Association of AGTR1 (A1166C) and ACE (I/D) Polymorphisms with Breast Cancer Risk in North Indian Population

Anukriti Singh*,†, Nidhi Srivastava†, Sonal Amit‡, S.N. Prasad§, M.P. Misra§ and Bushra Ateeq*

* Molecular Oncology Lab, Department of Biological Sciences and Bioengineering, Indian Institute of Technology, Kanpur, 208016, U.P., India; † Department of Bioscience and Biotechnology, Banasthali University, Tonk, 304022, Rajasthan, India; ‡ Path-Way Diagnostics Centre, Kanpur, 208005, U.P., India; § J. K. Cancer Institute, Kanpur, 208001, U.P., India

Abstract

Renin angiotensin system (RAS) comprising Angiotensin converting enzyme (ACE), Angiotensin II (Ang II) and its receptor Angiotensin II receptor type I (AGTR1), plays a critical role in several diseases including cancer. A single nucleotide polymorphism (SNP) A1166C located in 3’ untranslated region (UTR) of AGTR1 and an insertion/deletion (I/D) polymorphism present in intron 16 of ACE gene have been associated with many diseases, but their association with Breast cancer (BCa) is still debatable. Here, we for the first time investigated the association of these polymorphisms in a North Indian BCa cohort including 161 patients and 152 healthy women. The polymorphisms were evaluated by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) respectively. The association between these polymorphisms and BCa risk was estimated by calculating Odds Ratio (OR) and chi-square ($\chi^2$) test. The DD genotype/D allele of ACE (I/D) polymorphism and “AC and CC” genotype/C allele of AGTR1 (A1166C) polymorphism were associated with higher risk of BCa when evaluated independently. Furthermore, interaction analysis of “AC and CC” and DD genotype and combination of “C and D” alleles of both polymorphisms revealed significantly greater BCa risk than that observed independently. Conclusively, women harboring “AC or CC” genotype/C allele for AGTR1 (A1166C) polymorphism and DD genotype/D allele for ACE (I/D) polymorphisms have a predisposition to develop more aggressive disease with advanced staging and larger tumor size. Our study indicates importance of genetic screening based on these polymorphisms for women, who may have higher risk of BCa.

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Introduction

Breast cancer (BCa) is the most common cause of cancer associated death among women worldwide [1]. More than 1,300,000 cases and 450,000 deaths related with BCa are reported every year globally [2]. In 2017 alone, the incidence of invasive BCa and mortality in American women is projected to be 252,710 and 40,610 respectively [3]. A latest survey conducted by Indian Council of Medical Research (ICMR) estimated 150,000 new cases of BCa in the year 2016 from India [4]. Thus, rise in both BCa incidence and mortality evokes a need for clinical management strategies and early detection methods.
need to examine risk factors associated with this disease. The classification systems based on molecular subtypes characterized by the presence or absence of Estrogen receptor (ER), Progesterone receptor (PR) and Human epidermal growth factor receptor 2 (HER2) may have certain limitations [5]. While, subtypes such as Luminal A (ER+, PR+, HER2−) and HER2+ (ER−, PR−, HER2+) are known to have good prognosis and effective targeted treatment options such as tamoxifen and Herceptin respectively. Other subtypes such as basal-like group with ~70–80% triple negative and Luminal B which has higher recurrence and lower survival rate are still in need for additional biomarkers [5].

RAS is mainly involved in the systemic regulation of cardiovascular homeostasis [6] and is known to be expressed in multiple cancer types including breast [7]. Overexpression of AGTR1 and ACE has been often reported in most of the neoplastic stages [8]. In humans, the gene encoding ACE is located on chromosome 17 (17q23), spanning 21 kb and comprising 26 exons and 25 introns [6]. ACE is a zinc dependent dipeptidyl carboxypeptidase that catalyzes the conversion of inactive decapetide Angiotensin I (Ang I) to active octapeptide Ang II [6]. Ang II mediates its complex physiological effects by binding to two subtypes of receptors, AGTR1 and Angiotensin II receptor type II (AGTR2), that belong to a superfamily of G-protein-coupled receptors (GPCRs). Both receptors have about 32% structural homology, different tissue distribution and distinct intracellular signaling pathways [9]. Interestingly, the variable expression of ACE is mostly associated with the polymorphisms in ACE gene, among them the most studied is ACE (I/D) polymorphism (NCBI reference ID: rs1799752). The presence of a 287 bp Alu sequence of DNA in the intron 16 of ACE gene is represented by “Insertion” or “I”, and absence of the same denotes “Deletion” or “D”. The enzymatic activity of ACE was found to be approximately double in the DD carriers as compared to II carriers and intermediate in ID carriers, indicating codominance among the alleles [10–12]. It has been proposed that ACE (I/D) polymorphism might play a role in altered transcriptional regulation and/or in the splicing of ACE pre-mRNA [10,12]. However, the mechanism on how this polymorphism affects ACE activity levels is still debated.

The AGTR1 gene is located at 3q21-q25 and extending over 55 kb segment, comprising of five exons. Interestingly, 3′ untranslated region (3′UTR) of AGTR1 harbors A1166C (NCBI reference SNP id: rs5186), a single nucleotide polymorphism (SNP) with an A/C nucleotide transversion at 1166 position. Moreover, A1166C SNP has been associated with T810A variant which is localized in the promoter region of AGTR1 and affects transcription factor binding [12]. An elevated sensitivity to Ang II has been detected in individuals harboring 1166C allele [13]. AGTR1 signaling is known to induce cellular proliferation, angiogenesis and inflammatory response along with anti-apoptotic effects [14,15], while AGTR2 functionally antagonizes many of these actions [16]. Furthermore, overexpression of AGTR1 (~10–20% of cases) exclusively in ERBB2 negative BCA patients has been reported and is known to play a role in cell invasion and tumorigenesis [17]. Interestingly, both ACE (I/D) and AGTR1 (A1166C) polymorphisms have been associated with various diseases such as hypertension and a variety of cancers including breast [6,12]. Many epidemiological studies worldwide have evaluated the role of these polymorphisms with respect to BCA risk. A study of ACE gene on Chinese women in Singapore demonstrated that individuals carrying AA (A240T) and II (ACE I/D) genotypes predispose them to lower plasma concentrations of ACE, and significantly reduced (~50%) BCA risk [7]. Conversely, a reverse association of D allele with BCA risk was observed in a multi-ethnic cohort study, suggesting its association with low ACE levels in certain populations [18]. Similarly, AGTR1 (A1166C) polymorphism has also been studied with respect to BCA risk in several populations, for example a higher frequency of C allele was observed in post-menopausal Egyptian women with BCa than controls, indicating C allele as the high risk allele [6]. Conversely, studies carried out in Brazilian and Iranian BCa cohorts failed to exhibit any association of the C allele to BCa risk [11,19]. Thus far, numerous studies have investigated the relationship between ACE (I/D), AGTR1 (A1166C) polymorphisms and their association with BCa risk. Nevertheless, the outcomes remain inconclusive, even conflictive, leading towards the inference that association of ACE (I/D) and AGTR1 (A1166C) polymorphisms with BCa risk might be a population specific phenomenon. Thus, it was imperative to conduct a population specific study and access the BCa conferred by these polymorphisms among Indian women. Here, we elucidate the role of AGTR1 and ACE on the pathobiology of this disease by investigating the correlation of AGTR1 (A1166C) and ACE (I/D) polymorphisms with BCa risk. To the best of our knowledge this is the first study exploring the association of both AGTR1 (A1166C) and ACE (I/D) polymorphisms in North Indian population.

Material and methods

Patients and healthy individuals

The study was conducted in Kanpur (North India) in compliance to all relevant procedures and guidelines. For exploring the association of AGTR1 (A1166C) and ACE (I/D) polymorphisms in North Indian population, both the controls and cases were derived from the same population. The sample size was calculated using Quanto 1.2.4 software. All the specimens (161 BCa patients and 152 healthy women as controls) were procured from the J.K. Cancer Institute, GSVM Medical College and Path-Way diagnostics, Kanpur. The study subjects were mostly but not necessarily inhabitants of Kanpur city. In order to avoid confounding variables, we used inclusion criteria for the study subjects, 161 BCa patients and 152 healthy females, who had no history of alcohol or smoking and no family history of breast cancer. Moreover, the participants included in the study also had no history of cardiovascular disease or hypertension and never used ARBs or ACEi which can act as confounding factors for the statistical analyses. A written informed consent and personal details were duly obtained from all the subjects before taking the samples. Approximately 2 ml blood was taken from healthy women (ages 20–75 years) in EDTA vacuum tubes as control samples. For BCa patients (ages 20–72 years) the inclusion criteria were the women with BCa whereas the male BCa patients were excluded from the study. BCa patient samples were taken retrospectively in the form of formalin fixed paraffin embedded (FFPE) tissues from year 2012 to 2016 and a few were taken prospectively in the form of Fine-needle aspiration cytology (FNAC). All the specimens were collected after getting consent from the patients and approval from the Institutional human ethical committees of the J.K. Cancer Institute, Kanpur (reference number: ECJKCI/FEB/2016/05 and Indian Institute of Technology, Kanpur (reference number: IITK/IEC/2017–18 I/11) in accordance with the code of ethics of World Medical Association (Declaration of Helsinki) for experiments involving humans. All pathologic information about tumor characteristics including number
of lymph nodes, histologic grade, tumor size, TNM staging, ER, PR, HER2 expression and menopausal status of women were obtained from patients’ medical record files and pathology reports. Since the study was mainly retrospective in nature, therefore it was not possible to obtain blood samples from a majority of BCa patients. Hence, FFPE tissues were used to extract genomic DNA. Numerous studies for multiple cancer types provide a convincing evidence that genotyping results using DNA isolated from peripheral blood and tumor tissues show remarkable similarity, depicting virtually 100% concordance in germline and tumor DNA genotypes [20–22]. Thus, in the current study we used FFPE tissues for isolating genomic DNA for our BCa cohort, which can be utilized as a valid proxy for comparing germline DNA isolated from normal women for the retrospective SNP association studies [23].

**DNA extraction**

DNA was extracted from peripheral blood mononuclear cells (PBMCs) isolated from fresh blood samples of healthy women using QIAamp DNA Blood mini kit (Qiagen). For patients’ FFPE and FNAC samples, DNA was extracted using QIAamp DNA FFPE Tissue kit (Qiagen) and QIAamp DNA Mini kit (Qiagen) as per manufacturer’s instructions.

**Genotyping**

The genotype AGTR1 (A1166C) was determined by performing PCR-RFLP using sequence specific primers (Supplementary Table S1) [24]. Briefly, for 25 μl of PCR reaction, about 125 ng of genomic DNA was taken as template, 10 pmols of each primer, 2.5 μl of 10X PCR buffer, 0.1 mM of each deoxyribonucleoside triphosphate (dNTP), 0.25 U of Taq DNA polymerase (Taq Pol) was used. The PCR thermal cycling conditions were followed as described by Behravan et al. [24]. The resulting PCR product was subject to restriction digestion using 0.5 U of Ddel enzyme (R01755, NEB) at 37 °C overnight. The restriction enzyme digested product was run on 2% agarose gel and visualized using ethidium bromide (EtBr). NEB cutter was used to ascertain exact sizes of bands on the agarose gel [25]. The presence of SNP resulted in 412 bp, 118 bp and a 10 bp (often not visible on agarose gel) bands; however, its absence was indicated by the presence of only a 530 bp band [25]. As DNA extracted from FFPE samples is fragmented [26], hence we designed a different set of primers (Supplementary Table S1) which yielded a 250 bp amplicon. Here, the presence of SNP was confirmed by the presence of two bands of 129 bp and 121 bp and its absence marked by only a single band of 250 bp (Figure 1). The conditions used for PCR reaction were exactly similar for all the patients and control samples. Finally, to further validate our PCR-RFLP findings, a representative batch of samples were sequenced using Sanger sequencing (Macrogen, Seoul, Korea) (Supplementary Fig. S1).

The genotype of ACE (I/D) was ascertained by PCR via sequence specific primers (Supplementary Table S1) [27]. The 25 μl PCR reaction of 2.5 μl 10X PCR buffer, 0.05 mM dNTP mix, 5 pmol of both primers and 0.5 U of Taq Pol was used. For patients’ samples PCR reaction consisting of 2.5 μl 10X PCR buffer, 0.2 mM dNTP mix, 10 pmols of both primers and 1 U of Taq Pol., 0.1 mg/ml BSA and 5% DMSO. The thermal cycling conditions for control samples were followed as described by Namazi et al. [28]. Notwithstanding, the thermal cycling conditions for patient samples were as follows: 3 min initial denaturation at 94 °C followed by 35 cycles at 94 °C for 60s (denaturation), 59.8 °C for 60s (annealing), 72 °C for 90s (extension), finally a terminal extension of 72 °C for 10 min. The amplified fragments were visualized on 1.8% agarose gel and EtBr staining. The I allele was characterized by the presence of by 490 bp fragment (with 300 bp insertion) and D allele by 190 bp fragment (Figure 1). To avoid any chance of mistyping of ID heterozygotes as DD homozygotes [29], we further performed nested PCR with another set of primers (Supplementary Table S1) for all the samples with DD genotype with PCR conditions same as used for control samples except for annealing temperature of 67 °C [28]. The samples with DD genotype will not show any bands; however, presence of I allele (ID genotype) should result in a 335 bp amplicon.

**Statistical analysis**

The statistical analysis of data was performed using “Statistical Package for the Social Sciences (SPSS) 19.0 for Windows” statistical package. The quantitative data was shown as frequency or percentage. The association of AGTR1 (A1166C) and ACE (I/D) polymorphisms with the prognostic factors was evaluated using χ² test or Fischer’s exact test. P ≤ .05 was considered statistically significant and all tests were two sided. For both SNPs deviation of genotype frequencies in controls and cases from the HWE was assessed by χ² test with one degree of freedom (df) using the Michael H. Court’s (2005–2008) calculator [30]. For 95% confidence interval, P = .05 and χ² = 3.84; therefore, if the χ² ≤ 3.84 and the corresponding P ≥ .05 then the population is in HWE.

The statistical power was analyzed by G Power software 3.1 [31]. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated using SNP tools, an add in for MS excel developed by Chen et al. [32]. Allele combination analysis was done using SNPassoc software [33]. Linkage Disequilibrium (LD) analysis was done by PLINK software v1.07 [34]. MDR_3.0.2 (SourceForge, Inc., SourceForge.net) was used to detect gene–gene (SNP-SNP) interactions between AGTR1 (A1166C) and ACE (I/D) SNPs to detect the epistatic interaction between the two SNPs. Multifactor Dimensionality Reduction (MDR) is a non-parametric and model-free approach which can be used for detection of interactions between SNPs in disease associated epidemiologic studies especially cancer [35]. This method is effective for relatively small sample sizes with more than 80% statistical power for identification of gene–gene or gene–environment interactions even with small sample size less than 200 cases and/or controls or samples with 5% missing data and 5% genotyping error [36]. The fitness of an MDR model was predicted by determination of testing accuracy and cross validation consistency (CVC). A testing accuracy of 0.5 is expected under null hypothesis [36]. The best model has a maximum testing accuracy and CVC. Statistical significance was estimated by employing a 1000-fold permutation test that compared observed testing accuracies with those expected under null hypothesis of no association. The details of MDR have already been described in previous publications [35,36].

**Results**

**Genotype frequencies for AGTR1 (A1166C) and ACE (I/D) polymorphisms**

The Hardy–Weinberg equilibrium (HWE) test confirmed that the genotypic frequencies were balanced for both healthy individuals (controls) and BCa patients for ACE (I/D) polymorphism, while frequencies of AGTR1 (A1166C) polymorphism for BCa patients diverged significantly from the equilibrium, thereby indicating disease
association (Table 1). The power of the study was 1 for both ACE (I/D) and AGTR1 (A1166C) polymorphisms. Among healthy women \(n = 152\), the AGTR1 (A1166C) polymorphism showed diverse frequencies for AA (88%), AC (12%), CC (0%) genotypes (Figure 1A). However, among 161 BCa patients AGTR1 (A1166C) polymorphism showed deviation in genotype frequencies from healthy individuals, AA (26%), AC (68%); CC (6%) genotypes (Figure 1B). For ACE I/D polymorphism, 6.5% of BCa patients showed II, 38% had ID and 55.5% harbor DD genotype (Figure 1D). While the genotype frequencies of II, ID, DD among healthy women were 31.3%, 49.3%, and 19.3% respectively (Figure 1C).
**Table 1.** Hardy–Weinberg equilibrium (HWE) for AGTR1 (A1166C) and ACE (I/D) polymorphisms in case and control groups according to expected (E) and observed (O) values

| Control | AA | AC | CC | Total | HWE P value |
|---------|----|----|----|-------|-------------|
| E       | 134(88) | 17(11) | 0.5(0) | 152(100) | 0.44 |
| O       | 134(88) | 18(12) | 0(0) | 152(100) | |

| Case | AA | AC | CC | Total | HWE P value |
|------|----|----|----|-------|-------------|
| E    | 57(36) | 78(48) | 26(16) | 161(100) | 0 |
| O    | 41(26) | 110(68) | 10(6) | 161(100) | |

**Table 2.** Comparison of AGTR1 (A1166C) and ACE (I/D) genotype and allele frequencies among breast cancer patients and healthy women

| AGTR1 (A1166C) | Control (n = 152) | Case (n = 161) | Odds ratio (95%CI), P value |
|----------------|-------------------|----------------|---------------------------|
| CC            | n (%)             | n (%)           | χ², P value                |
|               | 0(0)              | 10(6)           | 125.39, P < .0001          |
| AC            | 18(12)            | 110(68)         |                            |
| AA            | 134(88)           | 41(26)          |                            |
| Total         | 152(100)          | 161(100)        |                            |

| ACE (I/D) | Control (n = 150) | Case (n = 155) | Odds ratio (95%CI), P value |
|-----------|-------------------|----------------|---------------------------|
| DD        | n (%)             | n (%)           | χ², P value                |
|           | 29(19.3)          | 86(55.5)        | 53.89, P < .0001          |
| ID        | 74(49.3)          | 59(38.0)        |                            |
| II        | 47(31.3)          | 10(6.5)         |                            |
| Total     | 150(100)          | 155(100)        |                            |

**Single locus analysis for AGTR1 (A1166C) and ACE (I/D) polymorphisms**

Higher frequency of CC (6% vs. 0%), and AC (68% vs. 12%) genotype for AGTR1 (A1166C) SNP was observed in BCa patients as compared to healthy women (Table 2). While, lower frequency of wild genotype AA (26% vs. 88%) was recorded in BCa patients than in healthy women. A statistically significant difference (P < .0001) in AGTR1 (A1166C) genotype frequencies between BCa patients and control group was observed. Moreover, a statistically significant association (P < .0001) between genotype distributions of the A1166C SNP with BCa risk was found as illustrated in Table 2. An Odds ratio of 19.9 (95% CI: 10.9–36.2) (Supplementary Table S2, P < .0001) indicates a strong association of AC genotype with increased BCa risk when compared to AA genotype (Supplementary Table S2).

The genotype frequencies (%) of ACE I/D polymorphism observed in BCa patients' vs. healthy women were 55.5% vs. 19.3% for polymorphic DD, 38.0% vs. 49.3% for heterozygous ID and 6.5% vs. 31.3% for wild type II (Table 2). Of note, the frequencies of DD genotype were significantly higher (P < .0001) and that of II and ID were lower in BCa patients than in healthy individuals. Moreover, the association of genotype distribution of ACE I/D polymorphism with BCa was found to be statistically significant (χ² = 53.89, P < .0001) (Table 2). While, DD genotype was strongly associated with increased BCa risk as compared to ID and II genotypes with an Odds ratio of 3.7 (95% CI: 2.2–6.4) and 13.9 (95% CI: 6.2–31) respectively. When comparing ID genotype to II, an Odds ratio of 3.7 (95% CI: 1.7–8.0) indicates a greater association of ID genotype with BCa risk as compared to II genotype (Supplementary Table S2).

The allele frequencies of AGTR1 (A1166C) SNP showed significant statistical difference (P < .0001) between BCa patients and healthy women (Table 2). BCa patients displayed higher frequency of allele C than control individuals (59.8% vs. 12.2%; P < .0001), indicating an increase in BCa risk with an Odds ratio of 10.76 (95% CI: 5.39–19.96), while ACE (I/D) polymorphism also showed a significant statistical difference (P < .0001) between BCa patients and controls. Here, the D allele had higher frequency in women with BCa than healthy women (74.5% vs. 44%), exhibiting increased BCa risk with an Odds ratio of 3.72 (95% CI: 2.64–5.24; P < .0001).

The dominant model of inheritance displayed a higher frequency of risk genotypes “AC and CC” in BCa patients when compared to normal individuals (74% vs. 12% respectively; P < .0001) (Supplementary Table S3). A significant increase in BCa risk (P < .0001) was also observed in individuals with “AC and CC” genotypes showing an Odds ratio of 21.8 (95% CI: 11.9–39.9) as compared to control group AA. While, the recessive model was not relevant due to lower number of CC genotype in BCa women, and due to absence of this genotype in the control group. Similarly, testing the dominant model for ACE (I/D) polymorphism revealed a statistically higher frequency of ID and DD genotypes within women with BCa than healthy women (94% vs. 69%, respectively). Here, an odds ratio of 6.62
(95%CI: 3.2–13.6; $P < .0001$) showed increased BCa risk in women with ID and DD genotype when compared to II genotype. Next, testing the recessive model for DD vs. ID and II, we observed higher frequency of DD genotype in BCa patients as compared to healthy controls (56% vs. 19% respectively; $P < .0001$). Moreover, a statistically increased BCa risk ($P < .0001$) was observed in individuals harboring DD genotype with an odds ratio of 5.2 (95% CI: 3.1–8.7) as compared to ID and II (Supplementary Table S3). No significant association was observed for prognostic factors associated with BCa for $ACE$ (I/D) or $AGTR1$ (A1166C) polymorphisms independently.

**Allele combination analysis for $AGTR1$ (A1166C) and $ACE$ (I/D) polymorphisms**

To further explore the cumulative effect of $ACE$ (I/D) or $AGTR1$ (A1166C) polymorphisms on the BCa risk, we calculated the frequencies of allele combinations and compared them between BCa patients and healthy women (Table 3). The D and C alleles showed a cumulative effect on BCa risk with an Odds ratio of 24.44 (CI: 7.98–74.88; $P < .0001$). Taken together, our analysis indicates that the women harboring C allele of $AGTR1$ (A1166C) and D allele of $ACE$ (I/D) polymorphisms are at higher risk of developing breast cancer.

**Interaction analysis of $AGTR1$ (A1166C) and $ACE$ (I/D) polymorphisms**

The combined genotypic distribution of $AGTR1$ (A1166C) and $ACE$ (I/D) polymorphisms was analyzed by calculating contingency coefficient. Where contingency coefficient for BCa patients (0.255; $P < .001$) show significant correlation with BCa risk as compared to healthy controls (0.128; $P = .115$) (Table 3). It was observed that the subjects harboring both risk genotypes “AC and CC” for $AGTR1$ (A1166C) and DD for $ACE$ (I/D) polymorphism were much higher in patient population as compared to control group (83.8% vs. 3.4% respectively; $P < .0001$) with an Odds ratio of 258 (95% CI: 13.0–238; $P < .0001$). The women harboring a combination of high risk genotype for $AGTR1$ gene, while low activity genotype for $ACE$ gene showed an Odds ratio of 9.2 (95% CI: 4.6–18.8; $P < .0001$) in comparison to low risk genotypes of both polymorphisms. Also, women with combination of high activity genotype for $ACE$ gene and low risk genotype for $AGTR1$ gene vs. women with low risk genotype for both polymorphisms exhibited an Odds ratio of 1.8 (95% CI: 0.82–3.91, $P = .142$). This finding suggests that high risk genotype of $AGTR1$ (A1166C) polymorphism has increased odds of BCa as compared to high activity genotype of $ACE$ (I/D) polymorphism.

The interaction analysis of $AGTR1$ (A1166C) and $ACE$ (I/D) was repeated after stratification of subjects with prognostic factors as baseline. A significant ($P < .001$) association between $AGTR1$ and $ACE$ gene polymorphisms with BCa risk was found in patients with higher staging when Tumor Node Metastasis (TNM) status was taken as baseline (Supplementary Table S4). While on taking menopausal status as baseline, a significant association between the

![Figure 2. Epistatic interaction between $AGTR1$ (A1166C) and $ACE$ (I/D) polymorphisms using MDR. The black bar in each cell represents frequency of BCa patients’ cases and white bar represents frequency of healthy individual controls. High risk genotype combinations are represented by dark gray shade cells, while light gray shade cells represent low risk genotype combinations. Cells with no shading or white cells represent genotype combination for which no data is observed.](image-url)
two polymorphisms and BCa risk was seen for postmenopausal women \( (P = .007) \) (Supplementary Table S5). Also, in context of the two polymorphisms an enhanced BCa risk was associated with larger tumor size \( (P < .001) \); (Supplementary Table S6).

The epistatic interaction between \textit{AGTR1} (A1166C) and \textit{ACE} (I/D) polymorphism was studied using MDR, a promising data mining analytical approach. The best model of each order was evaluated by testing accuracy, cross-validation consistency (CVC) and significance level as determined by permutation testing. The overall best model for interaction of \textit{AGTR1} and \textit{ACE} polymorphisms demonstrate a maximal testing accuracy (0.8047) with a CVC of 10 out of 10 \( (P = .0008) \), indicating that this model was observed once out of 1000 permutations which is unlikely under hypothesis of null association. Moreover, the graphical image generated from MDR involves the representation of two polymorphisms, each with three genotypes and nine two locus combinations. Further, within each multifactor cell (square box), the ratio of number of cases with disease to number of controls was calculated. As shown in Figure 2, if the ratio exceeds a certain threshold \( (T) \), for example, a cell within nine dimensional space, with \( T > 1 \) is labeled as high risk (darker shade of gray) than the cells labeled as low risk \( (T < 1) \) indicated in light gray shade. The interaction of AC with DD, ID and II along with that of CC with DD and ID is labeled as high risk. However, the interaction of AA with DD, ID and II is labeled as low risk (Figure 2). The linkage disequilibrium (LD) analysis between \textit{AGTR1} (A1166C) and \textit{ACE} (I/D) SNPs \( (D' = 0.6, r^2 = 0.086) \), indicates that these SNPs are co-inherited 60% of the time. Also, IA and DC were found to be in phase alleles meaning that they occur together more than expected by chance, indicating that AGTR1 (A1166C) and \textit{ACE} (I/D) polymorphisms are in LD.

### Discussion

In this study, we have explored the association of \textit{AGTR1} (A1166C) and \textit{ACE} (I/D) polymorphisms with BCa in North Indian population. To our knowledge, this is the first study elucidating the correlation between \textit{ACE} (I/D) and \textit{AGTR1} (A1166C) polymorphisms both independently and synergistically with BCa risk in North Indian cohort. It was observed that the “AC and CC” genotype and C allele for \textit{AGTR1} (A1166C) polymorphism as well as DD genotype and D allele for \textit{ACE} (I/D) polymorphism conferred higher disease risk as compared to other genotypes. Also, the two polymorphisms were observed to be in LD, where D and C alleles showed co-occurrence more than expected by chance. Our findings not only improve our understanding that complex genetic

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**Figure 3.** Illustration showing genotypic combinations of \textit{AGTR1} (A1166C) and \textit{ACE} (I/D) polymorphisms and their respective odds ratio for breast cancer risk.
interactions might lead to BCa risk, but also provide strong evidence implicating \textit{AGTR1} (A1166C) and \textit{ACE} (I/D) polymorphisms in the pathobiology of this disease.

Several studies explored the association of \textit{ACE} (I/D) polymorphism with BCa in various cohorts; however, the risk lines up with different alleles. In the present study, an association between \textit{ACE} (I/D) polymorphism and BCa risk was observed, where the DD genotype and D allele significantly conferred increased BCa risk as compared to ID/II genotype (Figure 3) and I allele respectively. While, II genotype plays a protective role for BCa risk as compared to DD and ID genotype. In agreement with the present study, the association of D allele with BCa risk in Mexican and Ukrainian women (age 34–45 years) has been observed [12,37]. Conversely, in a multi-ethnic cohort study a marginal increase in BCa risk for women with II genotype was observed [18]. While, no association of the polymorphism with BCa was noticed among post-menopausal Egyptian females [6]. Conversely, a study on Kashmiri population reported II genotype to be significantly associated with BCa and protective role of ID genotype against BCa [38].

Physiologically, the D allele is associated with increased ACE levels in tissue and circulation on comparing with I allele [10], thereby resulting in higher levels of Ang II [39]. Moreover, \textit{ACE} (I/D) polymorphism may also regulate ACE activity by being in LD with other functional polymorphisms and causal mutations. For example, Zhu et al. demonstrated that \textit{ACE} (I/D) polymorphism is in LD with SNP (A/G) in exon 17 and SNP (A/T) in the 5’ UTR of \textit{ACE} gene, which are responsible for variation in serum levels of ACE. Since, \textit{AGTR1} and \textit{ACE} are present in the secretory epithelium of normal breast tissue, suggesting that Ang II is produced in cells where it acts [40], we also contemplate that the variants of these genes could interact via local paracrine mechanisms, as ACE is relatively nonspecific and its action is a non-limiting step for Ang II production in plasma [41].

Furthermore, the A1166C polymorphism of \textit{AGTR1} has also been studied across different cohorts. In the present study, we observed an association of A1166C polymorphism with BCa, wherein the “AC and CC” genotype and C allele were significantly correlated with BCa risk when compared to AA genotype (Figure 3) and A allele respectively. In concordance to our results, Shakaryaw et al. reported association of C allele with BCa risk [6]. Similarly, in Ukrainian cohort, AC genotype was associated with BCa in women older than 54 years [12]. While, in Mexicans and Caucasians, C allele carriers showed reduced risk for BCa [37,42]. Nevertheless, no association of A1166C polymorphism with BCa was observed in few other studies [11,19].

Functionally, \textit{AGTR1} (A1166C) SNP is known to be involved in RAS over activation [43]. It may be in LD with a functional mutation that alters responsiveness to Ang II [13]. Also, increased expression of \textit{AGTR1} might be related to lack of complementarity of C allele with the seed sequence of miRNA-155 which interacts with A allele [44]. Moreover, the presence of C allele results in increased expression of \textit{AGTR1} [43], which has been associated with stimulation of downstream signaling pathways leading to proliferation, migration hypertrophy and EMT [45].

We have detected a remarkable epistatic interaction via MDR between \textit{ACE} (I/D) and \textit{AGTR1} (A1166C) polymorphisms. To substantiate our findings we also employed the model of epistatic interaction used by Dhillon et al. [46]. Interestingly we observed that the odds of BCa risk significantly increased to 258 times in BCa patients with respect to controls (Figure 3), when the epistatic interaction between “AC and CC” genotype of \textit{AGTR1} (A1166C) and DD genotype of \textit{ACE} (I/D) polymorphism was considered, inferring that women harboring the risk genotypes for both polymorphisms are at much higher risk as compared to women having a single polymorphism. Also, women harboring C allele of \textit{AGTR1} (A1166C) and D allele of \textit{ACE} (I/D) polymorphism are at a greater exposure to the disease in comparison to A and I allele which present a protective allele combination.

Independently \textit{AGTR1} (A1166C) and \textit{ACE} (I/D) polymorphisms did not seem to be associated with prognostic factors of BCa susceptibility (Supplementary Table S7) but on interaction of these two polymorphisms an association with higher TNM staging, postmenopausal status and greater tumor size in BCa patients was observed. In concordance to Iranian [19] and postmenopausal women in Brazilian cohorts [11], we also found an association of \textit{AGTR1} (A1166C) polymorphism with higher TNM staging among BCa patients. Taken together, these findings suggest that while independently both the polymorphisms seem to show no association with the prognostic factors linked to BCa, but on interaction an association with BCa risk emerges. Furthermore, these findings also suggest that postmenopausal women harboring the risk genotypes of both polymorphisms are at greater risk for BCa than premenopausal women.

Potential limitations of our study were that it was majorly retrospective which limited our access to patient data, confounding our comparative analysis of association studies with other prognostic factors (Supplementary Table S7) for BCa. Due to high genetic heterogeneity in the Indian population, we restricted our study to Kanpur, Northern India; however, a multicentric association study of polymorphisms across India is highly recommended.

Though we have not studied the effect of these polymorphisms on levels of \textit{AGTR1} or \textit{ACE} activity in this study, however it has been suggested in previous reports that CC genotype/C allele of \textit{AGTR1}(A1166C) polymorphism is associated with increased responsiveness to Ang II [13] and elevated \textit{AGTR1} levels [44]. Moreover, DD genotype of \textit{ACE} (I/D) polymorphism is associated with higher plasma levels of ACE [10]. Existing literature suggests that ACE inhibitors (ACEi) and/or Angiotensin receptor blockers (ARB’s) could possibly counteract the deleterious effect of \textit{AGTR1} (A1166C) and \textit{ACE} (I/D) polymorphisms. ARBs and ACEi have been known to exert anti-tumorigenic role in different cancers [45], for example, colorectal cancer mouse model for liver metastases show that Irbesartan (ARB) and Captopril (ACEi) treatment resulted in decreased tumor growth, tumor associated angiogenesis and metastases [47]. Dual blockade by ACEi and ARBs was observed to have higher efficacy than monotherapy [48]. Alternatively, the use of calcium channel blockers with ACEi, renin inhibitors and aldosterone receptor antagonists might be considered [49].

Conclusions

Our findings from North India BCa cohort suggest a significant association of \textit{AGTR1} (A1166C) and \textit{ACE} (I/D) polymorphism with BCa risk. While, a remarkable interaction between both the polymorphisms was observed in predisposition to BCa susceptibility, individuals harboring DD genotype/D allele of \textit{ACE} (I/D) and AC or CC genotype/C allele of \textit{AGTR1} (A1166C) polymorphism showed increased risk of BCa. Taken together, our study provides a rationale for developing a genetic screen for these polymorphisms for women.
who are at a higher risk for BCa and might benefit by therapeutic intervention. Both ACEi and ARBs have been proved efficacious in the treatment of hypertension. Thus, repurposing these drugs and other RAS blockers as co-adjuvants in cancer treatment, chemo-prophylactic agents or coupling their use with specific signaling inhibitors might prove to be an effective therapeutic intervention. Conclusively, consideration of SNP genotype along with phenotypic factors namely, postmenopausal status, tumor size and TNM staging will prove beneficial for better tailored treatment strategies for these patients.

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Authors’ contributions

AS and BA designed and directed the study. AS coordinated blood samples and patient specimens’ collection. AS performed DNA extraction, SNP genotyping and statistical analysis. AS and BA interpreted the results and wrote the manuscript. NS helped with data analysis. SA, MPM and SNP provided control and patient specimens. All authors read and approved the final manuscript.

Competing Interests

The authors declare no conflicts of interest or disclosures. Declarations of interest: none.

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