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RESEARCH ARTICLE

D Allele Frequency in Insertion/Deletion Polymorphism of the Angiotensin Converting Enzyme (ACE) Gene is Associated with Development of Breast Cancer Risk in Indian Women

Shashank Kumar[1,$], Syed Rizwan Hussain[1], Mohammad Waseem[1], Farzana Mahdi[2], Chery Bansal[3] and Mohammad Kaleem Ahmad[1*]

[1]Molecular Cell Biology Lab, Department of Biochemistry, King George's Medical University, Lucknow, Uttar Pradesh 226 003, India; [2]Department of Biochemistry, Era's Lucknow Medical College and Hospital, Lucknow 226 003, Uttar Pradesh, India; [3]Department of Pathology, Era's Lucknow Medical College and Hospital, Lucknow 226 003, Uttar Pradesh, India; [4]Department of Zoology, Lucknow University, Lucknow, Uttar Pradesh 226020, India

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Abstract: ***Aims*:** Breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women. Scientific literature has hypothesized the association of ACE I/D polymorphism with breast cancer for several decades. Unfortunately the outcomes of studies are inconsistent. Thus the present study was designed to evaluate the association of ACE gene (I/D) polymorphism with breast cancer in Indian population.

***Main Methods*:** Genotyping was performed by PCR (polymerase chain reaction), using genomic DNA extracted from peripheral blood of subjects, with (213 cases) or without (213 controls) breast cancer.

***Key Findings*:** The distribution of ACE genotype frequencies i.e. II, DD and ID in patients was 43.19%, 16.43% and 40.38% respectively. In healthy control group II, DD and ID frequencies were 52.58%, 11.27% and 36.15% respectively. The frequencies of D and I alleles were 29.34% and 70.66% in the healthy subjects, while 36.62% and 63.38% among the patient group. Frequency of D allele was significantly different (p=0.0287) between control and case subjects.

***Significance*:** The present study showed an association of D allele of ACE gene with increased genetic risk factor for breast cancer in Indian women. 0.2% increased disease risk was found in patients carrying D allele.

M. K. Ahmad

Keywords: Angiotensin converting enzyme (ACE), Breast cancer, Genotype, Indian population, Polymorphism.

1. INTRODUCTION

Breast gland is particularly prominent in females as the hallmark of pubertal development. Breast cancer (BCa) constitutes a major public health issue globally being the most common cause of cancer mortality in women worldwide [1, 2]. BCa development is a multistep process that includes genetic alterations and transformation of normal mammary epithelial cells into highly malignant derivatives which may be invasive or non invasive [2, 3].

The angiotensin-converting enzyme converts the inactive angiotensin I to the active angiotensin II, involved in the variety of biological functions via G-protein-coupled receptors. Ang II is secreted by pulmonary and renal endothelial cells and is the active product of the renin-angiotensin system (RAS). Ang II has been known to induce hypertrophy and hyperplasia by means of angiogenesis, tumour metastasis, and cell proliferation. These activities predict a possible role of Ang II in the etiology or progression of cancer [4-7].

Mutations are involved in cancer via deregulation of the cell growth and proliferation, including angiogenesis [8, 9]. There is increase in scientific interest to understand the association of ACE gene polymorphism and breast cancer during last few decades [10]. More than 110 polymorphisms have been known in the ACE coding gene. It is well documented that the ACE gene insertion/deletion (I/D) polymorphism has been linked to pathogenesis and progression of various malignancies [11]. Firstly Rigat*et al.* reported insertion (I) or deletion (D) of a 287-bp *Alu*DNA sequence mediated polymorphism in intron 16 of the ACE gene [12]. In contrast to II, individuals having DD homozygous allele have increased

*Address correspondence to this author at the Molecular Cell Biology Lab, Department of Biochemistry, King George's Medical University, Lucknow, Uttar Pradesh 226 003, Indi; E-mail: kaleembaksh@gmail.com

$ Present address: Centre for Biochemistry and Microbial Sciences, Central University of Punjab, Bathinda, 15001, Punjab, India

Table 1. Demographic details of controls and breast cancer patients.

Characteristics	Controls (n=213)	Cases (n=213)	p-value
Age	33.8 ± 5.73	38.4 ± 5.79	0.4398
BMI	24.8 ± 3.88	25.3 ±3.9	0.4702
Estrogen (+ / -)	91/110	125/88	0.0078*
Smoking	13.15% (n=28)	23.94% (n=51)	0.0197*
Alcohol consumption	16.43% (n=35)	29.11% (n=62)	0.0135*
Tobacco Chewing	20.66% (n=44)	35.21% (n=75)	0.0127*
**Tumor Stage 1 and 2 3 and 4	-	71.36% (n=152) 28.62% (n=61)	-
**Lymph Node Grade N0 N1+N2	-	81.69% (n=174) 18.31% (n=39)	-
**Tumour Grade Grade 1 >Grade 1	-	83.56% (n=178) 16.44% (n=35)	-
**Metastasis No Yes	-	92.96% (n=198) 7.04% (n=15)	-

* Significant values
**Characteristics values were mentioned only in case subjects as these were absent in control individuals.

ACE plasma levels which in turn stimulate Ang II production leading to neo-vascularization, cell proliferation, inflammation, and angiogenic effects. On the basis of linkage between ACE activity and Ang II in breast cancer it is hypothesized that the women carrying lower ACE levels and decreased Ang II synthesis would have lesser susceptibility towards breast cancer development [13]. Several publications indicate ACE gene polymorphism as a risk factor of breast cancer but the results of the studies were inconsistent [10]. Literature revealed that the role of ACE gene I/D polymorphism in breast cancer is unexplored. Thus the present case control study was designed to evaluate the relationship between ACE gene I/D polymorphism and breast cancer susceptibility in the Indian population.

2. MATERIAL AND METHODS

2.1. Subjects

A case-control study was performed with the comparison of frequencies of ACE genotypes of 426 eligible women: 213 cases with surgically and histopathologically confirmed breast cancer and 213 normal control subjects. Blood samples of breast cancer cases and healthy controls were collected from the Department of Pathology, of Era's Lucknow Medical College & Hospital, Lucknow with prior consent. Data collection was done for each patient on clinical variables including age, alcohol consumption, body mass index, cigarette smoking, and family history etc. Ethical committee's clearances were obtained from the respective departments, earlier to the recruitment of subjects in this study.

2.2. DNA Extraction

Peripheral blood was collected from all the subjects in 0.5M EDTA tubes. Genomic DNA was isolated from whole blood using the standard phenol-chloroform extraction method [14]. The quantity and quality of DNA was checked by NanoDrop and gel electrophoresis respectively. The DNA was stored at -20°C till further study.

2.3. ACE Genotyping

Polymerase chain reaction (PCR) was employed for genotyping of the *ACE* I/D polymorphism. The primer sequences used for amplification were FP 5'-CTGGAGACCACTCCCATCCTTTCT-3' and RP 5'-GATGTCGCCATCACATTCGTCAGAT- 3' [15]. Final concentration of the PCR mixture contained 1.5mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% gelatin, 1% Triton X-100, 0.3 mM each of dNTPs, and 2U Taq DNA polymerase in each reaction tube. PCR amplification was carried out under the conditions: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 1.15 minutes, extension at 72°C for 2.30 minutes and final extension at 72°C for 5 minutes. PCR products were 490 bp for allele I and 190 bp for allele D (Fig. **1**). The products were separated on a 2.0% agarose gel and visualized by ethidium bromide staining.

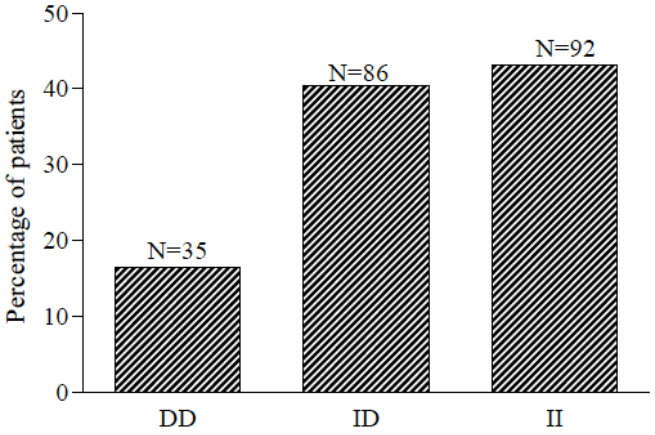

Fig. (1). Frequency of ACE genotype in breast cancer cases.

3. STATISTICAL ANALYSIS

Demographic and clinical data between groups were compared by chi-square test. ACE I/D genotype and allele frequencies of the breast cancer patients were compared to the respective frequencies of the control groups using the chi-square test. Odds ratios (ORs) were calculated to estimate relative risk conferred by a particular allele and genotype. ORs are given with 95 % confidence interval (CI). P value was considered significant at <0.05.

4. RESULTS

4.1. Characteristics of the Study Population

Distribution of selected demographic characteristics and risk factors in control subjects and breast cancer patients are

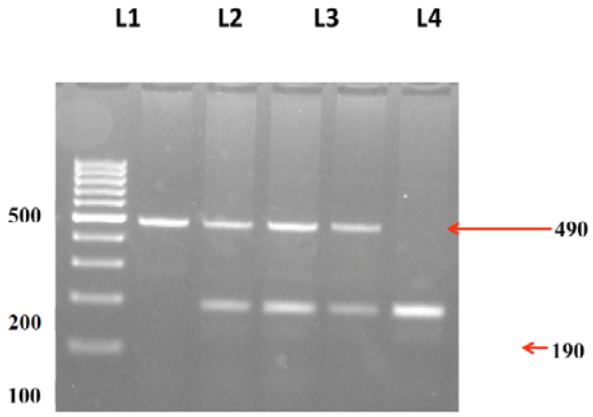

Fig. (2). Analysis of ACE gene polymorphism in breast cases. Lane 1 ladder 100 bp, Lane 2 genotypes Insetion/Insertion 490 bp, Lane 3, 4 and 5 genotypes Insertion/Deletion 490,190 bp and Lane 6 genotypes Deletion/Deletion 190 bp.

shown in Table **1**. The demographic profile included age, estrogen (positive/negative), body mass index, tumor stage/grade and various habitual risk factors involved in the progression of breast cancer. The mean age of breast cancer patients and healthy controls at the time of diagnosis was 38.4 ±5.79 and 33.8 ±5.73 years respectively. Estrogen (+ve), smoking, alcohol consumption and tobacco chewing frequencies were comparatively higher in case group and are associated with breast cancer (Table **1**). Body mass index was approximately same in both study groups. Estrogen positive individuals were more in case group and were significantly differ from control subjects (p=0.0078). The frequency of various tumor stage, lymph node grade, tumor grade and metastasis among the patients are shown in Table **1**. Out of 213 breast cancer subjects, 152 (71.36%) had early tumor stage (1 and 2), and 61 (28.62 %) had advanced tumor stage (3 and 4). About 81% breast cancer patients had N0 grade lymph node while N1+N2 reside in ≈18% case subjects. Thirty five patients had intermediate to high histological grade (>Grade I) cancer and 81.69% case subjects had grade I tumor. Out of 213 breast cancer patients metastasis was confined in only 15 (7.04%) subjects (Table **1**).

4.2. The Genotype and Allele Frequencies of ACE Genotype

The frequencies of the ACE genotype and alleles in breast cancer patients as well as control subjects are shown in Table **2**. The frequencies of the II, DD and ID genotypes of ACE I/D were 43.19%, 16.43% and 40.38% in patients and in healthy control group the frequencies were 52.58%, 11.27%, and 36.15% respectively. There was no significant difference found among the genotype frequencies of ACE I/D polymorphism between breast cancer patient and control group. The frequencies of D and I alleles were 29.34% and 70.66% in the healthy subjects, while 36.62% and 63.38% among the patient group (Table **2**). Frequency of D allele was increased significantly as compared with I allele (OR 1.319, 95 % CI 1.044–1.854, p=0.0287) in case subjects (Table **2**).

4.3. Association Between Breast Cancer and Genotypes with Environmental Factors

In this study, we also explored the possible association of environmental risk factors with ACE I/D gene polymorphisms on breast cancer susceptibility. Association of ACE genotype and alleles with different environmental factors (smoking, alcoholism and tobacco chewing) is shown in Table **3**. The environmental factors included in the study population were differentially associated with ACE genotypes and alleles. ID genotype was significantly (p>0.05) associated with patient having smoke, alcohol and tobacco chewing. In addition, D allele was also found to be significantly associated (p>0.05) with tobacco chewing patients (Table **3**).

4.4. Association of Genotypes with Pathological Conditions

The patients were stratified into two categories *viz.*, low-risk and high-risk breast cancer groups. Low risk group comprised of tumor stage 1 & 2, grade 1, lymph node N0, and no metastasis, where as high risk group involved patients with tumor stage 3 & 4, grade >1, lymph node N1 + N2, and metastasis (Table **3**). Low risk breast cancer entities were taken as reference. Frequency of ID genotype was higher than DD and II genotypes in all tumor stage (1, 2, 3 and 4), lymph node status (N0, N1+N2) and metastasis/non-metastasis in breast cancer subjects (Table **3**). DD genotype frequency was slightly higher in patients with tumor grade >1 while in grade 1, ID frequency was more prominent than other genotypes. Study demonstrate that frequency of D allele was greater than I allele in all the pathological parameters *viz.*, tumor stage, lymph node, tumor grade and metastasis/non-metastasis (p=0.8269, OR=0.9475, CI=0.62-1.45); (p=0.8742, OR=0.9380, CI=0.50-1.75); (p=0.8412,

Table 2. Genotype and allele frequencies of ACE gene I/D polymorphism in the north Indian population.

ACE gene		Control (n=213)	Patients (n=213)	P-value	Odds Ratio	95% CI
Genotype	II	112	92	-	-	-
	DD	24	35	0.0565	1.775	0.9859-3.197
	ID	77	86	0.1718	1.360	0.8995-2.055
Allele	I	301	270	-	-	-
	D	125	156	0.0287*	1.319	1.044-1.854

*p<0.05

Table 3. Association between breast cancer genotypes with environmental factors and pathological conditions.

ACE Genotyping					
Smoking	**Control (n=28)**	**Patients (n=51)**	**P-value**	**Odds Ratio**	**95% CI**
II	9 (32.14%)	7(13.73%)	-	-	-
DD	11(39.29%)	16(31.37%)	0.3610	1.870	0.5351-6.536
ID	8(28.57%)	28(54.90%)	0.0250*	4.500	1.273-15.903
I	26(46.43%)	42(41.18%)	-	-	-
D	30(53.57%)	60(58.82%)	0.6147	1.238	0.6419-2.388
Alcohol consumption	**Control (n=35)**	**Patients (n=62)**	**P-value**	**Odds Ratio**	**95% CI**
II	9(25.71%)	10(16.13%)	-	-	-
DD	16(45.71%)	8(12.90%)	0.2304	0.4500	0.1305-1.552
ID	10(28.57%)	44(70.97%)	0.0303*	3.960	1.276-12.292
I	28(40.00%)	64(51.61%)	-	-	-
D	42(60.00%)	60(48.39%)	0.1358	0.6250	0.3451-1.132
Tobacco Chewing	**Control (n=44)**	**Patients (n=75)**	**P-value**	**Odds Ratio**	**95% CI**
II	6 (13.64%)	8(10.67%)	-	-	-
DD	29 (65.91%)	1722.67(%)	0.2232	0.4397	0.1303-1.484
ID	9 (20.45%)	50(66.67%)	0.0320*	4.167	1.165-14.907
I	34(38.64%)	60 (53.57%)	-	-	-
D	54(61.36%)	52(46.43%)	0.0021*	0.3989	0.2218-0.7174
Tumor Stage	**1 and 2 (n=152)**	**3 and 4 (n=61)**	**P-value**	**Odds Ratio**	**95% CI**
II	35 (23.03%)	11 (18.03%)	-	-	-
DD	54 (35.53%)	17 (27.87%)	1.000	1.002	0.4198-2.390
ID	63(41.45%)	33 (54.10%)	0.2472	1.667	0.7504-3.702
I	133(43.75%)	55(45.08%)	-	-	-
D	171 (56.25%)	67 (54.92%)	0.8296	0.9475	0.6209-1.446
Lymph Node Grade	**N0 (n=191)**	**N1+N2 (n=22)**			
II	49 (25.65%)	5 (22.73%)	-	-	-
DD	61 (31.94%)	6 (27.27%)	1.000	0.9639	0.2775-3.349
ID	81(42.41%)	11 (50.00%)	0.7855	1.331	0.4363-4.060
I	179 (46.13%)	21 (47.73%)	-	-	-
D	209 (53.87%)	23 (52.27%)	0.8742	0.9380	0.5023-1.752

Table 3. contd…

ACE Genotyping					
Tumor Grade	**Grade 1 (n=200)**	**>Grade 1 (n=13)**			
II	45 (22.50%)	4 (30.77%)	-	-	-
DD	69 (34.50%)	5 (38.46%)	1.000	0.8152	0.2076-3.201
ID	86 (43.00%)	4 (30.77%)	0.4523	0.5233	0.1249-2.192
I	176 (44.00%)	12 (46.15%)	-	-	-
D	224 (56.00%)	14 (53.85%)	0.8412	0.9167	0.4135-2.032
Metastasis	**No (n=30)**	**Yes (n=143)**			
II	8 (26.67%)	29 (20.28%)	-	-	-
DD	9 (30.00%)	49 (34.27%)	0.5841	1.502	0.5216-4.325
ID	13 (43.33%)	65 (45.46%)	0.6070	1.379	0.5157-3.689
I	29 (48.33%)	123 (43.01%)	-	-	-
D	31 (51.67%)	163 (56.99%)	0.4765	1.240	0.7096-2.166

*p<0.05

OR=0.9167, CI=0.41); and (p=0.4765, OR=1.240, CI=0.71-2.2) respectively. There was also no correlation between the number of lymph node metastasis and ACE genotype or alleles (Table **3**).

5. DISCUSSION

ACE gene polymorphism is known to be associated with various cancers. In the present study we tried to determine whether the correlations occur between ACE genotype/alleles frequency with breast cancer subjects in Indian population. Result demonstrates that the individuals having DD (homozygote variant) and ID (heterozygote) genotypes for ACE polymorphism have 5.16% and 4.23% higher frequencies in breast cancer subjects in comparison to normal control. This indicates DD and ID genotype for *D/I* gene polymorphism in ACE gene as a risk for breast cancer. At the same time study also showed that DD genotype showed 0.93% greater frequency than ID genotype in Indian breast cancer population. The present study corroborates with the other findings which showed that the individuals having DD genotype for ACE polymorphism are at greater risk towards breast cancer [13]. Moreover, we found that D allele is significantly associated (0.0287) with the case subjects, which indicates its possible involvement in the pathogenesis of human breast cancer. Some of the studies have been done previously around the world, on the association of ACE gene polymorphism in breast cancer. But the findings were inconsistent [16].

The association of ACE polymorphism has been studied in other disease/disorders *viz.,* schizophrenia, obstructive sleep apnea, type-2 diabetes mellitus, hypertension, oral carcinoma, etc. Angiotensin II (converted from Angiotensin I by the activity of ACE enzyme) act as a potent angiogenic factor in the promotion and development of tumor genesis [17, 18]. In endothelial cells it up regulates the NADPH oxidase which in turn involved in the generation of reactive oxygen species (ROS), which in turn induces angiogenesis via vascular endothelial growth factor (VEGF) mediated signaling pathway [19, 20]. VEGF has also been reported to associate with the promotion of neo-vascularisation in human breast cancer [21].

More than one million new breast cancer cases are being diagnosed annually, resulting in over 400,000 annual deaths and about 4.4 million women living with the disease. The precise etiology and molecular pathogenesis of breast cancer is largely unknown, but several risk factors have been identified such as age, height, obesity, high body mass index, diet, alcohol, smoking, tobacco chewing, underlying genetic difference, geographic variations, hormone/pregnancy related factors, previous breast disease and environmental exposures (*e.g.* ionizing radiations) etc [22, 23]. Thus, breast cancer is a heterogeneous disease involving several risk factors in its development. ACE gene polymorphism (I/D) leads to variation in its activity. Polymorphic homozygous segment (II); homozygous deletion (DD), and heterozygote (ID) genotype are reported to their respective serum ACE levels of 299/393/494 μg/mL [24]. This indicates that in ID genotype, more ACE will be present in serum to convert angiotensin I to angiotensin II. As a result angiotensin II will induce more efficiently theangiogenesis as well as promotion and development of tumor genesis [17, 18]. Present study revealed that the ID genotype is significantly associated with smoking status (p=0.025), alcohol consumption (p=0.303) and tobacco chewing (p=0.002). Additionally, D allele is also significantly linked to tobacco chewing individuals (p=0.002). This demonstrates that smoking, alcoholism and tobacco chewing might be a risk factor for breast cancer by virtue of their ability to enhance serum ACE level (ID genotype and/or D allele). Furthermore, together with smoking, alcohol consumption and tobacco chewing, ACE is also known

Table 4. Worldwide ethnicity of ACE genotype in breast cancer.

Ethnicity (Country/Continent)	**ACE Genotyping**						
Japan (Asia)	**Genotype/ Allele**	**Control n=284**	**Case n=357**	**OR**	**P-value**	**95% CI**	**Reference**
	II	119	154	-	-		[10]
	DD	37	43	0.8980	0.702	0.544-1.481	
	ID	128	160	0.9659	0.8651	0.6919-1.348	
	I	366	468	-	-		
	D	202	246	0.9524	0.6803	0.7560-1.200	
Iran (Asia)		**Control n=70**	**Case n=70**	**OR**	**P-value**	**95% CI**	[10]
	II	8	7	-	-	-	
	DD	20	29	1.657	0.5531	0.5176-5.306	
	ID	42	34	0.9252	1.000	0.3046-2.810	
	I	58	48	-	-	-	
	D	82	92	1.356	0.2674	0.8349-2.201	
Neitherland (Europe)		**Control n=153**	**Case n=655**	**OR**	**P-value**	**95% CI**	[10]
	II	32	141	-	-	-	
	DD	54	185	0.7775	0.3284	0.4767-1.268	
	ID	67	329	1.114	0.6330	0.6999-1.775	
	I	131	611				
	D	175	699	0.8564	0.2515	0.6662-1.101	
Ukraine (Europe)		**Control n=131**	**Case n=102**	**OR**	**P-value**	**95% CI**	[10]
	II	37	31	-	-	-	
	DD	41	21	0.6113	0.2107	0.3004-1.244	
	ID	53	50	1.126	0.7555	0.6092-2.081	
	I	127	112	-	-	-	
	D	135	92	0.7728	0.1911	0.5353-1.116	
Mexico (North America)		**Control n=63**	**Case n=288**	**OR**	**P-value**	**95% CI**	[10]
	II	4	74	-	-	-	
	DD	53	63	0.06425	<0.0001	0.0220-0.1874	
	ID	6	151	1.360	0.7342	0.3723-4.970	
	I	14	299	-	-	-	
	D	112	277	0.1158	<0.0001	0.06488-0.2067	

Table 4. contd…

Ethnicity (Country/Continent)	ACE Genotyping						
Brazil (South America)		**Control n=101**	**Case n=307**	**OR**	**P-value**	**95% CI**	[26]
	II	20	53	-	-	-	
	DD	61	141	0.8723	0.7647	0.4807-1.583	
	ID	20	113	2.132	0.0423	1.058-4.296	
	I	60	219	-	-	-	
	D	142	395	0.7621	0.1248	0.5402-1.075	

to increase oxidative stress, thereby causing cancer [25]. It might be inferred that the ACE polymorphism and free radical biology both are involved in the breast cancer. Tumor stage (1 and 2), lymph node grade N0, tumor grade 1 and metastasis showed higher frequencies (42.74%, 63.38%, 67.12% and 85.92% respectively) among case subjects in comparison with their respective properties (Table **1**). We did not find significant association of ACE gene polymorphism with different pathological parameters *viz.*, tumor stage, lymph node status, tumor grade and metastasis in breast cancer subjects (Table **3**).

A number of studies have been carried out in different populations for potential association of ACE gene polymorphism with breast cancer (Table **4**) [10]. Studies from Japan, Iran, Neitherland, and Ukraine did not show any significant difference in distribution of ACE I/D gene polymorphism between breast cancer patients and healthy controls. These studies also did not support the role of SNP in breast cancer [10]. In a Brazilian study, researchers showed the significant association of DD genotype and D allele with ACE I/D polymorphism in breast cancer [26]. Similarly ID genotype was also found to be associated with breast cancer in a Mexican study [10]. Overall, these contradictory implications from studies on different populations imply that the role of D allele in susceptibility to breast cancer might depend on ethnic or geographic factors.

CONCLUSION

To the best of our knowledge, no study has been carried out to assess the association of ACE gene insertion/deletion polymorphism in breast cancer subjects in an Indian population. The study concludes that there is an association of D allele with the risk of breast cancer in Indian population. Moreover, the study emphasizes that environmental factors *viz.*, smoking, alcohol consumption and tobacco chewing also play an important role in progression of breast cancer.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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