Association of low penetrance vitamin D receptor Tru9I (rs757343) gene polymorphism with risk of premenopausal breast cancer

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Abstract
Objective: The aim of this study was to determine whether a novel polymorphism (Tru9I) in the low penetrance vitamin D receptor (VDR) gene is associated with risk of premenopausal breast cancer (BC).
Methods: This case-control study included 228 patients with BC and 503 healthy women living in Pakistan who were analyzed for the VDR Tru9I (rs757343) single nucleotide polymorphism. BC cases were histopathologically confirmed, and all healthy controls were age-matched with patients (age range, 20–45 years). DNA was extracted, and the polymerase chain reaction and restriction fragment length polymorphism assays were performed.
Results: The VDR Tru9I polymorphism was not significantly associated with premenopausal BC. However, the risk of BC was associated with the ‘uu’ genotype (odds ratio [OR], 1.141; 95% confidence interval [95% CI], 0.206–6.317). Further, mutant Tru9I was significantly associated with Grade IV carcinoma (OR, 5.36; 95% CI, 1.181–24.338).
Conclusion: The VDR Tru9I ‘uu’ genotype may increase the risk of premenopausal BC.

Keywords
Vitamin D receptor, Tru9I, rs757343, premenopausal, breast cancer, Pakistan

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Introduction

Breast cancer (BC) is the most prevalent cancer and a major cause of death among women worldwide. In Pakistan, the incidence of BC is approximately 34.6% in women. Among risk factors, lifestyle, alcoholism, high birth weight, and abdominal adiposity intensify the risk of BC. Diet may modify the incidence of BC, although many dietary components such as vitamin D are categorized as “Limited or no conclusion.” Vitamin D plays a major role in mineral homeostasis; however, evidence indicates that its deficiency exacerbates the risk of BC. Moreover, higher vitamin D levels are weakly associated with the low BC risk.”

Vitamin D derived from diet and sun exposure of the skin undergoes activation within the liver through 25 hydroxylation, and in the kidney through 1-α hydroxylation, to form 1,25(OH)2D3 (active vitamin D). The classical role of vitamin D is to regulate calcium and phosphate metabolism to maintain bone mineral density (BMD). However, there are wide ranges of nonclassical functions of vitamin D within the body. Vitamin D is activated by forming a complex with the vitamin D receptor (VDR) that is involved in the regulation of many target genes that contribute to cell differentiation, cell growth, programmed cell death, angiogenesis, inflammation, and immune responses. Vitamin D, in concert with growth factors, arrests the cell cycle by preventing entry into S phase. Vitamin D induces apoptosis through down-regulation of the anti-apoptotic protein B-cell lymphoma 2 (BCL2), inhibits the expression of P-cadherin and increases the expression E-cadherin, which may contribute to the anti-invasive or antimetastatic effects of vitamin D. Further, vitamin D induces the expression of the tumor suppressor BRCA1, because the binding sites for the vitamin D/VDR/retinoid X receptor heterodimer complex are present within the BRCA1 promoter region. Although VDR is present in many body tissues, including normal and cancerous breast tissues, its expression is low in mammary cancers cells, which may be caused by variations in VDR.

There are many polymorphic regions in VDR, among which Fok1, Bsm1, Apal, and Taq1 are the most extensively studied in American (Hispanic white, nonhispanic white, and Caucasian) and Asians (Iranian and Egyptian) populations. Attention focuses on the role of single nucleotide polymorphisms (SNPs) in VDR that are associated with the development of a wide range of pathological conditions, including type 2 diabetes mellitus, prostate cancer, and BC. However, limited studies are available on the Pakistani population. Studies of Jordanian populations reveal a statistically significant difference between VDR Taq1 variants and 25(OH)D among women with BC. Bsm1 and Cdx2 polymorphisms are significantly associated with women with BC living in Southeastern Iran. The Fok1 SNP in BC plays a protective role, whereas the Bsm1 SNP is not associated with BC among women living in the Iranian city of Urmia. Among Egyptian women, the Bsm1 “B” allele and “Bb” variants of VDR may represent a risk factor for susceptibility to the development of BC. In contrast, a meta-analysis shows no association of VDR SNPs (Fok1, Bsm1, Taq1, and Apa1) with risk of BC in a general population or in a Caucasian population. Among Pakistanis who are BRCA1/2 non-carriers, the Bsm1 “b” allele is associated with an increased risk of BC. Although the associations of VDR Fok1 and Bsm1 SNPs with the risk of BC have been investigated, no study, to our knowledge, investigated the relationship between the VDR Tru9I polymorphism and the
premenopausal risk of BC. Therefore, the present study was designed to detect an association between the novel low penetrance VDR Tru9I SNP and BC among premenopausal women of Pakistan.

**Materials and methods**

**Ethics statement**

The study was approved by the Bioethics Committee, Board of Advance Studies & Research, University of Karachi (ethics approval number [10(27) 28032012] and conducted in accordance with the Helsinki Declaration. Each subject provided written informed consent before providing a blood sample.

**Study design and study population**

The study included 228 patients with BC and 503 control subjects who were premenopausal ethnic Pakistanis aged 20–45 years. The sample size was calculated using OpenEpi (http://www.openepi.com/Menu/OE_Menu.htm) with 80% statistical power and 95% confidence intervals (95% CIs). Patients with BC, who were randomly selected from two tertiary hospitals in Sindh, Pakistan from 2012 to 2015, were histopathologically diagnosed. The medical information (types, grades, estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2, and TNM classification) of patients with BC was obtained from histopathological reports and hospital records. All healthy volunteers were age-matched (1:2) to premenopausal healthy subjects acquired from the general population. Whole blood (3 mL) was collected from each subject. We used a self-structured questionnaire to acquire along with information about personal demographics, detailed history, and BC-related risk factors.

**DNA isolation**

DNA was extracted using a DNA isolation kit (Gene JET Genomic DNA Purification Kit, Thermo Fisher Scientific Baltics UAB, and Vilnius, Lithuania) following the manufacturer’s protocols. Samples were stored at −86°C.

**Primer selection and genotyping**

VDR Tru9I (rs757343) genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism analysis. The forward primer F: 5’-GCAGGGTACAAAAACTTTGGAG-3’ and the reverse primer R: 5’-CCTCATCACCACA CATCATGTC-3’ were used to amplify the isolated DNA. Amplification was performed using a thermal cycler (Veriti; Applied Biosystems, Foster City, CA, USA) in a final reaction volume (50 μL) containing 25 μL of 2X GoTaq Green Master Mix (Promega, Madison, WI, USA), 4 μL of 12 pmol of both primers, 5 μL of DNA, and 12 μL of nuclease-free water. Polymerase chain reaction analysis was performed as follows: 94°C, initial denaturation (5 minutes); 40 cycles of denaturation at 94°C (30 s); annealing at 59°C (30 s), and extension at 72°C (30 s). The final extension was performed at 72°C for 7 minutes. The 177-bp amplicons were digested using 5 units of MseI (37°C for 1 hour). Amplicons and restriction fragment length polymorphism fragments, which were separated using 2.5% agarose gel electrophoresis, were detected using ethidium bromide and visualized using a ChemiDoc-It2 imaging system with VisionWorks LS Analysis Software (version 7.1) (UVP, Cambridge, UK). The results were coded as U-u, where the Upper case letter is denoted as the absence and lowercase letter is designated as the presence of the restriction site, respectively. The call and concordance rates of the VDR genotypes were 98% and 100%, respectively. The accuracy of the genotypes
was determined by subjecting samples (15 cases and 15 controls) to nucleotide sequence analysis.

Data analysis

Statistical analysis was performed using SPSS software version 22 (IBM Corporation, Armonk, NY, USA) with significance indicated by $P < 0.05$. The Shapiro–Wilk and Kolmogorov–Smirnov tests were used to determine whether the variables were normally distributed. The goodness-of-fit ($\chi^2$) test was used to test whether the controls exhibited Hardy–Weinberg equilibrium to determine deviation of their genotype distribution. Comparisons between groups of $VDR$ $Tru9I$ genotypic and allelic distributions were calculated using Person’s $\chi^2$ test. Odds ratios (ORs) along with 95% CIs were determined using binary and multinomial logistic regression analysis of the $Tru9I$ SNP to determine the risk of BC. The significance of ORs was determined using Wald’s statistics and defined as $P < 0.05$.

Results

Characteristics of patients with BC

Patients’ characteristics are presented in Table 1, which shows that the parameters were not normally distributed among

| Characteristics                                      | Patients n (%) |
|------------------------------------------------------|----------------|
| **Affected area**                                    |                |
| Right*                                                | 123 (53.9)     |
| Left*                                                 | 99 (43.2)      |
| Both*                                                 | 6 (2.6)        |
| **Types of BC**                                       |                |
| Invasive ductal carcinoma (IDC)*                      | 197 (93.4)     |
| Invasive lobular carcinoma (ILC)*                     | 13 (5.7)       |

(continued)
patients according to the Shapiro–Wilk and Kolmogorov–Smirnov tests \((P < 0.01)\).

**Association of the VDR Tru9I SNP with BC**

The genotype distributions of Tru9I among patients (228/228) and healthy controls (497/503) are presented in Table 2. The distribution of Tru9I in the control population did not deviate \((\chi^2 = 0.416, P > 0.05)\) from the Hardy–Weinberg equilibrium. There was no significant difference \((\chi^2 = 0.238)\) between the VDR Tru9I genotype among patients and controls. However, an 11.2% increase in OR \((1.112; 95\% \text{ CI}, 0.202–6.125)\) was observed among ‘uu’ carriers compared with ‘UU and Uu’ carriers. After adjusting for age, the VDR Tru9I ‘uu’ genotype exhibited a 14.1% increase in the OR \((1.141; 95\% \text{ CI}, 0.206–6.317)\) (Table 2).

**Association of the VDR Tru9I SNP with specific pathological characteristics of patients with BC**

We next investigated the association of the VDR Tru9I SNP with BC risk through immunohistochemical subtyping of BC tissues and TNM classification (Table 3). A direct association with risk of BC was found for at least one ‘u’ allele, which was more evident and significant for G-IV stage carcinoma \((OR, 5.36; 95\% \text{ CI}, 1.181–24.338)\).

**Relationship of the VDR Tru9I SNP and risk factors associated with BC**

The possible relationship of the Tru9I SNP and other contributing factors to the risk of BC are presented in Table 4. Those with the mutant \((Uu+uu)\) genotype along with Sindhi and Baluchi ethnicity and marital age greater than 20 years were at significantly increased risk of developing BC; however, there were no significant associations with other contributing factors and BC.

Nine models of contributing factors were prepared in which the Tru9I genotype was adjusted by sequentially adding individual contributing factors (Appendix 1). This analysis shows that the Tru9I ‘uu’ genotype in model 8 significantly increased the risk of BC \((OR, 1.288; 95\% \text{ CI}, 0.16–10.398)\).

**Discussion**

Among Asian countries, Pakistan has the highest incidence of BC, which is a

| Genotypes | Alternate designation | BC cases n (%) | Controls n (%) | X^2 | P value | UOR (95% CI) | AOR (95% CI) |
|-----------|-----------------------|----------------|----------------|-----|----------|---------------|---------------|
| GG        | UU                    | 179 (78.5)     | 398 (80)       | 0.238 | 0.888    | Ref           | Ref           |
| GA        | Uu                    | 47 (20.6)      | 95 (19.1)      |     |          | 1.1 (0.744–1.627) | 1.085 (0.731–1.611) |
| AA        | uu                    | 2 (0.88)       | 4 (0.8)        |     |          | 1.112 (0.202–6.125) | 1.141 (0.206–6.317) |
| Total     |                       | 228            | 497            |     |          |               |               |
| GG        | UU                    | 179 (78.5)     | 398 (80)       |     |          | Ref           | Ref           |
| GA+AA     | Uu+uu                | 49 (21.4)      | 99 (19.6)      |     |          | 1.101 (0.749–1.617) | 1.087 (0.737–1.604) |
| Total     |                       | 228            | 497            |     |          |               |               |
| G allele  | U allele             | 405 (88.8)     | 891 (89.6)     | 0.223 | 0.637    | 1.089 (0.763–1.554) | 1.086 (0.76–1.55) |
| A allele  | u allele             | 51 (11.1)      | 103 (10.3)     |     |          | Ref           | Ref           |
| Total     |                       | 456            | 994            |     |          |               |               |

DNA was amplified from 228/228 patients’ samples and 497/503 control subjects’ samples. BC, breast cancer; UOR, unadjusted odds ratio; AOR, odds ratio adjusted for age and time of blood collection, CI, confidence interval.
Table 3: Associations of the VDR Tru9I single nucleotide polymorphism and risk of premenopausal BC according to the immunohistochemical and pathological characteristics of patients with BC.

| BC characteristics | Tru9I genotype | UU (GG) | OR (95% CI) | Uu+uu (GA+AA) | OR (95% CI) |
|--------------------|----------------|---------|-------------|----------------|-------------|
| BC characteristics | Cases/Controls | OR (95% CI) | Cases/Controls | OR (95% CI) | 
| Types of BC | | | | | |
| IDC | 152/398 Ref | 45/99 | 1.19 (0.799–1.774) | |
| ILC | 13/398 Ref | 0/99 | 1.67 × 10⁻⁹ | (1.67 × 10⁻⁹–1.67 × 10⁻⁹) |
| DCIS | 5/398 Ref | 1/99 | 0.804 (0.93–6.96) | |
| Other | 9/398 Ref | 3/99 | 1.34 (0.356–5.042) | |
| Grades of BC | | | | | |
| I | 5/398 Ref | 1/99 | 0.804 (0.093–6.96) | |
| II | 131/398 Ref | 33/99 | 1.02 (0.66–1.578) | |
| III | 35/398 Ref | 10/99 | 1.149 (0.55–2.399) | |
| IV | 3/398 Ref | 4/99 | 5.36 (1.181–24.338)* | |
| II & III | 2/398 Ref | 0/99 | 12 × 10⁻⁹ | (12 × 10⁻⁹–12 × 10⁻⁹) |
| Unknown | 3/398 Ref | 1/99 | 1.34 (0.138–13.02) | |
| Immunohistochemical classification | | | | | |
| Estrogen receptor status | | | | | |
| Positive (0 to +9) | 103/398 Ref | 23/99 | 0.898 (0.543–1.484) | |
| Negative (−1 to −2) | 25/398 Ref | 9/99 | 1.447 (0.655–3.199) | |
| None | 51/398 Ref | 17/99 | 1.34 (0.742–2.421) | |
| Progesterone receptor status | | | | | |
| Positive (0 to +8) | 92/398 Ref | 22/99 | 0.961 (0.575–1.608) | |
| Positive (−1 to −2) | 30/398 Ref | 8/99 | 1.072 (0.477–2.411) | |
| None | 57/398 Ref | 19/99 | 1.34 (0.762–2.355) | |
| Human epidermal growth factor receptor 2 status | | | | | |
| Positive (0 to +3) | 78/398 Ref | 20/99 | 1.031 (0.602–1.766) | |
| Negative (−1 to −2) | 18/398 Ref | 2/99 | 0.447 (0.102–1.957) | |
| None | 83/398 Ref | 27/99 | 1.308 (0.804–2.128) | |
| TNM classification (For invasive cases) | | | | | |
| Size of primary tumor (T stage) | | | | | |
| Tx | 1/398 Ref | 1/99 | 4.681 (0.277–79.215) | |
| Tis | 3/398 Ref | 1/99 | 1.281 (0.13–12.598) | |
| T1 | 0/398 Ref | 0/99 | NC | |
| T2 | 27/398 Ref | 9/99 | 1.412 (0.639–3.121) | |
| T3 | 25/398 Ref | 6/99 | 0.896 (0.354–2.267) | |
| T4 | 23/398 Ref | 3/99 | 0.521 (0.152–1.783) | |
| No involvement | 100/398 Ref | 29/99 | 1.135 (0.706–1.823) | |
| Metastasize to regional lymph node (N stage) | | | | | |
| Nx | 4/398 Ref | 2/99 | 1.821 (0.325–10.218) | |
| N0 | 20/398 Ref | 3/99 | 0.593 (0.172–2.048) | |

(continued)
heterogeneous group of diseases associated with various risk factors. The most common risk factor is exposure to endogenous hormones such as estradiol.\textsuperscript{28} Approximately 15\% of BC is caused by genetic changes,\textsuperscript{29} including those associated with high penetrance genes such as BRCA\textsubscript{1/2},\textsuperscript{30} and low-to-moderate penetrance genes such as checkpoint kinase-2 (CHEK2) and VDR.\textsuperscript{20,31} VDR contributes to the prevention of BC, and the vitamin D/VDR complex induces apoptosis, reduces angiogenesis, proliferation, and invasion and increases cell differentiation.\textsuperscript{32} Evidence indicates that the VDR mediates most of the activities of vitamin D. Analyses of common VDR SNPs (Fok1, Bsm1, Apa1, Taq1, and Poly-A) show associations between VDR SNPs and BC, although the results are inconsistent among different types of exposure.\textsuperscript{20,22,33,34} The Fok1 ‘ff’ genotype may increase the risk of BC.\textsuperscript{35,36} However, evidence suggests that Fok1 significantly correlates with human epidermal growth factor receptor 2 status\textsuperscript{20} and is significantly associated with estrogen receptor status of patients with BC.\textsuperscript{37} One study shows that the Bsm1 ‘b’ allele is associated with risk of BC,\textsuperscript{20} although another study reports the opposite results.\textsuperscript{22} To our knowledge, the present study is the first to determine the association of the low penetrance VDR Tru9I polymorphism with premenopausal BC. The VDR Tru9I is caused by an A to G substitution that was first identified in 2000.\textsuperscript{38} VDR Tru9I resides in intron 8, +443-bp from the end of exon 8, and may be involved in the regulation of gene expression through its affect on enhancer affinity and target area.\textsuperscript{39} The present study shows that the Tru9I mutant ‘uu’ genotype was associated with an increased risk of developing premenopausal BC. There are few association studies of Tru9I in other diseases such as coronary heart disease (CHD), colorectal adenoma, and prostate cancer.\textsuperscript{26,39,40} However, Tru9I is not associated with CHD\textsuperscript{39} and prostate cancer\textsuperscript{40} among the Chinese Han population, whereas Tru9I lowers the risk of colorectal carcinoma

### Table 3 Continued.

| Tru9I genotype | BC characteristics | Cases/Controls | OR (95\% CI) | Cases/Controls | OR (95\% CI) |
|---------------|-------------------|---------------|-------------|---------------|-------------|
| UU (GG)       | N1                | 25/398        | Ref         | 11/99         | 1.796 (0.847–3.806) |
|               | N2                | 16/398        | Ref         | 3/99          | 0.737 (0.209–2.598)  |
|               | N3                | 14/398        | Ref         | 1/99          | 0.297 (0.038–2.304)  |
|               | No involvement   | 100/398       | Ref         | 29/99         | 1.134 (0.706–1.822)  |
| Uu+uu (GA+AA) | Mx                | 29/398        | 10/99       | 1.386 (0.654–2.94)  |
|               | M0                | 39/398        | 9/99        | 0.928 (0.435–1.979)  |
|               | M1                | 11/398        | 1/99        | 0.365 (0.047–2.864)  |
|               | No involvement   | 100/398       | 29/99       | 1.166 (0.73–1.862)   |

BC, breast cancer; CI, confidence interval; OR, odds ratio. VDR Tru9I “Uu” and “uu” genotypes were combined, because there were very few subjects with “uu.” ORs were calculated using multinomial logistic regression analysis adjusted for age and time of blood collection. *\(P < 0.05\).
Table 4 Combined associations of the VDR Tru9I single nucleotide polymorphism and factors contributing to the risk of premenopausal BC.

| BC risk factors          | Combined cases/controls | OR (95% CI) | Cases/controls | OR (95% CI) | Uu+uu cases/controls | OR (95% CI) |
|--------------------------|-------------------------|-------------|----------------|-------------|----------------------|-------------|
| **Age (years)**          |                         |             |                |             |                      |             |
| 20–26 (Younger)          | 20/58                   | Ref         | 16/51          | Ref         | 4/5                  | Ref         |
| 27–33                    | 34/78                   | 1.264 (0.661–2.418) | 23/53          | 1.383 (0.667–2.914) | 11/23     | 0.598 (0.134–2.675) |
| 34–40                    | 110/225                 | 1.418 (0.812–2.475) | 83/179         | 1.478 (0.796–2.745) | 27/44     | 0.767 (0.189–3.109) |
| 41–45 (Older)            | 64/142                  | 1.307 (0.726–2.352) | 57/115         | 1.58 (0.829–3.012)  | 7/27      | 0.324 (0.068–1.535) |
| **Total**                | 228/503                 |              | 179/398        |             | 49/99                |             |
| **Ethnicity/Race**       |                         |             |                |             |                      |             |
| Urdu-Speaking            | 94/252                  | Ref         | 78/199         | Ref         | 16/51                | Ref         |
| Sindhi                   | 41/12                   | 9.41 (4.719–18.767)* | 29/9           | 8.456 (3.809–18.772)* | 12/3      | 14.934 (3.573–62.418)* |
| Panjabi                  | 20/104                  | 0.522 (0.305–0.891)* | 16/86          | 0.493 (0.272–0.897)* | 4/17      | 0.675 (0.193–2.335) |
| Bukhtoon                 | 18/47                   | 0.996 (0.549–1.806) | 15/39          | 0.946 (0.492–1.821) | 3/8       | 0.97 (0.22–4.283)   |
| Baluchi                  | 19/4                    | 12.926 (4.265–39.171)* | 12/3.          | 10.875 (2.963–39.908)* | 7/1       | 19.323 (2.15–173.641)* |
| Others                   | 36/84                   | 1.17 (0.738–1.854) | 29/62          | 1.188 (0.709–1.992) | 7/19      | 1.179 (0.408–3.41)  |
| **Total**                | 228/503                 |              | 179/398        |             | 49/99                |             |
| **Marital status**       |                         |             |                |             |                      |             |
| Yes                      | 208/363                 | Ref         | 162/286        | Ref         | 45/75                | Ref         |
| No                       | 20/140                  | 0.139 (0.071–0.273)* | 16/112        | 0.15 (0.071–0.318)* | 4/24.     | 0.111 (0.024–0.524)* |
| **Total**                | 228/503                 |              | 179/398        |             | 49/99                |             |
| **Marital age (years)**  |                         |             |                |             |                      |             |
| <20                      | 105/244                 | Ref         | 84/186         | Ref         | 21/56                | Ref         |
| 20 to 30                 | 95/117                  | 3.343 (2.157–5.18)* | 71/98          | 2.48 (1.525–4.033)* | 24/19     | 10.645 (3.473–32.623)* |
| >30                      | 8/2.                    | 20.996 (4.15–106.236)* | 8/2.           | 15.914 (3.138–80.698)* | 0/0      | NC                     |
| **Total**                | 208/363                 |              | 163/286        |             | 45/75                |             |
| **Parity**               |                         |             |                |             |                      |             |
| Parous                   | 191/342                 | Ref         | 149/267        | Ref         | 40/71                | Ref         |
| Nulliparous              | 37/161                  | 0.347 (0.216–0.557)* | 30/131        | 0.366 (0.216–0.619)* | 9/28      | 0.395 (0.145–1.071) |
| **Total**                | 228/503                 |              | 179/398        |             | 45/99                |             |

(continued)
| BC risk factors | Combined | UU | Uu+uu |
|----------------|----------|----|-------|
| **Age at first child birth (years)** | | | |
| Below 20 | 49/63 Ref | 41/55 Ref | 8/8 Ref |
| 20 to 24 | 81/118 0.528 (0.33–0.845)* | 64/87 0.515 (0.302–0.879)* | 17/30 0.582 (0.208–1.627) |
| 25 to 29 | 39/115 0.621 (0.328–1.179) | 30/88 0.458 (0.215–0.977)* | 9/26 1.533 (0.41–5.731) |
| >29 | 17/43 1.218 (0.753–1.969) | 11/36 1.086 (0.639–1.846) | 6/7 2.006 (0.604–6.661) |
| Total | 186/339 | 146/266 | 40/71 |
| **History of breast feeding** | | | |
| Yes | 186/339 Ref | 146/266 Ref | 40/71 Ref |
| No | 22/24 1.674 (0.901–3.107) | 17/20 1.54 (0.773–3.068) | 5/4 2.269 (0.528–9.748) |
| Total | 208/363 | 163/286 | 45/75 |
| **Age at menarche (years)** | | | |
| 12 to 14 | 186/423 Ref | 148/334 Ref | 38/83 Ref |
| >14 | 28/45 0.913 (0.479–1.74) | 13/29 0.995 (0.5–1.978) | 1/6 0.306 (0.035–2.662) |
| <12 | 14/35 1.389 (0.838–2.303) | 18/35 1.111 (0.606–2.035) | 10/10 2.567 (0.946–6.966) |
| Total | 228/503 | 179/398 | 49/99 |
| **Use of hormone replacement therapy** | | | |
| No | 209/481 Ref | 168/382 Ref | 41/93 Ref |
| Yes | 19/22 1.954 (1.034–3.691)* | 11/16 1.547 (0.702–3.412) | 8/6 3.114 (0.99–9.798) |
| Total | 228/503 | 179/398 | 49/99 |
| **Use of oral contraceptive** | | | |
| No | 197/454 Ref | 155/361 Ref | 42/87 Ref |
| Yes | 31/49 1.473 (0.91–2.383) | 24/37 1.521 (0.878–2.635) | 7/12 1.17 (0.423–3.234) |
| Total | 228/503 | 179/398 | 49/99 |
| **Waist to hip ratio** | | | |
| Good (<0.8) | 27/92 Ref | 20/71 Ref | 7/19 Ref |
| Average (0.8–0.85) | 15/173 0.296 (0.15–0.585)* | 12/134 0.317 (0.146–0.685)* | 3/37 0.252 (0.057–1.12) |
| High (>0.85) | 186/238 2.687 (1.672–4.317)* | 147/193 2.727 (1.58–4.707)* | 39/43 2.568 (0.949–6.944) |
| Total | 228/503 | 179/398 | 49/99 |
Table 4 Continued.

| BC risk factors           | Combined Cases/ Controls | OR (95% CI) | UU Cases/ Controls | OR (95% CI) | Uu+uu Cases/ Controls | OR (95% CI) |
|---------------------------|--------------------------|-------------|--------------------|-------------|------------------------|-------------|
| BMI (kg/m²)               |                          |             |                    |             |                        |             |
| Normal/healthy weight     | 56/157                   | Ref         | 43/127             | Ref         | 13/29                  | Ref         |
| weight (18.6–23)          |                          |             |                    |             |                        |             |
| Underweight (>18.5)       | 22/64                    | 0.966 (0.544–1.717) | 19/48             | 1.196 (0.633–2.261) | 3/14 | 0.383 (0.09–1.637) |
| Overweight (23.1–27)      | 79/158                   | 1.381 (0.909–2.097) | 63/132             | 1.374 (0.858–2.2)  | 16/23 | 1.689 (0.654–4.364) |
| Obese (>27)               | 71/124                   | 1.589 (1.028–2.456) | 54/91             | 1.708 (1.036–2.815)* | 17/33 | 1.183 (0.474–2.953) |
| Total                     | 228/503                  | 179/398     | 42/88              | 1.348 (0.473–3.841) | 49/99 | |
| Family history of BC      |                          |             |                    |             |                        |             |
| No                        | 191/455                  | 149/361     | Ref                | 42/88       | Ref                    |            |
| Yes                       | 37/48                    | 1.83 (1.153–2.906)* | 30/37           | 1.947 (1.156–3.28)* | 7/11  | 1.348 (0.473–3.841) |
| Total                     | 228/503                  | 179/398     |                     |             |                        |             |

VDR Tru9I “Uu” and “uu” genotypes were combined because there were very few subjects in the “uu” category. DNA was amplified from 228/228 patients’ samples and 497/503 control subjects’ samples. ORs were adjusted for age and time of blood collection. *P < 0.05.

#Crude/unadjusted ORs for age were calculated. BC, breast cancer; BMI, body mass index; NC, not calculated, OR, odds ratio.
among women living in North Carolina.\textsuperscript{26} We show further that mutant \emph{Tru9I} may be associated with the development of Grade IV carcinoma, whereas another study shows the opposite relationship with colorectal adenoma, which is more pronounced for multiple, larger, and sessile adenomas, and perhaps with dysplasia.\textsuperscript{26}

This study has certain limitations. First, the sample size was relatively small. Large-scale studies are therefore required to support our conclusions. Second, we did not study environmental factors including diet and lifestyle, which may modify the association between the \emph{VDR Tru9I} polymorphism and BC. Finally, other \emph{VDR} SNPs and serum vitamin D levels were not studied. However, further research is required to confirm our findings and explore the possible mechanisms of BC underlying the association of \emph{VDR Tru9I} with oncogenesis to facilitate the development of more effective treatment.

We conclude that the \emph{VDR Tru9I} ‘uu’ genotype may predict a high risk for premenopausal BC.

\textbf{Declaration of conflicting interest}

The authors declare that there is no conflict of interest.

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\textbf{References}

1. Shaukat U, Ismail M and Mehmood N. Epidemiology, major risk factors and genetic predisposition for breast cancer in the Pakistani population. \textit{Asian Pac J Cancer Prev} 2013; 14: 5625–5629.

2. Ferlay J, Soerjomataram I, Dokshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. \textit{Int J Cancer} 2015; 136: E359–E386. DOI: 10.1002/ijc.29210.

3. Hirko KA, Chen WY, Willett WC, et al. Alcohol consumption and risk of breast cancer by molecular subtype: Prospective analysis of the nurses’ health study after 26 years of follow-up. \textit{Int J Cancer} 2016; 138: 1094–1101. DOI: 10.1002/ijc.29861.

4. Nagrani R, Mhatre S, Rajaraman P, et al. Central obesity increases risk of breast cancer irrespective of menopausal and hormonal receptor status in women of South Asian Ethnicity. \textit{Eur J Cancer} 2016; 66: 153–161. DOI: \texttt{http://dx.doi.org/10.1016/j.ejca.2016.07.022}.

5. Bassett JK, Hodge AM, English DR, et al. Plasma phospholipids fatty acids, dietary fatty acids, and breast cancer risk. \textit{Cancer Causes Control} 2016; 27: 759–773. DOI: 10.1007/s10552–016-0753–2.

6. Eisman JA and Bouillon R. Vitamin D: direct effects of vitamin D metabolites on bone: lessons from genetically modified mice. \textit{BoneKEy Rep} 2014; 3: 499. DOI: 10.1038/bonekey.2013.233.

7. Bauer SR, Hankinson SE, Bertone-Johnson ER, et al. Plasma vitamin D levels, menopause, and risk of breast cancer: dose-response meta-analysis of prospective studies. \textit{Medicine (Baltimore)} 2013; 92: 123–131. DOI: 10.1097/MD.0b013e3182943bc2.

8. Williams JD, Aggarwal A, Swami S, et al. Tumor autonomous effects of Vitamin D deficiency promote breast cancer metastasis. \textit{Endocrinology} 2016; 157: 1341–1347. DOI: 10.1210/en.2015–2036.

9. Kim Y and Je Y. Vitamin D intake, blood 25 (OH)D levels, and breast cancer risk or mortality: a meta-analysis. \textit{Br J Cancer} 2014; 110: 2772–2784. DOI: 10.1038/bjc.2014.175.

10. Kuhn T, Kaaks R, Becker S, et al. Plasma 25-hydroxyvitamin D and the risk of breast cancer in the European prospective investigation into cancer and nutrition: a nested case-control study. \textit{Int J Cancer} 2013; 133: 1689–1700. DOI: 10.1002/ijc.28172.
11. Lin R and White JH. The pleiotropic actions of vitamin D. *BioEssays* 2004; 26: 21–28. DOI: 10.1002/bies.10368.

12. Rai V, Abdo J, Agrawal S, et al. Vitamin D Receptor Polymorphism and Cancer: An Update. *Anticancer Research* 2017; 37:3991–4003. DOI: 10.21873/anticancerres.11784. Available at: https://www.ncbi.nlm.nih.gov/pubmed/28739681

13. Deeb KK, Trump DL and Johnson CS. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nat Rev Cancer* 2007; 7: 684–700. DOI: 10.1038/nrc2196.

14. Pendas-Franco N, Gonzalez-Sancho JM, Suarez Y, et al. Vitamin D regulates the phenotype of human breast cancer cells. *Differentiation* 2007; 75: 193–207. DOI: 10.1111/j.1432-0436.2006.00131.x.

15. Lopes N, Carvalho J, Duraes C, et al. 1Alpha,25-dihydroxyvitamin D3 induces de novo E-cadherin expression in triple-negative breast cancer cells by CDH1-promoter demethylation. *Anticancer Res* 2012; 32: 249–257.

16. Campbell MJ, Gombart AF, Kwok SH, et al. The anti-proliferative effects of 1alpha,25(OH)2D3 on breast and prostate cancer cells are associated with induction of BRCA1 gene expression. *Oncogene* 2000; 19: 5091–5097. DOI: 10.1038/sj.onc.1203888.

17. Lopes N, Sousa B, Martins D, et al. Alterations in Vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions. *BMC Cancer* 2010; 10: 483. DOI: 10.1186/1471–2407-10-483.

18. Bertocci L, Sentinelli F, Leonetti F, et al. The vitamin D receptor functional variant rs2228570 (C>T) does not associate with type 2 diabetes mellitus. *Endocr Res* 2017; 42: 1–5. DOI: 10.1080/07435800.2017.1305965.

19. Torkko KC, van Bokhoven A, Mai P, et al. VDR and SRD5A2 polymorphisms combine to increase risk for prostate cancer in both non-Hispanic White and Hispanic White men. *Clin Cancer Res* 2008; 14: 3223–3229. DOI: 10.1158/1078–0432.ccr-07–4894.

20. Hashemi SM, Arjabi N, Hashemi M, et al. Association between VDR Gene Polymorphisms (rs 1544410, rs 7975232, rs 2228570, rs 731236 and rs 11568820) and Susceptibility to Breast Cancer in a Sample of Southeastern Iranian Population. *Int J Cancer Manag* 2017; 10: e8807.

21. Atoum MF and Al-Khatib YM. Association between Serum 25-hydroxy Vitamin D Concentration and TaqI Vitamin D Receptor Gene Polymorphism among Jordanian females with breast cancer. *Chin Med J (Engl)* 2017; 130: 1074–1078. DOI: 10.4103/0366–6999.204933.

22. Talaneh S, Ghorbani A, Oghabi Bakhshaiesh T, et al. FokI and BsmI Polymorphisms of the VDR Gene and Breast Cancer Risk. *MCI* 2017; 1: 21–25.

23. Elzehery RR, Baiomy AA, Hegazy MAF, et al. Vitamin D status, receptor gene BsmI (A/G) polymorphism and breast cancer in a group of Egyptian females. *Egyptian Journal of Medical Human Genetics* 2017; 18: 269–273. DOI: https://doi.org/10.1016/j. ejmhg.2016.11.003.

24. Lu D, Jing L and Zhang S. Vitamin D Receptor Polymorphism and Breast Cancer Risk: A Meta-Analysis. *Medicine* 2016; 95: e3555. DOI: 10.1097/md.0000000000003535.

25. Rashid MU, Muzaffar M, Khan FA, et al. Association between the BsmI Polymorphism in the Vitamin D Receptor Gene and Breast Cancer Risk: Results from a Pakistani Case-Control Study. *PloS One* 2015; 10: e0141562. DOI: 10.1371/journal.pone.0141562.

26. Gong YL, Xie DW, Deng ZL, et al. Vitamin D status, receptor gene BsmI (A/G) polymorphism and breast cancer in a group of Egyptian females. *World J Gastroenterol* 2005; 11: 4794–4799.

27. Sarwar MR and Saqib A. Cancer prevalence, incidence and mortality rates in Pakistan in 2012. *Cogent Medicine* 2017; 4: 1288773. DOI: 10.1080/2331205X.2017.1288773.

28. Wu H, Chen Y, Liang J, et al. Hypomethylation-linked activation of PAX2 mediates tamoxifen-stimulated endometrial carcinogenesis. *Nature* 2005; 438: 981–987. DOI: doi:10.1038/nature04225

29. Saslow D, Boetes C, Burke W, et al. American Cancer Society guidelines for
breast screening with MRI as an adjunct to mammography. *CA Cancer J Clin* 2007; 57: 75–89.

30. Peterlongo P, Chang-Claude J, Moysich KB, et al. Candidate genetic modifiers for breast and ovarian cancer risk in BRCA1 and BRCA2 mutation carriers. *Cancer Epidemiol Biomarkers Prev* 2015; 24: 308–316. DOI: 10.1158/1055–9965.epi–0532.

31. Desrichard A, Bidet Y, Uhrhammer N, et al. CHEK2 contribution to hereditary breast cancer in non-BRCA families. *Breast Cancer Res* 2011; 13: R119. DOI: 10.1186/ber3062.

32. Krishnan AV and Feldman D. Mechanisms of the anti-cancer and anti-inflammatory actions of vitamin D. *Annu Rev Pharmacol Toxicol* 2011; 51: 311–336. DOI: 10.1146/annurev-pharmtox–010510–100611.

33. Guo B, Jiang X, Hu X, et al. Association between vitamin D receptor gene polymorphisms and breast cancer in a Chinese population. *Int J Clin Exp Med* 2015; 8: 8020–8024.

34. Reimers LL, Crew KD, Bradshaw PT, et al. Vitamin D-related gene polymorphisms, plasma 25-hydroxyvitamin D, and breast cancer risk. *Cancer Causes Control* 2015; 26: 187–203. DOI: 10.1007/s10552–014–0497–9.

35. Engel LS, Orlow I, Sima CS, et al. Vitamin D receptor gene haplotypes and polymorphisms and risk of breast cancer: a nested case-control study. *Cancer Epidemiol Biomarkers Prev* 2012; 21: 1856–1867. DOI: 10.1158/1055–9965.epi–0551.

36. Mishra DK, Wu Y, Sarkissyan M, et al. Vitamin D receptor gene polymorphisms and prognosis of breast cancer among African-American and Hispanic women. *PloS One* 2013; 8: e57967. DOI: 10.1371/journal.pone.0057967.

37. Nemenqani DM, Karam RA, Amer MG, et al. Vitamin D receptor gene polymorphisms and steroid receptor status among Saudi women with breast cancer. *Gene* 2015; 558: 215–219. DOI: 10.1016/j.gene.2014.12.065.

38. Ye WZ, Reis AF and Velho G. Identification of a novel Tru9 I polymorphism in the human vitamin D receptor gene. *J Hum Genet* 2000; 45: 56–57. DOI: 10.1007/s100380050011.

39. He L and Wang M. Association of vitamin d receptor-a gene polymorphisms with coronary heart disease in Han Chinese. *Int J Clin Exp Med* 2015; 8: 6224–6229.

40. Bai Y, Yu Y, Yu B, et al. Association of vitamin D receptor polymorphisms with the risk of prostate cancer in the Han population of Southern China. *BMC Med Gen* 2009; 10: 125–125. DOI: 10.1186/1471–2350-10–125.

**Appendix 1** Association of the Tru9I single nucleotide polymorphism with early onset BC after adjustment for multiple contributing factors.

| Models | Contributing factors | Beta coefficient (β) | Wald | P value | OR | 95% CI | Lower | Upper |
|--------|----------------------|----------------------|------|--------|----|--------|-------|-------|
| Model 8 | Tru9I uu | 0.253 | 0.057 | 0.812 | 1.288 | 0.16 | 10.398 |
|        | Tru9I Uu | 0.074 | 0.078 | 0.78 | 1.076 | 0.642 | 1.805 |
|        | Age 27–33 years | –21.417 | 0.000 | 0.999 | 0.000 | 0.000 | NC |
|        | Age 34–40 years | –21.577 | 0.000 | 0.999 | 0.000 | 0.000 | NC |
|        | Age 41–45 years | –22.593 | 0.000 | 0.998 | 0.000 | 0.000 | NC |
|        | Marital age 20–30 years | 0.854 | 10.332 | 0.001* | 2.349 | 1.396 | 3.955 |
|        | Marital age >30 years | 2.917 | 10.134 | 0.001* | 18.492 | 3.068 | 111.444 |

(continued)
Continued.

| Models                  | Contributing factors                  | Beta coefficient (β) | Wald | P value | OR     | 95% CI          |
|-------------------------|---------------------------------------|----------------------|------|---------|--------|-----------------|
| Age at first live birth | 20–24 years                           | −0.435               | 2.616| 0.106   | 0.647  | 0.382 – 1.097   |
| Age at first live birth 25–29 years | −0.457               | 1.546               | 0.214| 0.633   | 0.308  | 1.301          |
| Age at first live birth >29 years | −0.044               | 0.025               | 0.873| 0.957   | 0.554  | 1.653          |
| No lactation            | NC                                    | NC                  | NC   | NC      | NC     | NC              |
| Age at menarche >14 years| 0.025                  | 0.004               | 0.951| 1.025   | 0.46   | 2.287          |
| Age at menarche <12 years| 0.236                  | 0.541               | 0.462| 1.266   | 0.676  | 2.372          |
| BMI >18.5 kg/m²         | 0.527                  | 1.493               | 0.222| 1.694   | 0.727  | 3.944          |
| BMI 23.1–27 kg/m²       | 0.149                  | 0.282               | 0.595| 1.16    | 0.67   | 2.009          |
| BMI >27 kg/m²           | 0.329                  | 1.217               | 0.27 | 1.39    | 0.774  | 2.496          |
| WHR 0.8–0.85            | −1.315                 | 9.386               | 0.002*| 0.269   | 0.116  | 0.623          |
| WHR >0.85               | 0.813                  | 6.885               | 0.009*| 2.254   | 1.228  | 4.137          |
| OC use                  | 0.437                  | 1.761               | 0.185| 1.548   | 0.812  | 2.953          |

BC, breast cancer; BMI, body mass index; WHR, waist to hip ratio; OC, oral contraceptive use; HRT, hormone replacement therapy. *Significant association (Wald’s statistics); \( P < 0.05 \). Data are shown only for model 8.