Polymorphisms of HER2 Ile655Val and cyclin D1 (CCND1) G870A are not associated with breast cancer risk but polymorphic allele of HER2 is associated with nodal metastases

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The HER2 codon Ile655Val and Cyclin D1 (CCND1) G870A polymorphisms were analyzed in a hospital-based Malaysian population using PCR-RFLP method. Peripheral blood samples were collected from 230 breast cancer patients, and 200 normal and healthy women who had no history of breast disease or breast cancer. We evaluated the association between HER2 or CCND1 polymorphisms and breast cancer risk, and clinico-pathological parameters in the population. The genotype and allele frequencies of HER2 (P=0.163 vs P=0.0622) and CCND1 (P=0.377 vs P=0.284) polymorphisms were not significantly different between the breast cancer cases and normal subjects, respectively. Women who were Ile/Val heterozygotes (OR=1.48; 95% CI, 0.91-2.43), Val/Val homozygotes (OR=1.93; 95% CI, 0.51-7.77) and carriers of Val allele genotype (OR=1.53; 95% CI, 0.95-2.45) were not significantly associated with increased breast cancer risk. Similarly, women who were homozygous (OR=1.34; 95% CI, 0.77-2.34) or heterozygous (OR=0.98; 95% CI, 0.60-1.60) for A allele, or carriers of A allele genotype (OR=1.10; 95% CI, 0.70-1.73) were not associated with breast cancer risk. Analysis on clinico-pathological parameters showed that Val allele genotype was significantly correlated with nodal metastases but A allele genotype was not associated with any of the variables. Our findings suggest that the polymorphic alleles of HER2 and CCND1 may not play an important role as genetic markers for breast cancer risk, but presence of Val allele may be useful for tumor prognosis.

Keywords: HER2, Cyclin D1, genetic polymorphism, breast cancer.

The human HER2 proto-oncogene was mapped to chromosome 17q21.1 and encodes a 185 kDa cell surface glycoprotein with tyrosine kinase activity [1]. The transmembrane protein is highly homologous to other members of the type I tyrosine kinase receptor family [2]. HER2 is the preferred partner for heterodimerization for other members of the receptor family. Binding of ligand to the receptor leads to activation of tyrosine kinase which subsequently activates downstream signaling events regulating many cellular functions, such as cell proliferation and differentiation [3,4].

Amplification of the HER2 gene and overexpression of the gene product have been implicated in the pathogenesis of human breast cancer and the clinical significance of the receptor has been extensively studied in breast carcinogenesis. HER2 gene amplification and/or overexpression was detected between 20-30% of human breast carcinomas [5]. Clinical studies have shown that HER2 gene alteration was associated with reduced response to chemotherapy and hormonal therapy, and shorter overall survival [6]. It has been well documented that HER2 abnormalities were strongly associated with poor prognosis and aggressive behaviour in breast carcinomas [5]. An earlier study have demonstrated that the substitution of valine amino acid to glutamine at codon 664 of transmembrane region of the rat neu gene leads to constitutive neu dimerization and increases its enzymatic activity [2,7]. The single nucleotide polymorphism (SNP) in the human HER2 was identified in the transmembrane coding region of the gene at codon 655, encoding either isoleucine (ATC) or valine (GTC) [8]. A recent study reported that the presence of Val

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allele may enhance dimerization of HER2, resulting in increased autophosphorylation, tyrosine kinase activation and subsequently leading to cell transformation [9]. Xie *et al.* [10] was the first to report that the Val allele was significantly associated with an increased risk of breast cancer development, particularly among younger women. Subsequent studies have shown that this association is controversial with some studies showed presence of association between Val allele and breast cancer risk [10-12] whereas others demonstrated no association in breast cancer development [13-16]. The functional importance of Val allele in breast carcinogenesis has not been fully understood.

Cyclin D1 (CCND1), a member of the D-type cyclin proteins, is involved in the regulation of cell cycle progression from G1 into the S phase. Elevated levels of cyclin D1 disrupt normal cell cycle function, and may promote development of cancer [17]. Increased expression of CCND1 has been associated with increased cell proliferation and may lead to premature entrance into S phase of the cell cycle. Overexpression and/or amplification of cyclin D1 has been detected in different cancers, particularly in breast cancer [17]. Cyclin D1 is amplified and/or overexpressed in approximately 20 to 50% of breast carcinomas. Increased expression of CCND1 has been associated with good prognostic factors such as estrogen receptor positivity and well differentiated tumors, whereas amplification of the gene has been correlated with poor prognostic factors and early recurrence [18-22]. The common site for CCND1 polymorphism has been found at nucleotide 870 in codon 242 located in the conserved splice donor region of the exon 4 of the gene [23]. The A/G substitution has been shown to be associated with splicing of CCND1 mRNA but does not alter the amino acid. The polymorphism was shown to produce 2 types of transcripts [23]. The transcript-a from the CCND1 G allele is produced by normal splicing of the exon 5. The alternate transcript, transcript-b, arising from the CCND1 A allele does not splice at the exon 4-intron 4 boundary and has a longer half-life. Several studies have reported association between the CCND1 G870A polymorphisms and an increased risk in different types of cancer such as colorectal cancer [24-26], squamous cell carcinoma (SCC) of the esophagus [27], lung cancer [28], SCC of the head and neck [29], bladder cancer [30] and cervical cancer [31]. However, some investigators found no association with increased risk in colorectal carcinoma, SCC of the esophagus, gastric cancer and breast carcinomas [32-36].

Female breast cancer is the most common cancer among women in Malaysia. The data from the National Cancer Registry of Malaysia 2003 indicated that the life time risk of developing breast cancer for a woman in Malaysia is 1 in 20 [37]. It was accounted for 31.0% of newly diagnosed female breast cancer in Malaysian women. Breast cancer is a disease of multifactorial background and the influence of some factors such as genetic and environmental factors may differ according to geographic localization. To the best of our knowledge, association of HER2 Ile655Val and CCND1 G870A polymorphisms with breast cancer risk have not been studied in Malaysian population. In the present study, we determined the genotype and allele frequencies of HER2 and CCND1 polymorphisms in breast cancer cases and normal controls, and evaluated the association between these polymorphisms and breast cancer risk in a hospital-based Malaysian population, and also with established clinico-pathological parameters such as ER status, nodal status and histological grading. The patients were stratified by age at diagnosis to determine any association between the polymorphisms and breast cancer risk among women younger or older than 50 years old.

Materials and Methods

Patients and tissues. The study population consists of 230 women who were confirmed as having breast cancer, and 200 women who were healthy and had no history of breast disease or a family history of breast cancer as normal controls. The present study was approved by the Medical Ethics Committee of University Malaya Medical Centre, University of Malaya, Malaysia. Written informed consent was obtained from these patients before proceeding further for collection of peripheral blood. Histopathological evaluation of the tissues confirmed that 230 patients had invasive ductal carcinoma. The relevant clinico-pathological information was obtained from the pathology report of each patient which has been reported previously [38]. In Malaysia, the age pattern in 2003 showed that the age specific incidence rate peaks in the 50-59 years age group [37]. The patients were divided according to the age at diagnosis: age < 50 years and age ≥ 50 years. A total of 104 patients and 96 normal controls were less than 50 years old, and 126 patients and 104 normal controls were 50 years and above. The mean ages of breast cancer patients and normal controls were 52.04 and 50.30 years, respectively.

Genotyping at codon 655 of HER2 and nucleotide 870 of Cyclin D1 genes. Genomic DNA was isolated from the peripheral blood samples according to the method provided by the manufacturer with some modification using PUREGENE Genomic DNA Purification kit (Gentra, USA) as described previously [38].

The polymorphisms at codon 655 of HER2 and nucleotide 870 of CCND1 genes were analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as described by Xie *et al.* [10] and McKay *et al.* [36], respectively. The primer sequences used to amplify the polymorphic sites of HER2 (148 bp) and CCND1 (167 bp) genes were as follows: HER2fwd: 5'-AGA GCG CCA GCC CTC TGA CGT CCA T-3'; HER2rev: 5'-TCC GTT TCC TGC AGC AGT CTC CGC A-3'; CCND1fwd:. 5'-GTG AAG TTC ATT TCC AAT CCG C -3'; CCND1rev: 5'-GGG ACA TCA CCC TCA CTT AC-3' The primer sequences for HER2 and CCND1 have been published previously [10,36].

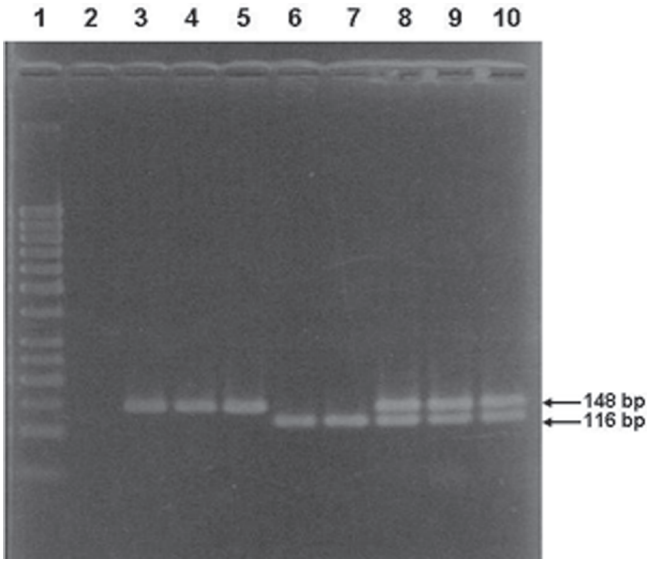

Figure 1. PCR-RFLP analysis at codon 655 of HER2 gene was performed on DNA extracted from peripheral blood samples of breast cancer patients, and normal healthy controls. Each lane shows the presence or absence of nondigested 148 bp fragments and/or digested 116 bp fragments. The 32 bp fragment is not clearly visualized. Lane 1: 50 bp DNA ladder molecular weight marker. Lane 2: negative control of deionized water. Lanes 3,4,5: homozygous for Ile allele; Lane 6,7: homozygous for Val allele; Lane 8,9,10 heterozygous for Ile and Val alleles.

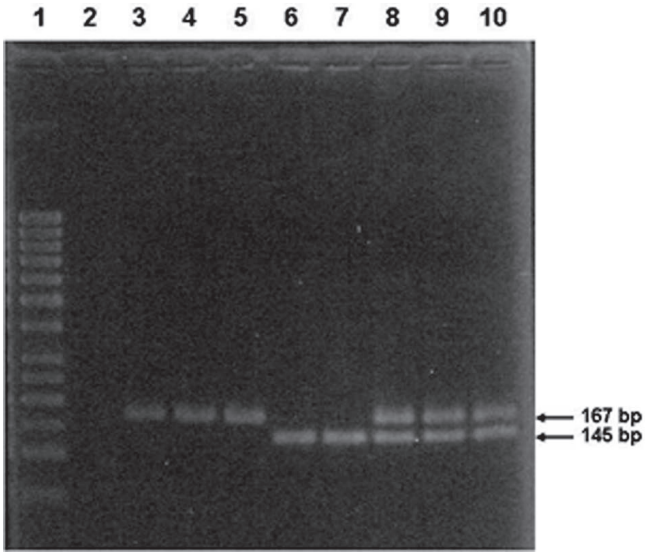

Figure 2. PCR-RFLP analysis at nucleotide 870 of CCND1 gene was performed on DNA extracted from peripheral blood samples of breast cancer patients, and normal healthy controls. Each lane shows the presence or absence of nondigested 167 bp fragments and/or digested 145 bp fragments. The 22 bp fragment is not clearly visualized. Lane 1: 50 bp DNA ladder molecular weight marker. Lane 2: negative control of deionized water. Lanes 3,4,5: homozygous for A allele; Lanes 6,7: homozygous for G allele. Lane 8,9,10: heterozygous for G and A alleles.

Each PCR amplification was performed in a 50 µl reaction mixture containing 100 ng genomic DNA, 1X PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$], 250 µM each of dGTP, dCTP, dTTP and dATP, 0.2 µM of each primers and 2.5 U of Ampli*Taq* DNA polymerase (Fermentas, Lithuania). The samples were amplified in a thermalcycler (Eppendorf, Hamburg, Germany). The cycling parameters for HER2 was 94°C for 4 min. followed by 35 cycles of 94°C for 30 sec., 60 °C for 30 sec., 72 °C for 30 sec. and final cycle of 72 °C for 7 min, and CCND1 was 94°C for 4 min. followed by 35 cycles of 94°C for 1 min., 56 °C for 1 min., 72 °C for 1 min, and final cycle of 72 °C for 7 min. In each PCR run, DNA was substituted with sterile deionized water as a negative control. The 7 µl amplified HER2 and CCND1 PCR products were digested with *BsmA1* (New England BioLabs, Beverly, MA, USA) at 55°C for 3 hrs and *ScrF*1 (New England BioLabs, Beverly, MA, USA) at 37°C for 3 hrs, respectively. The 116 bp and 32 bp fragments indicates presence of G (GTC) for the Val allele and 148 bp fragment in the presence of A (ATC) for the Ile allele at codon 655 of the HER2 gene. The 167 bp amplicons were not digested if A allele is present, but cleaved into 145 bp and 22 bp fragments when G allele is present at nucleotide 870 of the CCND1 gene.

Immunohistochemistry. The immunohistochemistry of estrogen receptor was performed using the DAKO EnVision™ System (Dako, Denmark) according to the manufacturer's guidelines. The immunostaining method has been described previously [38].

Statistical analysis

Analysis of data was performed using Epi Info (version 6.0, Atlanta, USA). The odds ratio (OR) and its 95% confidence interval (CI) was used to determine the correlation between the genotypes or alleles of HER2 Ile655Val and CCND1 G870A polymorphisms and breast cancer risk. The association between the genotypes of the genes and breast cancer risk among women younger than 50 years, and 50 years and older were also analyzed. The significance of association between the observed and expected number of the genotypes for a population in the Hardy-Weinberg equilibrium was analyzed using the chi-square test. The test was also used to demonstrate the significant difference of genotype and allele frequencies between the breast cancer cases and normal controls, and also association between the genotype frequencies and clinico-pathological parameters. We used the Yates corrected chi-square test to calculate the P values. The 0.05 (5%) level of significance was used throughout the statistical test.

Results

The restriction analysis at HER2 Ile655Val and CCND1 G870A polymorphisms were shown in Figure 1 and 2, respectively. The genotype and allele frequencies of HER2 and CCND1 polymorphisms in the breast cancer cases and normal controls in a hospital-based Malaysian population were

Table 1. Distribution of HER2 alleles and genotype frequencies in breast cancer cases and the control group.

	Cases No. (%)	Controls No. (%)	OR (95%CI)	χ^2 P*-value
HER2 Genotype	(n=230)	(n=200)		
Ile/Ile	165 (71.7)	159 (79.5)*	1.00 (reference)	P=0.163+
Ile/Val	57 (24.8)	37 (18.5)	1.48 (0.91-2.43)	P=0.123
Val/Val	8 (3.5)	4 (2.0)	1.93 (0.51-7.77)	P=0.437
Val Carrier (Ile/Val + Val/Val)	65 (28.3)	41 (20.5)	1.53 (0.95-2.45)	P=0.0801
Age at Diagnosis				
< 50 years	(n=104)	(n=96)		
Ile/Ile	75 (72.1)	77 (80.2)	1.00 (reference)	
Val carrier	29 (27.9)	19 (19.8)	1.57 (0.77-3.20)	P=0.241
≥ 50 years	(n=126)	(n=104)		
Ile/Ile	90 (71.4)	82 (78.8)	1.00 (reference)	
Val carrier	36 (28.6)	22 (21.2)	1.49 (0.78-2.87)	P=0.255
HER2 Alleles	No. of alleles (n=460)	No. of alleles (n=400)		
Ile	387 (84.1)	355 (88.8)	1.00 (reference)	
Val	73 (15.9)	45 (11.2)	1.49 (0.98-2.26)	P=0.0622

Note: '+' represents chi-square analysis between breast cancer cases and normal controls for HER2 genotypes
'*' represents significance at P<0.05

Table 2. Distribution of CCND1 alleles and genotype frequencies in breast cancer cases and the control group.

	Cases No. (%)	Controls No. (%)	OR (95%CI)	χ^2 P*-value
CCND1 Genotype	(n=230)	(n=200)		
GG	58 (25.2)	54 (27.0)	1.00 (reference)	P=0.377+
GA	103 (44.8)	98 (49.0)	0.98 (0.60-1.60)	P=0.979
AA	69 (30.0)	48 (24.0)	1.34 (0.77-2.34)	P=0.336
A carrier (GA+AA)	172 (74.8)	146 (73.0)	1.10 (0.70-1.73)	P=0.757
Age at Diagnosis				
< 50 years	(n=104)	(n=96)		
GG	28 (26.9)	26 (27.1)	1.00 (reference)	
A carrier	76 (73.1)	70 (72.9)	1.01 (0.52-1.97)	P=0.893
≥ 50 years	(n=126)	(n=104)		
GG	30 (23.8)	28 (26.9)	1.00 (reference)	
A carrier	96 (76.2)	76 (73.1)	1.18 (0.62-2.23)	P=0.697
CCND1 Alleles	No. of alleles (n=460)	No. of alleles (n=400)		
G	219 (47.6)	206 (51.5)	1.00 (reference)	
A	241 (52.4)	194 (48.5)	1.17 (0.89-1.54)	P=0.284

Note: '+' represents chi-square analysis between breast cancer cases and normal controls for CCND1 genotypes
'*' represents significance at P<0.05

shown in Table I and Table II, respectively. The genotype frequencies of HER2 was conformed to the Hardy-Weinberg equilibrium in both the cases (P=0.849) and controls (P=0.675). The distribution of CCND1 genotypes was also consistent with Hardy-Weinberg equilibrium in cases (P=0.533) and controls (P=0.969).

The genotype frequencies of HER2 polymorphism were not significantly different between the breast cancer cases and normal subjects (P=0.163). Although the distribution of HER2 genotypes lack statistical significance, the frequencies of Ile/Val (24.8%), Val/Val (3.5%) and Val allele genotype (Ile/Val + Val/Val) (28.3%) in breast cancer cases were elevated compared to the frequencies of Ile/Val (18.5%), Val/Val (2.0%) and Val allele genotype (Ile/Val+Val/Val) (20.5%) observed in normal individuals. Our data showed that women who were homozygous (OR=1.93; 95% CI, 0.51-7.77) or heterozygous

Table 3. Association between genotype frequencies of the HER2 Ile655Val polymorphism and clinico-pathological parameters of the breast cancer patients.

Clinico-pathological parameters	Total no. of cases (n=230)	Ile/Ile Genotype (%) (n=165)	Val allele genotype (Ile/Val+Val/Val) (%) (n=65)	χ^2 P*-value
Estrogen Receptor (ER) Status				
ER+	116	90 (54.5)	26 (40.0)	
ER-	105	70 (42.4)	35 (53.8)	P=0.0964
N/A+	9	5 (3.1)	4 (6.2)	
Lymph Node (N) Status				
N+	96	61 (37.0)	35 (53.8)	
N-	124	98 (59.4)	26 (40.0)	P=0.0167
N/A+	10	6 (3.6)	4 (6.2)	
Histological Grade				
Grade I	26	17 (10.3)	9 (13.8)	
Grade II	91	68 (41.2)	23 (35.4)	P=0.641
Grade III	70	51 (30.9)	19 (29.2)	
N/A+	43	29 (17.6)	14 (21.6)	

Note: N/A: not available
'+' represents values that are not included in the chi-square analysis.
'*' represents significance at P<0.05

(OR=1.48; 95% CI, 0.91-2.43) for Val allele or carriers of Val allele genotype (OR=1.53; 95% CI, 0.95-2.45) were not significantly associated with breast cancer risk. The distribution of Ile and Val allele frequencies were not significantly different between breast cancer patients and the normal individuals (P=0.0622). The data showed that the individuals who were carriers of Val allele (OR=1.49; 95% CI, 0.98-2.26) were not associated with an increased risk of breast cancer. In the age stratified analysis, we observed no significant correlation between Val allele genotype and breast cancer risk among women younger than 50 years (OR=1.57; 95% CI, 0.77-3.20) or 50 years and older (OR=1.49; 95% CI, 0.78-2.87) at diagnosis.

The distribution of CCND1 genotypes between the cancer cases and normal individuals showed lack of statistical significance (P=0.377). We noted that the frequencies of AA (30%) and A allele genotype (GA+AA) (74.8%) were higher in breast cancer cases compared to the frequencies of AA (24.0%) and A allele genotype (73.0%) seen in normal individuals. However, GA genotype is increased in normal women (49.0%) as compared to the cancer cases (44.8%). Individuals with the homozygous AA (OR=1.34; 95% CI, 0.77-2.34) and heterozygous GA (OR=0.98; 95% CI, 0.60-1.60) genotypes were not significantly associated with breast cancer risk. Similarly, women who were carriers of A allele genotype (GA+AA) (OR=1.10; 95% CI, 0.70-1.73) also showed no significant risk of developing breast cancer. Analysis on allele distribution showed that the frequency of allele A and G was not significantly different between the cancer cases and the controls (P=0.284). Individuals who were carriers of A allele (OR=1.17; 95% CI, 0.89-1.54) were not associated with an increased risk of breast cancer. Age stratification analysis showed that women carrying G allele genotype who were less than 50 years old (OR=1.01; 95% CI, 0.52-1.97) or 50 years old and above (OR=1.18; 95% CI, 0.62-2.23) were not significantly associated with increased risk of breast cancer.

Table III and IV summarize the relationship between the HER2 Ile655Val or CCND1 G870A genotypes and clinico-pathological parameters such as ER status, lymph node involvement and histological grade. Patients who were carriers of Val allele genotype showed significant association with lymph node metastases than those with Ile/Ile genotype (P=0.0167). We noted that the frequencies of the Val allele genotype were higher in the lymph node positive (53.8%) than in the lymph node negative (40.0%) patients whereas Ile/Ile genotype was represented more frequently in node negative (59.4%) compared with node positive (37.0%) patients. Significant difference was not observed in patients with ER status (P=0.0964) and histological grade (P=0.641). With regard to CCND1 A allele genotype, no significant relationship was seen with ER status (P=0.258), nodal status (P=0.158) and tumor grade (P=0.510).

Discussion

Our findings demonstrated that the genotype and allele frequencies of HER2 Ile655Val polymorphism among the breast cancer cases were not significantly different from the normal subject groups. However, we found that the frequencies of Ile/Val and Val/Val were higher in breast cancers (24.8% vs 3.5%) than in the controls (18.5% vs 2.0%), respectively. Similarly, there was higher representation of the Val allele (15.9%) among the cases compared with the normal individuals (11.2%). In the present study we also found that individuals who were heterozygous for Val allele or carriers of Val allele

Table 4. Association between genotype frequencies of the CCND1 G870A polymorphism and clinico-pathological parameters of the breast cancer patients.

Clinico-pathological parameters	Total no. of cases (n=230)	GG Genotype (%) (n=58)	A allele genotype (GA+AA) (%) (n=172)	χ^2 P*-value
Estrogen Receptor (ER) Status				
ER+	116	33 (56.9)	83 (48.3)	
ER-	105	22 (38.0)	83 (48.3)	P=0.258
N/A+	9	3 (5.1)	6 (3.4)	
Lymph Node (N) Status				
N+	96	29 (50.0)	67 (40.0)	
N-	124	26 (44.8)	98 (57.0)	P=0.158
N/A+	10	3 (5.2)	7 (3.0)	
Histological Grade				
Grade I	26	8 (13.8)	18 (10.5)	
Grade II	91	23 (39.7)	68 (39.5)	P=0.510
Grade III	70	14 (24.1)	56 (32.5)	
N/A+	43	13 (22.4)	30 (17.5)	

Note: N/A: not available
'+' represents values that are not included in the chi-square analysis.
'*' represents significance at P<0.05

genotype were not significantly associated with increased risk of breast cancer. Individuals who were Val/Val homozygotes also showed no association with breast cancer risk. Xie and colleagues [10] were the first to report that women homozygous for the Val/Val genotype were at increased risk of breast cancer in Shanghai-Chinese population but no association was noted in women heterozygous for Val allele. The authors also found that women who were less than 45 years old with Val/Val genotype were at higher risk of developing breast cancer compared with women older than 45 years. Montgomery and coauthors [39] showed increased risk among Australian women less than 40 years old. Rutter and coinvestigators [40] noted an increased risk of breast cancer for Val allele carriers at younger age in Ashkenazim population, particularly among women with a family history of breast cancer. In contrast, Pinto and colleagues [12] reported an association between increased risk of breast cancer and Val allele genotype among Portuguese women who were older than 46 years old. Wang-Gohrke and Chang-Claude [14] demonstrated that Val/Val and Ile/Val genotypes were not linked to breast cancer risk among German Caucasians. However, when the data were stratified the authors noted that the Val allele genotype increased the risk of breast cancer in women with a first–degree family history of breast cancer. In a Hawaii and Los Angeles Multiethnic Cohort study, McKean-Cowdin *et al.* [11] showed that women with Val allele genotype were associated with increased risk of localized breast cancer but not with regional or metastatic disease. In a recent study, Zubor and colleagues [41] noted that women who were Val/Val homozygotes were associated with breast cancer risk in Slovakian populations. However, others studies showed no significant association between HER2 polymorphism and breast cancer risk in several populations such as Japanese [13], Turkish [42], Iranian [43], Korean [44], New York-Latinas [45], New York-Caucasian [45], Northern Greek [46], British [47], North Carolina African-American and North Carolina Caucasian [15]. The data from some of the published studies were not comparable because some of the studies have examined Ile/Val genotypes separately from the Val/Val genotype whereas some authors have combined both the genotypes, which may alter the significance of association with breast cancer risk.

Using a computational exploration, Fleishman *et al.* [9] reported that the transmembrane region of HER2 homodimer can exists in two stable conformations either as an active or inactive form. The authors found that substitution of Val for Ile in this position of the transmembrane region will destabilize the formation of active HER2 dimers. This results in reduced receptor activation and tyrosine kinase activity, even during overexpression of HER2 receptor. However, presence of the Val allele was associated with the formation of active dimers of the transmembrane domain which result in increased autophosphorylation, tyrosine kinase activation and cell transformation. Based on computational analysis, presence of Val allele in the transmembrane region of HER2 gene indicates that the gene could be a strong candidate for breast cancer susceptibility. However, the data from the present study do not support the association between Val allele and breast cancer risk. These findings indicate that HER2 polymorphism at codon 655 may not play an important role in breast cancer susceptibility in Malaysian population.

In the present study, we found that genotype and allele frequencies of the CCND1 G870A polymorphism were not significantly different between breast cancer cases and normal subjects. Although the genotype and allele distribution

were not statistically significant, the AA genotype and allele A were more frequent in cancer cases (30% vs 52.4%) than in controls (24% vs 48.5%), respectively. Some studies have reported lower frequency of AA genotype in breast cancer cases [34,35,48] while others have noted higher frequency of AA genotype in cancer cases [49,50] as compared to the controls. The frequencies of AA genotype in cancer cases as reported by Kripp *et al.* [35] (27.8%), Frosti *et al.* [48] (21.5%) and Grieu *et al.* [34] (21%) were lower, but the frequencies demonstrated by Shu *et al.* [49] (31.5%) and Ceschi *et al.* [50] (40.4%) were higher compared to the frequency found in the present study (30%). However, when GG homozygote was used as the reference, we noted that individuals who were AA homozygote, GA heterozygote or carriers of A allele genotype were not associated with breast cancer risk. Similarly other investigators were not able to demonstrate any link between the polymorphic CCND1 locus and breast cancer risk in other populations including the Australians [34], Austrians [35], Finnish [48] and Singapore Chinese [50]. Among the Shanghai-Chinese population, Shu *et al.* [49] demonstrated that individuals who were homozygous for A allele and carriers of A allele genotype were not associated with breast cancer risk, but weak association was seen in those who are GA carriers. Our data suggests individuals who are carrying allele A may not be susceptible to breast cancer in Malaysian population.

In contrast to breast cancer, several studies have linked CCND1 A allele to increased susceptibility to a variety of cancers including squamous cell carcinoma (SCC) of the head and neck [29,51], non-small cell lung cancer [23,28], hepatocellular carcinoma [52], endometrial carcinoma [53], prostate cancer [54], colorectal carcinoma [24,25,55,56], esophageal and gastric cardiac carcinoma [27], SCC of the upper aerodigestive system [57] and urinary bladder carcinoma [30]. On the other hand, discordant results were seen between CCND1 polymorphism and the risk of developing SCC of the uterine cervix [58], SCC of the esophagus [33], oral cancer [59], gastric cancer [32] and colorectal carcinoma [36]. In some studies CCND1 polymorphism has also been associated to prognosis in pituitary adenomas [60], ovarian carcinomas [61], non-small cell lung carcinomas [23] and SCC of the head and neck [62]. Bettichar *et al.* [23] reported that individuals with AA genotype produce altered transcript, the transcript-b, that may have longer half-life. Therefore, cells with damaged DNA carrying allele A may bypass the G1/S phase checkpoint easily compared with individuals not carrying the polymorphism. Sawa *et al.* [63] demonstrated that high levels of normal transcript, transcript-a, inhibit entry into and completion of S phase. The authors also reported that patients with GG genotype had longer disease-free survival than patients with the polymorphism. These observations suggest that the expression level of CCND1 polymorphism transcript may influence the biological behaviour of cells, thus alters the risk of developing different types of cancers.

Stratification based on age at diagnosis revealed no evidence of association between carriers of Val allele genotype or A allele genotype and breast cancer risk among women younger than 50 or 50 years and older. Other studies did not reveal any correlation between Val allele genotype [16,43,44] or A allele genotype [34,35] and patients' age. However, Shu and coinvestigators [49] noted that CCND1 polymorphism was weakly associated with breast cancer risk in younger women. Although the age specific incidence rate is the highest in the 50-59 age group, no statistical association was observed between Val allele or A allele and risk of breast cancer among women younger or older than 50 years at diagnosis suggesting that breast cancer risk by these alleles is unrelated to patients' age. A clinico-pathological analysis showed that Val allele genotype was associated with lymph node metastases but not with estrogen receptor status and histological grade. Furthermore, we also noted that Ile/Ile genotype was found more frequent in node negative compared with node positive patients. It has been suggested that Val allele might enhance but Ile allele might reduce tyrosine kinase activity [9]. Benlimame *et al.* [64] reported that increased activity of tyrosine kinase is essential to induce invasion and metastases. This probably explains the positive correlation between Val allele and nodal metastases noted in the present study. Based on our observation Val allele might have an important role for tumor metastatic capacity. Kamali-Sarvestani *et al.* [43] found that Val allele was not associated with steroid status but no analysis was performed on nodal status and tumor grade. To the best of our knowledge majority of the studies did not compare association between HER2 polymorphism and nodal status [11-16,39-42, 44-47]. Significant correlation was not observed between the clinico-pathological parameters and A allele genotype in breast cancer patients. Similar findings were also reported by other investigators where the authors found no link with overall survival, tumor size, histological grade, lymph node involvement and estrogen receptor status [34,35]. It has been well documented that tumors lacking of estrogen receptors, demonstrate nodal metastases and poorly differentiated were associated with poor prognosis and worse clinical outcome. In the present study, an association with nodal metastases suggests that Val allele may have the potential to be an indicator for poor prognosis but A allele may not be a useful marker for tumor prognosis.

The conflicting reports between the studies could be due to factors such as selection bias, sample size, heterogenous ethnic background, and genetic factors within the ethnic groups that predispose them to breast cancer. In addition, small number of individuals with Val/Val or AA genotype can decrease statistical power to determine the association between the polymorphisms and cancer risk. In conclusion, our data shows that HER2 Ile655Val and CCND1 G870A gene polymorphisms may not be potential markers for breast cancer risk but Val allele of HER2 gene may have the potential to be a genetic marker for tumor prognosis at least in a hospital-based Malaysian population.

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