	-
AAC	36.8(36.8)	19.5(19.5)	0.004	0.40[0.21-0.77]
AAA	12.2(12.2)	12.5(12.5)	0.99	1.00[0.43-2.32]

*Alleles from left to right are rs1800469, rs1800470 and rs4803455, respectively.

differentiation and division which regulate mammary development. Variation to the TGF-ß might alter the normal homeostasis and determine the density of the breast. According to the BIRADS classification, individuals with BIRADS 1 and BIRADS 2 consist of less breast dense detected and analysed by mammography whilst individuals with BIRADS 3 and BIRADS 4 are prone to predispose to breast cancer risk as the breast density is higher than BIRADS 1 & BIRADS 2. In an attempt to elucidate the role of TGF-ß1, two variants (rs1800469 and rs1800470) have shown their association with breast density according to the BIRADS clasification. A non-synonymous variant Leu10Pro substitution of rs1800470 and a promoter variant C509T of rs1800469 have been extensively studies in breast cancer risk. The minor allele of rs1800469 was shown to increase risk by giving the odd ratio of 2.30 in this study. Even though the odd ratio from association analysis of rs1800470 could not be calculated due to no heterogeneity, the variant showed a significant association with breast density. The data was consistent with other studies that the minor allele from this variant was significantly associated with a 4% and 5% increased of breast cancer risk (Broeks et al. 2011; Qiu et al. 2010). The data also projected the relative risk was higher in Asians compared to Caucasians with OR=1.11 (Qiu et al. 2010). The minor allele from both variants were shown association with higher TGF-ß1 serum level in which this study did not performed (Grainger et al. 1999). The third variant, rs4803455 was not significantly associated in this study with the minor allele conferred protective effect based on OR=0.61. The data was contradicted to Lee et al. (2013) in which the variant showed a fairly association with p value of 0.034 among Singapore Chinese women and the association was observed among nulliparous women. The contradictory might be due to ethnicity factor in which the women in this study was majority among 98% Malays.

Malay is the majority ethnic group (96.6%) in Terengganu that comprises of 54.7% in Malaysia, 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% (Malaysian National Cancer Registry Report 2007-2011). The data may provide evidence of breast density among Malay women with predicting the breast cancer incidence rate in Terengganu, particularly. However, future study in different ethnic is worth to be taken into account.

In the current study, the data postulated that individuals with a combination of GGA haplotypes were prone to predispose to higher breast dense (categorized as BIRADS 3 and BIRADS 4). The susceptible allele from rs4803455 which is A allele might confer a strong effect to this haplotype. An allele change from haplotype GAC to GAA strengthen the predisposing effect to breast density with odd ratio with 2.21[1.07-4.55]. However when haplotype GAC was changed to haplotype AAC, the allele A from rs1800470 conferred protection or reduced the breast density development as projected by odd ratio with 0.40[0.21-0.77]. Noteworthly allele A from rs4803455 and allele A from rs1800470 are the mutant or rare allele. To the best of our nowledge, our study is the first study conducted in Malaysia in assessing the association of TGF-ß1 polymorphisms with breast density accoding to the BIRADS classification. Even though the limitation of this study may arise from small number of sample size, this preliminary data may provide several avenues of future research with polymorphisms interaction within the same gene or gene-gene interaction might provide more accuracy in prediction of breast cancer risk. Thus, the single association and haplotype analysis of TGF-ß can be as a determinant risk factor for breast carcinoma. This data can provide an information about the role of TGF-ß in Malaysian population.

Other limitation may arise from smaller sample size that requires larger cohort to be tested and investigated in unraveling the role of genetic variants in well-characterized population-based study. Yet, this current study suggests that the variations of TGF- ß1 might alter the normal homeostasis of mammary parenchymal cells that can be as a potential biomarker in breast cancer risk and may provide baseline information to other researchers in exploring more evidences in the role of TGF-ß1 variants in breast density.

ACKNOWLEDGEMENT

The authors would like to thank all participants in this study who underwent the mammogram and all the staff members of Radiology Department of Sultanah Nur Zahirah of Kuala Terengganu. This study was supported by the Dana Penyelidikan Universiti (DPU) (No grant: UniSZA/2015/DPU/83) and approved by National Medical Research Registry (No grant: NMRR 15-1021-26292 (IIR).

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Received: 24 August 2017
Accepted: 6 October 2017