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ORIGINAL ARTICLE

Genetic polymorphisms in cell cycle regulatory genes CCND1 and CDK4 are associated with susceptibility to breast cancer

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Summary

Purpose: *This study was performed to screen cyclin D1 (CCND1) and cyclin dependent kinases (CDK4) genes in order to evaluate their association with breast carcinogenesis.*

Methods: *The germline screening of these genes was carried out by combining polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP), followed by DNA sequence analysis. A total of 400 individuals (200 breast cancer patients and 200 healthy controls) were recruited prospectively for this study.*

Results: *Sequence analyses of the coding region of CCND1 revealed 12 mutations. When analyzed, a significant association was found between CCND1 mutations and breast cancer. It was observed that a 6-fold increased breast cancer risk (odds ratio/OR=5.75, 95% confidence interval/CI=1.26-26.33) was associated with Cys7Tyr in breast cancer patients when compared with healthy controls. In addition, a 5-fold increased breast cancer risk was associated with Trp63Stop mutation (OR=5.44, 95% CI=1.82-16.23), 10861C>A (OR=4.84; 95% CI=1.60-14.58) and 7720insTT (OR=5.32, 95% CI=1.98-14.23) in breast cancer patients compared to healthy controls. Concerning CDK4 gene, 5 mutations were identified and a significant association was observed between CDK4 gene mutations and breast cancer. It was observed that a 6-fold increased risk of breast cancer (OR=5.71, 95% CI=0.29-4.65) was associated with 5693 T>A. In addition, a 5-fold increased risk of breast cancer (OR=5.05, 95% CI= 2.17-11.78) was associated with 5732 G>A in breast cancer patients compared to controls.*

Conclusion: *In this study, our results showed that CCND1 and CDK4 mutations are associated with an increased risk of breast cancer, and may serve as biomarkers for early diagnosis and detection of breast cancer.*

Key words: *breast carcinogenesis, CCND1, CDK4, cell cycle genes, SSCP*

Introduction

Breast cancer is the most commonly diagnosed malignancy in females worldwide, including Pakistan. The incidence rate of this cancer in Pakistan is 69.1/100,000, and every ninth Pakistani woman is expected to develop breast cancer, which is the highest incidence rate among Asian populations [1]. Development of breast cancer in Pakistan appears to be a multifactorial process associated with a variety of risk factors such as environmental factors, hormonal factors and genetic susceptibility [2]. Genetic variations in genes involved in different pathways such as metabolism, DNA repair and cell cycle have been implicated as susceptibility factors in cancer development including breast carcinogenesis. Involvement of genetic susceptibility of cell cycle genes such as CCND1 and CDK4 has also been studied for possible association with different cancers in Pakistan [3].

However, there is little data available regarding the association of these genes with breast cancer.

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Cyclin D1 is encoded by CCND1 proto-oncogene which is located on chromosome 11q13 in humans. It is a key regulator of cell cycle transition from G1-S phase [4]. A common single nucleotide polymorphism (SNP) (A870G) located at 242 codon on exon 4 results in two mRNA transcripts (isoform a and isoform b). Isoform b has been reported to cause mostly breast, colon and other types of cancers [5]. Another well-known mutation A/A has been associated with increased predisposition to breast and colon cancer [6]. The gene and its product have been examined in cases of cancer and there is significant evidence of its involvement in breast, lung, colon, head and neck, bladder and liver cancers, melanoma, oral squamous cell carcinomas and mantle cell lymphoma [7].

Cyclin dependent kinase (CDK) gene is another important member of the cell cycle pathway which belongs to serine/threonine kinase family. It makes a complex with cyclin D1 and phosphorylates RB gene. CDK4 is located on chromosome 12q13. Apart from the role of CDKs and cyclin D in cell cycle pathway, these genes are also involved in other cellular functions like cytoskeleton rearrangement and cell migration [8]. Mechanisms of activation of this gene include gene amplification, overexpression, decreased degradation and activating point mutations, whereas in case of genetic variations, rare mutations in CDK4 gene have been identified worldwide [9]. These reported mutations are located in exon 2, a region which codes for p16INK4A binding site [10]. CDK4 activity is deregulated in many human tumors including gliomas, sarcomas, head and neck cancers, breast tumors and colorectal carcinomas [11]. More research work is required to completely understand the role of this gene in breast carcinogenesis. In this study we tried to evaluate the contribution of CCND1 and CDK4 germline mutational spectrum to breast cancer in different ethnic groups of Pakistani populations.

Methods

Patients

The study group consisted of 200 patients with histologically confirmed breast cancer and 200 cancer-free healthy controls. All subjects were age- and gender-matched. Breast cancer patients were recruited at National Oncology and Radiotherapy Institute (NORI) during 2013-2014. The inclusion criteria for the controls were absence of prior history of cancer or pre-cancerous lesions. Patients and controls suffering from any other disease (diabetes, blood pressure and cardiovascular diseases etc) were excluded from this study. At the time of recruitment, informed consent was obtained from each subject and each participant was then interviewed to seek detailed information on demographic characteristics. The present study was conducted after approval from the Ethics Committees of both CIIT (COMSATS Institute of Information Technology, Islamabad) and NORI. The demographic data obtained from both patients and controls are given in Table 1.

Primer designing

Human CCND1 and CDK4 gene sequence was taken from Ensembl genome browser software (http://www.ensemble.org/index.html). Primers were designed by using primer 3 input software and BLAST. Exonic regions and their exon-intron boundaries of approximately 60 bp sequence of both genes were also investigated to identify any splice site variation.

DNA isolation and amplification

Genomic DNA was isolated from the blood using standard phenol–chloroform extraction method [12] and stored at - 20 °C for further processing. PCR was performed in a 20 µl reaction mixture containing approximately 20 ng DNA, 10 mM of each primer, 25 mM of each dNTP, 0.2 µl Taq DNA polymerase with 2 µl 109 reaction buffer. Amplification was carried out under the following conditions: an initial melting step of 5 min at 94 °C, followed by 35 cycles of 45 sec at 94 °C, 1 min for annealing temperature and 1 min at 72 °C, and a final extension step of 72 °C for 10 min and hold at 4 °C. PCR products were electrophoresed on a 2 % agarose gel and stained with ethidium bromide.

Detection of CCND1/CDK4 mutations

SSCP was used for the mutational analysis of the PCR products. Samples displaying an altered electrophoretic mobility were re-amplified in another reaction and were analyzed by direct sequencing to confirm and characterize the nature of mutations. Sequencing was carried out by MCLAB (USA). Healthy control samples were also sequenced along with cancer samples for comparison.

Statistics

Data were analyzed by using different statistical tools, which included two-tailed sample t-test (Student's t-test) and Fishers exact test at a level of $p<0.05$. For each gene (CCND1 and CDK4) mutations, allele and genotype frequencies were calculated and compared with controls according to Hardy Weinberg equilibrium. OR and 95% CI were calculated using unconditional logistic regression adjusted for age and sex. All statistical analyses were performed using Graph Pad PRISM statistical software, version 5.04.

Results

CCND1 mutations and associated breast cancer risk

In this study we screened the coding regions as well as intron/exon boundaries of CCND1 gene using PCR-SSCP and identified the samples with altered mobility patterns. These identified samples are then sequenced for detection of variations. In this regard, we observed 12 mutations in total in the CCND1 gene after DNA sequencing of identified variants. Among these mutations 4 missense mutations (Cys7Tyr, Glu9Lys, Ile13Val and Val27Met), one non-sense mutation (Trp63Stop) and one silent mutation (Ala20Ala) observed in exon 1. Two substitution mutations (1026G>T and 1030G>T) were observed in intron 1 of CCND1 gene. In addition, 4 mutations (7656 G>A, 7720insTT, 10811 C>T and 10861 C>A) were observed in the 3'UTR region of the CCND1 gene as shown in Table 2 and Figure 1.

Allele frequency of CCND1 polymorphisms were analyzed by Hardy Weinberg equilibrium method and are shown in Table 2. A 6-fold increase in breast cancer risk was associated with Cys7Tyr (847 G>A) mutation (OR=5.75, 95% CI=1.26-26.33; p<0.02) in breast cancer patients compared to controls. A 5-fold increase (OR=4.56, 95% CI=1.28-16.21) with Glu9Lys, 4-fold increase with Ile13Val (OR=4.26, 95% CI=1.39-12.98) and 4-fold increase with Val27Met (OR=3.68, 95% CI=1.1911.40) in breast cancer patients was observed, compared to healthy controls. A 5-fold increase in breast cancer risk was associated with nonsense mutation Trp63Stop (OR=5.44, 95% CI=1.82-16.23) in breast cancer patients compared to healthy controls. For silent mutation a 3-fold increase in breast cancer risk (OR=3.12, 95% CI=0.99-9.86) was associated with Ala20Ala in breast cancer patients compared to healthy controls. In case of substitution mutations, a 4-fold increase in breast cancer risk was associated with 1026G>T (OR=3.68, 95% CI=1.19-11.40) and 10811C>T (OR=3.78, 95% CI=2.09-6.81) mutation in breast cancer patients compared to healthy controls. Additionally a 3-fold increase was associated with 1030G>T (OR=3.39, 95% CI=1.32-8.68) and with 10811C>T (OR=3.59; 95% CI=1.41-9.14) mutation and a 5-fold increase was associated with 10861C>A (OR=4.84; 95% CI=1.60-14.58) in breast cancer patients compared to healthy controls. In the case of frameshift mutation a 5-fold increase in breast cancer risk (OR=5.32, 95% CI=1.98-14.23) was associated with 7720insTT in breast cancer patients compared to healthy controls (Table 3).

Table 1. Demographic characteristics of patients/controls included in this study

Characteristics	*Cases N (%)*	*Controls*
Age (years) Mean (±SD)	48.5 (±9.1)	45.22 (±0.7)
Gender Female	200	200
Family history		
Yes	34 (17)	
No	166 (83)	
Breast cancer location		
Right	88 (44)	
Left	97 (49)	
Bilateral	15 (7)	
Breast feeding		
Yes	168 (84)	
No	32 (16)	
Metastatic		
Yes	57 (29)	
No	143 (71)	
Age at menarche (years)		
< 13	126 (63)	
≥ 13	74 (37)	
Age at menopause (years)		
< 50	173 (86)	
≥ 50	27 (14)	

SD: standard deviation

The above-mentioned mutations were further correlated with demographic characteristics such as age at menarche and age at menopause as shown in Table 3. In case of age at menarche, a 11-fold increase in breast cancer risk was associated with Glu9Lys mutation (OR=10.8; 95% CI=2.85-41.1; p<0.0005), 3-fold increase with 1030 G>T mutation (OR=3; 95% CI=0.01-1.03; p<0.05) and 2-fold increase with 7656 G>A mutation (OR=2.37; 95% CI=0.89-6.28; p<0.0005) in patients with age at menarche >13 years compared to patients with age at menarche < 13 years. Concerning age at menopause, a 7-fold increase in breast cancer risk was associated with Glu9Lys mutation (OR=7.0; 95% CI=2.0-33.0; p<0.0009), a 15-fold increase with Ile13Val mutation (OR=15; 95% CI=3.0-71.0; p<0.01), a 12-fold increase with Val27Met mutation (OR=12; 95% CI=2.0-59.0; p<0.009) and a 7-fold increase with Trp63Stop mutation (OR=7; 95% CI=1.0-35.0; p<0.001) in patients with age at menopause < 50 years compared to patients with age at menopause >50 years. Moreover, a 5-fold increase in breast cancer risk was associated with 1026 G>T mutation (OR=5; 95 % CI=1.025.0; p<0.0002), a 17-fold increase with 7656 G>A mu-

Table 2. Association between the polymorphisms of CCND1 and risk of breast cancer patients and controls

Mutation	*No of controls (%)*	*Allele frequency*	*No of patients (%)*	*Allele frequency*	*OR (95% CI) ; p value*
Cys7Tyr (847 G>A) Exon 1	2 (3)	0.03,0.97	11 (5)	0.05, 0.05	5.75(1.26-26.33); 0.02
Glu9Lys (852 G>A) Exon 1	3 (5)	0.05, 0.95	13 (6)	0.06, 0.04	4.56 (1.28-16.21); 0.01
Ile13Val (864 A>G) Exon 1 (COSM1644302)	4 (6)	0.06, 0.04	14 (7)	0.07, 0.03	4.26 (1.39-12.98); 0.01
Ala20Ala (887 C>A) Exon 1 (rs143734358)	4 (6)	0.06, 0.04	12 (5)	0.05, 0.05	3.12 (0.99-9.86); 0.05
Val27Met (906 G>A) Exon 1	4 (6)	0.06, 0.04	14 (6)	0.06, 0.04	3.68 (1.19-11.40); 0.02
Trp63Stop (1016 G>A) Exon 1	4 (6)	0.06, 0.04	20 (9)	0.09, 0.01	5.44 (1.82-16.23); 0.002
1026 G>T Intron 1	4 (6)	0.06, 0.04	14 (6)	0.06, 0.04	3.68 (1.19-11.40); 0.02
1030 G>T Intron 1	6 (10)	0.09, 0.91	19 (7)	0.07, 0.03	3.39 (1.32-8.68); 0.01
7656 G>A Intron 4 (rs9344)	17 (28)	0.28, 0.72	52 (23)	0.23, 0.77	3.78 (2.09-6.81); 0.0001
7720insTT 3' UTR	5 (8)	0.08, 0.92	24 (10)	0.1, 0.9	5.32 (1.98-14.23); 0.0009
10811 C>T 3' UTR	6 (10)	0.1, 0.90	20 (9)	0.09, 0.01	3.59 (1.41-9.14); 0.007
10861 C>A 3' UTR, (rs7177)	4 (6)	0.06, 0.04	18 (7)	0.07, 0.03	4.84 (1.60-14.58); 0.005

Table 3. Assocation between the polymorphisms of CCND1 gene and age at menarche and menopause of breast cancer patients

Types of mutation	*Age at menarche*		*OR (95% CI); p value*	*Age at menopause*		*OR (95% CI); p value*
	<13years (%)	*≥13years (%)*		*<50years (%)*	*≥50years (%)*	
Cys7Tyr (847 G>A)	04 (8)	07 (4)	1.58 (0.44- 5.65); 0.4	09 (5)	02 (5)	0.21 (0.04- 1.23); 0.08
Glu9Lys (852 G>A)	03 (6)	10 (6)	10.8 (2.85-41.11); 0.0005	09 (5)	04 (9.5)	7 (2.0-33.0); 0.0009
Ile13Val (864 A>G)	06 (12)	08 (4)	2.1 (0.7-6.53); 0.1	11 (6)	03 (7)	15 (3.0 -71.0); 0.01
Ala20Ala (887 C>A)	03 (6)	09 (5)	1 (0.23-3.43); 0.8	10 (5)	02 (5)	0.25 (0.04-1.38); 0.11
Val27Met (906 G>A)	03 (6)	11 (6)	1 (0.19-2.69); 0.6	09 (5)	03 (7)	12 (2.0-59.0); 0.009
Trp63Stop (1016 G>A)	08 (16)	12 (7)	2 (0.74-5.04); 0.1	15 (8)	05 (12)	7 (1.0-35.0); 0.001
1026 G>T	03 (6)	11 (6)	1 (0.19-2.69); 0.6	11 (6)	03 (7)	5 (1.0-25.0); 0.0002
1030 G>T	01 (2)	18 (10)	3 (0.01-1.03); 0.05	17 (9)	02 (5)	0.4 (0.08-2.35); 0.33
7656 G>A	03 (6)	49 (27)	2.37(0.89-6.28); 0.0005	46 (24)	06 (14)	17 (3.0-91.0); 0.03
7720insTT	05 (10)	19 (10)	0.6(0.24-1.92); 0.4	22 (12)	02 (5)	0.59 (0.11-3.14); 0.5
10811 C>T	06 (12)	14 (8)	0.5 (0.11-2.46); 0.4	16 (9)	04 (9.5)	14 (3.0-60.0); 0.008
10861 C>A	05 (10)	13 (7)	1.0 (0.35-3.08); 0.9	12 (6)	06 (14)	36 (0.6-18.0); 0.0001

Table 4. Association between the polymorphisms of CDK4 and risk of breast cancer patients and controls

Mutation	*No in controls (%)*	*Allele frequency*	*No of patients (%)*	*Allele frequency*	*OR (95% CI); p value*
4289delT	5 (18)	0.18, 0.82	17 (16)	0.16, 0.84	3.62 (1.30-10.02); 0.01
4290delG	5 (18)	0.18, 0.82	17 (16)	0.16, 0.84	3.62 (1.30-10.02); 0.01
5332delT	8 (29)	0.28, 0.72	22 (21.5)	0.21, 0.79	2.96 (1.28-6.83); 0.01
5693 T>A	3 (10)	0.1, 0.9	16 (15.5)	0.15, 0.85	5.71 (0.29-4.65); 0.006
5732 G>A	7 (25)	0.25, 0.75	31 (30)	0.30, 0.70	5.05 (2.17-11.78); 0.0002

Table 5. Association between the polymorphisms of CDK4 gene and age at menarche and menopause of breast cancer patients

Types of mutation	*Age at menarche*		*OR (95% CI); p value*	*Age at menopause*		*OR (95% CI); p value*
	<13 years (%)	*≥13 years (%)*		*<50 years (%)*	*≥50 years (%)*	
4289delT	02 (10.5)	15 (18)	4 (7.0-21.0); 0.0002	13 (15)	04 (22)	11 (2.0-48.0); 0.003
4290delG	02 (10.5)	15 (18)	4 (7.0-21.0); 0.0002	12 (14)	05 (29)	5 (1.0-28.0); 0.0004
5332delT	03 (16)	19 (22)	2 (0.3-11.0); 0.0001	21 (25)	01 (5)	1.31 (0.15-11.22); 0.80
5693 T>A	02 (10.5)	14 (17)	4 (8.0-25.0); 0.002	12 (14)	04 (22)	9 (2.0-44.0); 0.002
5732 G>A	10 (52.5)	21 (25)	3 (0.3-25.0); 0.001	27 (32)	04 (22)	1.25 (0.05-1.08); 0.06

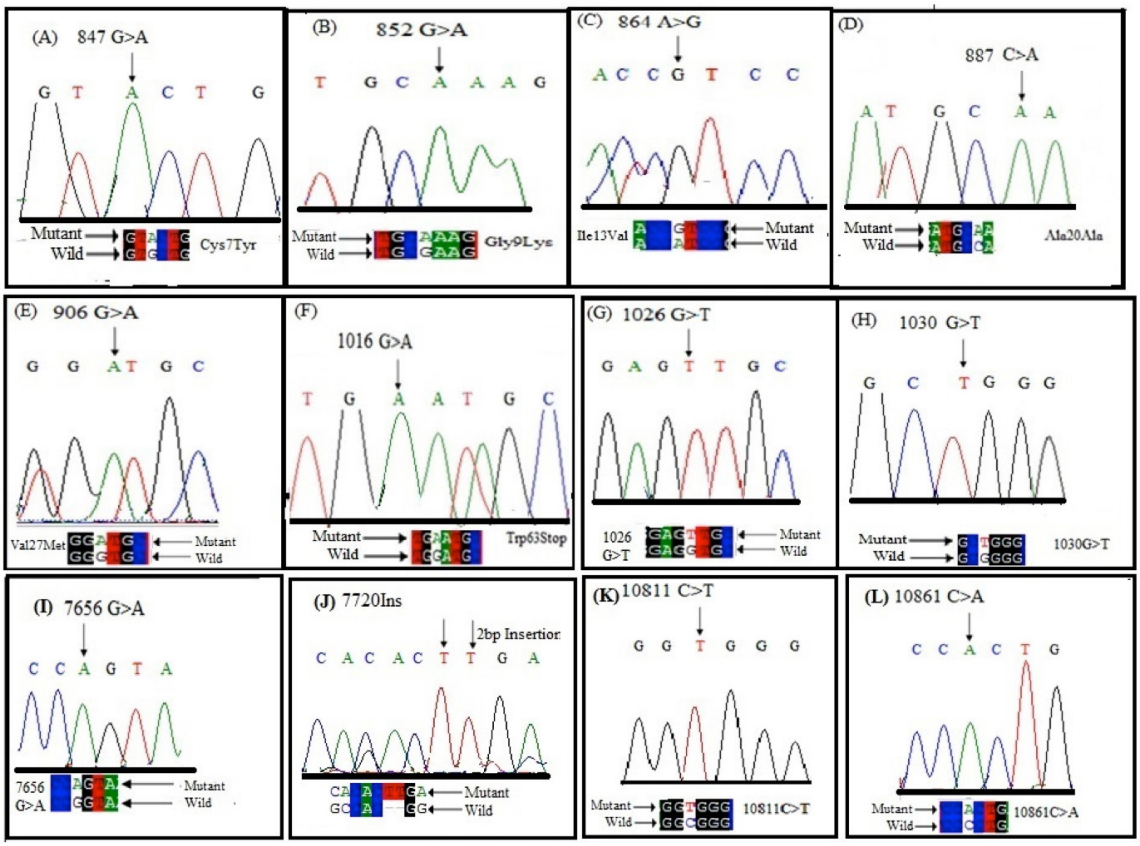

Figure 1. Sequencing electropherogram of mutations of CCND1 gene. **(A):** Missense mutation, Cys7Tyr (847 G>A); **(B):** Missense mutation, Glu9Lys (852 G>A); **(C):** Missense mutation, 864A>G (COSM1644302); **(D):** Silent mutation, Ala20Ala (887 C>A); **(E):** missense mutation, Val27Met (906 G>A); **(F):** Non-sense mutation, Trp-63Stop (1016 G>A); **(G):** substitution mutation, 1026 G>T; **(H):** substitution mutation, 1030 G>T; **(I):** Substitution mutation, 7656 G>A; **(J):** Frameshift mutation, 7720insTT; **(K):** Substitution mutation, 10811 C>T; **(L):** Substitution mutation, 10861 C>A .

tation (OR=17; 95% CI=3.0-91.0; p<0.03), a 14-fold increase with 10811 C>T mutation (OR=14; 95% CI=3.0-60.0; p< 0.008) and 36-fold increase with 10861 C>A mutation (OR=36; 95% CI=0.6-18.0; p<0.0001) in patients with age at menopause <50 years when compared to patients with age at menopause >50 years.

CDK4 mutations and associated breast cancer risk

PCR-SSCP and DNA sequence analysis of CDK4 gene in breast cancer patient samples and controls revealed 5 different types of mutations (3 frameshift and 2 substitution mutations) as shown in Table 4 and Figure 2. Among these mutations, 2 frameshift mutations (4289delT and 4290delG) were found in exon 1, one frameshift mutation (5332delT) was observed in exon 3, and 2 substitution mutations (5693T>A and 5732G>A)

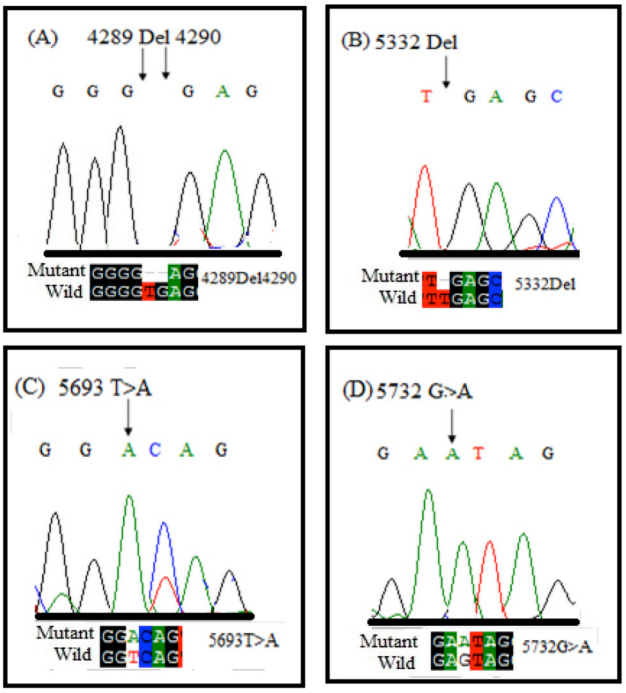

Figure 2. Sequencing electropherogram of mutations observed in CDK4 gene. **(A):** Frameshift mutations observed in exon 1 at position 4289 and 4290; **(B):** Frameshift mutation, 5332delT; **(C):** Substitution mutation, 5693T>A; **(D):** Substitution mutation, 5732G>A (rs2069502).

were observed on intron 4. The details of mutations are depicted in Table 4. Allele frequency of CDK4 mutations was observed by Hardy Weinberg equilibrium method and is shown in Table 4. In case of frameshift mutations a 4-fold increase in breast cancer risk was associated with 4289delG and 4290delG (OR=3.62, 95% CI=1.30-10.02) mutation in case of breast cancer patients compared to controls. However, a 2-fold increase in risk was associated with 5332delT mutation (OR=2.96, 95% CI=1.28-6.83) in patients when compared to controls. In case of intronic mutations observed in CDK4 gene, a 6-fold increase in breast cancer risk (OR=5.71, 95% CI=0.29-4.65) was associated with 5693T>A mutation and a 5-fold increase in risk

(OR=5.05, 95% CI= 2.17-11.78) was associated with the already reported substitution mutation (5732G>A, rs2069502) in breast cancer patients compared to healthy controls.

Furthermore, the above-mentioned mutations were correlated with demographic characteristics such as age at menarche and age at menopause as shown in Table 5. In case of age at menarche, a 4-fold increase in breast cancer risk was associated with 4289delT (OR=4, 95% CI=7.0-21.0; p<0.0002), with 4290delG (OR=4, 95% CI=7.0-21.0; p<0.0002) and with 5693 T>A (OR=4, 95% CI=8.0-25.0; p<0.002) mutation in patients with age at menarche >13 years when compared with patients <13 years. A 2- and 3-fold increase in breast cancer risk was associated with mutation 5332delT (OR=2, 95% CI=0.3-11.0; p<0.0001) and mutation 5732 G>A (OR=3, 95% CI=0.3-25.0; p>0.001), respectively in patients with age at menarche >13 years when compared to patients with age <13 years. In case of age at menopause, a 11-fold increase in breast cancer risk was associated with mutation 4289delT (OR=11, 95% CI=2.0-48.0; p<0.003), a 5-fold with mutation 4290delG (OR=5, 95% CI=1.028.0; p<0.0004) and a 9-fold with mutation 5693 T>A (OR=9, 95% CI=2.0-44.0; p<0.002) in patients with age at menopause <50 years compared with patients >50 years.

Discussion

In this study we genotyped breast cancer patients/control healthy individuals and investigated the association of CCND1 and CDK4 germline mutations with breast cancer risk. After mutational analysis of first selected gene CCND1, we identified 12 variants, 3 of which have already been reported while 9 are novel mutations. Among these mutations 2 missense mutations (Cys7Tyr, Glu-9Lys) were observed in retinoblastoma interacting site (RB interacting site) of CCND1 gene. RB interacting site of CCND1 is involved in binding and phosphorylation of RB gene. Early evidence suggested that the ability of cyclin D1 to promote RB binding and phosphorylation is crucial for its cell cycle function, as cancer cells that are defective in RB interacting site are refractory to cyclin D1 action [13]. Two missense mutations (Ile13Val and Val27Met) and one silent mutation (Ala20Ala) were also observed in exon 1. Missense mutations in the coding region result in amino acid substitution in the gene. Since the substitute and the original residues differ in chemical properties (polarity, hydrophobicity etc), these mutations may have a substantial effect on the conformation and consequently the functional activity of the gene [14]. In addition, the role of silent mutation (Ala20Ala) is yet to be explored; however, the silent mutation in conserved region can affect the timing of co-translational folding and consequent function [15].

In the present study, one nonsense mutation (Trp63Stop) was observed in cyclin box (exon 1) of the CCND1 gene. This site is specific for bind-

ing of CCND1 with CDKs to form CCND1/CDK complex. CyclinD:CDK complexes are believed to promote cell cycle progression through at least two-rate limiting steps in cell cycle pathway: by interacting with cell cycle inhibitors, such as p21 and p27, and by the phosphorylation of the retinoblastoma tumor suppressor [16]. This variant may be associated with a reduced ability to communicate with other cell cycle proteins such as CDKs as even a slight change in cell division capacity could be detrimental and many functional studies are underpowered to detect subtle changes. In addition to mutations observed in the coding region, 2 substitution mutations (1026 G>T and 1030 G>T) were also observed in the intronic (non-coding) region of the CCND1 gene. Unlike exonic mutations, intronic mutations do not usually result in aberrant expression of the gene. However, their impact on cancer risk may be present because of linkage with another susceptibility gene [17] or because of induction of aberrant splicing of mRNA, leading to mutant mRNA [18].

While screening the UTR region of CCND1 gene, 2 substitution mutations (10811 C>T and 10861 C>A) and one frameshift (7720insTT) mutation were observed in 3'UTR in this study. Mutations in this region can result in either exon skipping or cryptic splice site utilization and can possibly deregulate protein levels by affecting protein half life [19,20]. Mutations or loss of 3'UTR region of Cyclin D1 has been linked to over-expression of this proto-oncogene (Cyclin D1) in different cancers [21] and hyper-proliferation of Cyclin D1 in mantle cell lymphoma [22].

In the present study, the already reported mutation G870A [rs9344] in introns/exon boundary (introns 4/exon 5) of CCND1 gene was also observed. This specific polymorphism (G to A) is located in the splice site region and results in enhanced CCND1 alternate splice potential [23]. Several previous studies have reported the association between CCND1 G870A SNP and greater risk of a number of solid tumors including breast, head and neck, esophagus, lung, skin, kidney, colon, bladder and prostate cancer [24-27]. In addition, 3 substitution mutations (7733 G>T, 10811 C>T, and 10861 C>A) were observed in 3'UTR region of CCND1 gene. A mutation observed in the present study (10861C>A) has already been reported (rs7177) [28]. Although the mechanism of this polymorphism regulating the CCND1 gene is unknown, this is likely due to the fact that 3'UTR variants in regions of regulatory elements that control the nature and timing of gene expression, and their effects are only manifested under particular extracellular and/or intracellular stimuli [29]. As reported in earlier studies in some lymphoid tumors, rearrangement of the 3'UTR of CCND1 may play a role in its activation [30]. CCND1 mutation frequencies were also correlated with different clinico-pathological parameters and significant findings were observed. Higher mutation frequencies were found associated with early menopause and late menarche in present study. Butt et al. [31] reported increased incidence of breast carcinogenesis in patients with late menarche in Pakistani population. Younger age at first live birth decreased breast cancer risk in both pre- and post-menopausal women.

The second gene selected in the present study for germline analysis was CDK4, which is one the members of ser-thr protein kinase family. The results of PCR-SSCP and DNA sequencing in our study revealed 3 frameshift mutations and 2 substitution mutations in this gene. Two frameshift mutations in exon 1 (4289delT and 4290delG) and one frameshift mutation in exon 3 (5332delT) were observed in the CDK4 gene. Frameshift mutations observed in the coding region changes the whole downstream sequence of gene. As a result translation of wrong reading frame continues and the resultant premature RNA stability is compromised, so either the mRNA will not be stable or the protein will be degraded [3]. Kim et al. identified the frameshift mutations in cell-division cycle related genes. These mutations would affect folding and the stability of these cell cycle proteins and might inhibit their role in gastric and colorectal cancer [32]. In addition, 2 substitution mutations (5693T>A and 5732G>A) are reported in intron 4. 5732G>A (rs2069502) substitution mutation has already been reported in ovarian and breast cancer [33]. Substitution mutation in intronic region links with other susceptible genes or may result in aberrant gene expression [18]. Sequence variations occurring in exons or introns may affect the correct process of the mRNA by disrupting the splice site or altering the secondary structure of the mRNA [34]. CDK4 mutation frequencies were further correlated with different clinico-pathological parameters and significant findings were observed. Higher mutation frequencies were found associated with early menopause and late menarche in the present study.

In conclusion, our results suggest that CCND1 and CDK4 cell cycle genes are highly polymorphic

genes and their genetic variations are associated with breast carcinogenesis at least in Pakistani population. Additional research on these and other cell cycle gene mutations will be helpful to further explore the interaction between cell cycle pathways and carcinogenesis. It is obvious that further research on this topic will enlighten the complex landscape of cell division and cancer risk.

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