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ASSOCIATION OF INTRON 4 VNTR (4A/B) POLYMORPHISM OF THE ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE WITH THE INCIDENCE OF BREAST CANCER IN IRAQI WOMEN

Background. The VNTR 4a/b (rs61722009) polymorphism in intron 4 of the *NOS3* gene is crucial for various biological processes and has been linked to cancer. Evidence suggests this polymorphism affects *NOS3* gene expression and may promote tumor growth in the mammary gland. **Aim.** This study aimed to investigate the association between *NOS3* 4a/b polymorphism and breast cancer (BC) susceptibility in Iraqi women, and to evaluate the potential correlation between these genetic variants and serum cancer antigen 15-3 (CA15-3) levels as a prognostic marker. **Materials and Methods.** The role of the 4a/b polymorphism was examined using PCR genotyping on DNA extracted from participants, including 50 women with BC and an equal number of controls. The level of CA15-3 was measured in the patients as well. **Results.** The homozygous wild-type b/b genotype may confer a protective effect against BC, with a significantly lower frequency in patients (8%) compared to controls (72%) ($p < 0.01$). Conversely, the heterozygous a/b and homozygous mutant a/a genotypes were more frequent in patients (50% and 42%, respectively) than in controls (22% and 6%, respectively) ($p < 0.01$). Notably, the a/a genotype was significantly associated with increased BC risk (OR = 3.08, 95% CI: 1.19—5.47) and predominantly observed in the advanced pT2 stage. Additionally, the mean serum CA15-3 levels were significantly higher in patients with the a/a and a/b genotypes (15.66 U/mL and 22.91 U/mL, respectively) compared to those with the b/b genotype (6.37 U/mL) ($p < 0.01$). **Conclusion.** The differences in genotype and allele frequencies between BC patients and healthy controls, along with the association of polymorphisms with CA15-3 levels, suggest that this genetic marker could serve as a valuable tool for risk assessment as well as prognosis in BC ptients. Further investigations with larger and more diverse population samples are needed.

Keywords: VNTR 4 a/b, breast carcinogenesis, polymorphism.

Breast cancer (BC) is the most prevalent cancer among women worldwide, comprising 25% of new cancer cases [1]. In Iraq, it is the most common malignancy affecting women and the primary cause of cancer-related deaths among them [2, 3]. While high-resource countries have achieved sig-

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nificant advancements in BC survival rates, the risk remains substantial in low-income countries, resulting in higher mortality rates [4]. Nitric oxide synthase (NOS) exists in three forms: inducible NOS (iNOS/NOS2), neuronal NOS (nNOS/NOS1), and endothelial NOS (eNOS/NOS3). These enzymes belong to the NOS family and produce nitric oxide (NO), which functions as a signaling molecule and plays a crucial role in cancer development, either by promoting or inhibiting it. Furthermore, NO is involved in various physiological processes, including immunity, neurotransmission, endothelial vasodilation, and carcinogenesis [5, 6].

Numerous studies have highlighted the role of nitric oxide in carcinogenesis, emphasizing its dual effect on cancer development and progression [7]. Nitric oxide is a highly reactive molecule within biological systems, capable of interacting with other free radicals and causing DNA damage. Its role in cancer development involves the formation of peroxynitrite (ONOO-) and N_2O_3, which can result in apoptosis, DNA strand breaks, or the removal of DNA bases [5, 6]. In BC research, some studies suggest that the expression levels of both NOS2 and NOS3 are elevated in invasive tumors [8, 9].

The *NOS3* gene, located at locus 7q35-36 in humans, encodes a protein made up of 1,203 amino acids [10]. Among the polymorphisms of the *NOS3* gene, the 4a/b (rs61722009) polymorphism is one of the most common and widely studied worldwide [11—13], although studies involving Iraqi BC patients remain scarce. The *NOS3* gene 4a/b polymorphism is a 27-bp VNTR located in intron 4, with two common alleles: 4a and 4b. The 4a allele contains 4 repeats, while the 4b allele consists of 5 repeats. Additionally, 2 rarer alleles, 4y and 4c, have been identified in African and Colombian populations, containing 3 and 4 repeats, respectively [12, 14]. It was revealed that individuals with the 4a variant of the *NOS3* gene have lower plasma nitric oxide levels and reduced protein expression [12]. Endothelial cells with 5 copies of the VNTR show higher quantities of siRNA compared to cells with 4 copies [15].

Cancer antigen 15-3 (CA15-3) is a high-molecular-weight glycoprotein (300—450 kDa), typically produced by the apical surfaces of epithelial ducts and acinar cells in the breast, where it is secreted into milk. In cancer, alterations in the breast tissue structure enable CA15-3 to enter the bloodstream. Tracking CA15-3 levels can help assess the BC progression [16, 17]. However, since some types of BC cells do not overproduce this antigen, elevated CA15-3 levels may not be observed in all cases of the disease [18].

Many studies have reported differences in the frequency of alleles for the 4a/b polymorphism across various ethnic groups and have linked it to several diseases, including cancer [12]. However, other studies investigating the relationship between BC and the 4a/b polymorphism of the *NOS3* gene have not found statistically significant associations [19]. This suggests an ongoing debate. Given the lack of local studies in Iraq, the aim of our research was to explore the potential link between breast cancer and the 4a/b polymorphism of the *NOS3* gene in Iraqi women, as well as to examine its possible association with the plasma CA15-3 levels in patients.

Materials and Methods

Study samples. Blood samples (100 in total) were collected between February and June 2024, consisting of 50 samples from women with BC and 50 healthy women who volunteered as blood donors (control group). All participants were Iraqi nationals, nonsmokers. The mean age of the patients was 49.6 ± 11.5 years, while the controls had a mean age of 48.7 ± 11.6 years. Among the BC patients, 64% had a family history of the disease, with 40% in stage pT1 and 60% in stage pT2. The diagnoses were made by doctors and specialist surgeons at the Oncology Hospital and Baghdad Teaching Hospital in Baghdad, Iraq. Clinicopathological demographic information for the participants was gathered from hospital records for the patients and via a questionnaire for the healthy controls. Informed consent was obtained from all subjects, and the Ethics Committee of the National Cancer Research Center at the University of Baghdad approved conducting the study.

Genomic DNA isolation. Total genomic DNA was isolated from the whole fresh blood collected in EDTA K3-containing tubes for molecular studies. DNA extraction from the blood samples (both patients and controls) was performed directly using the Blood DNA Extraction Kit (46300-Norgen®, Canada), following the manufacturer's protocol.

Determination of genotypes for the 4a/b polymorphism of the NOS3 gene. Genotyping the *NOS* gene was conducted according to a protocol adapted from

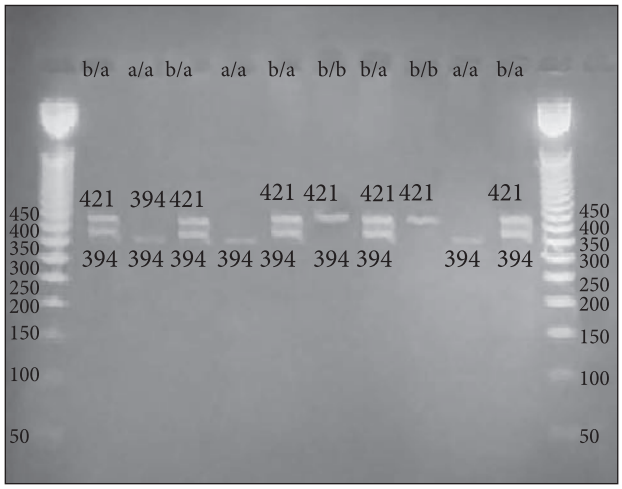

Fig. 1. Genotyping of the VNTR 27 polymorphism in intron 4 of the *NOS3* gene. The 421-bp band indicates five repeats of the 27-bp sequence, while the 394-bp band represents four repeats. Lanes 2, 4, 6, 8, and 11 show a/b heterozygous variants; lanes 7 and 9 are b/b homozygous wild type. Lanes 3, 5, and 10 show a/a homozygous mutant. Lanes 1 and 12 are 50-bp DNA markers

Kiffmeyer et al. [20] to our laboratory conditions. For this experiment, primer sequences were designed using an NCBI Primer-BLAST tool, and their specificity was checked in silico against the human genome reference sequence (RefSeq: NG_011992.1) to ensure a target-specific amplification. The sequences of the primers are listed in Table 1. The primers were synthesized by Alpha DNA (Canada) and provided as lyophilized products. They were reconstituted in DNase/RNase-free water to obtain a stock concentration of 100 pmol/μL and stored at −20 °C. A working solution of 10 pmol/μL was prepared by a 1:10 dilution in DNase/RNase-free water.

PCR amplification was performed in a 25 μL reaction volume using GoTaq® Green Master Mix (Promega, USA) according to the manufacturer's protocol. After preparing the reaction mixture, tubes were vortexed and placed into a thermal cycler. The PCR amplified products were subsequently separated by electrophoresis on a 2% agarose gel stained with 1 μL of a 10 mg/mL ethidium bromide solution; run at 75 V for 1 h in 1X TBE buffer. A total of 6 μl aliquots from each sample were loaded onto the gel, and 5 μL of a 50 bp DNA ladder was used as a marker. The 4b and 4a alleles were identified at 421 bp and 394 bp, respectively, as shown in Fig. 1.

Determination of serum CA15-3 levels. Serum levels of the tumor marker CA15-3 were quantified using a sandwich enzyme-linked immunosorbent assay (ELISA) (EIA5068R, DRG International, Inc., USA). The microtiter wells were pre-coated with a monoclonal mouse antibody that specifically targets a particular antigenic site on the CA15-3 protein. After incubating the patient samples on the solid phase, they were incubated with an enzyme conjugate, which consisted of horseradish peroxidase combined with an anti-CA15-3 antibody. Unbound molecules were removed through washing after incubation. The color intensity that developed after adding the substrate was measured at 450 nm using HumaReader HS, Germany.

Statistical analysis. The Statistical Analysis System (SAS) software, version 9.4, was used to analyze the data and assess the effect of different groups (patients and controls) on study parameters. The least significant difference (LSD) test and the *t*-test were used to compare means. The Chi-square (χ^2) test was used to compare frequencies. One-way analysis of variance (ANOVA) was used to compare the mean serum levels of CA15-3 among different *NOS3* gene polymorphism groups, as well as to compare its levels between different age groups of patients. In addition, odds ratio (OR) and confidence interval (CI) values were also calculated. The significance levels were set at $p < 0.05$.

Results

The present study investigated the distribution of the 4a/b polymorphism of the *NOS3* gene in both apparently healthy subjects and BC patients in a sample from the Iraqi population. The distribution of genotype and allele frequencies for the 4a/b polymorphism among Iraqi women with BC and healthy women is presented in Table 2.

The frequency of the wild-type b/b genotype was significantly lower in BC patients (8%) compared

Table 1. **Nucleotide sequence of primers VNTR, rs61722009**

SNP	Primers, probes	Sequence → (5–3)	Product size, bp	Tm, °C
rs61722009	Forward Reverse	AGGCCCTATGGTAGTGCCTTT TCTCTTAGTGCTGTGGTCAC	421	58

to healthy women (72%), $p < 0.01$, Conversely, both the a/b and a/a genotypes were significantly more frequent in patients, with the a/a genotype present in 42% of patients compared to 6% of controls, showing the strongest association ($p < 0.01$, OR = = 3.08 [1.19–5.47]). In addition, the 4a allele was substantially more prevalent in BC patients (67%) than in controls (17%).

Furthermore, our study revealed a significant difference in the distribution of *NOS3* genotypes among patients at the pT1 stage (tumors ≤2 cm) and those at the pT2 stage (tumors >2–≤5 cm) ($\chi^2 = 26.00$, $p < 0.001$). The a/a genotype was predominantly found in the more advanced pT2 stage, whereas the b/b genotype appeared exclusively in pT1 cases (20.0%), as shown in Table 3.

The difference in *NOS3* genotypes between younger patients (≤55 years) and older patients (>55 years) was evaluated in this study as well. The a/a genotype was notably more frequent in the younger group (48.5%) compared to the older group (29.4%), which might reveal a potential link between *NOS3* polymorphisms and BC progression or prognosis. However, this difference did not reach statistical significance ($\chi^2 = 5.21$, $p = 0.07$) (Table 4).

We estimated the marker CA15-3 serum levels [21] in the patient group and investigated the po-

Table 2. **Genotype distribution and allele frequencies of the 4a/b (rs1722009) VNTR polymorphism**

Genotypes	Control	Breast cancer	χ^2	*p*-value	OR (CI)
b/b	36 (72.0%)	4 (8.0%)	25.60	0.0002**	Ref. =1
a/b	11 (22.0%)	25 (50.0 %)	5.44	0.0196*	1.078 (0.92—1.84)
a/a	3 (6.0%)	21 (42.0%)	13.50	0.0002**	3.08 (1.19—5.47)
Total	50 (100.0%)	50 (100.0%)	—	—	—
Allele frequencies					
b	83 (83.0%)	33 (33.0%)	21.55	0.0001**	—
a	17 (17.9%)	67 (67.0%)	29.76	0.0001**	—

** $p < 0.05$, ** $p < 0.01$.*

Table 3. **Distribution of *NOS3* genotypes across breast cancer stages**

Genotypes	pT1	pT2	Total	χ^2	*p*-value
b/b	4 (20.0%)	0 (0.0 %)	4 (8.0%)	26.00	< 0.001**
a/b	16 (80.0%)	9 (30.0%)	25 (50.0%)		
a/a	0 (0.0%)	21 (70.0%)	21 (42.0%)		
Total	20 (100.0%)	30 (100.0%)	50 (100.0%)		

Table 4. **Distribution of *NOS3* genotypes across age categories in breast cancer patients**

Genotypes	Age ≤ 55	Age > 55	Total	χ^2	*p*-value
b/b	4 (12.1%)	0 (0.0%)	4 (8.0%)	5.21	0.07
a/b	13 (39.4%)	12 (70.6%)	25 (50.0%)		
a/a	16 (48.5%)	5 (29.4%)	21 (42.0%)		
Total	33 (100.0%)	17 (100.0%)	50 (100.0%)		

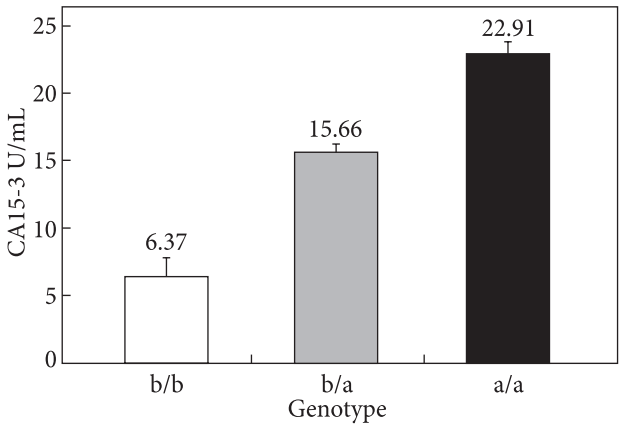

Fig. 2. Variation of CA15-3 serum levels in patients according to the *NOS3* gene polymorphism, $p < 0.01$, ANOVA

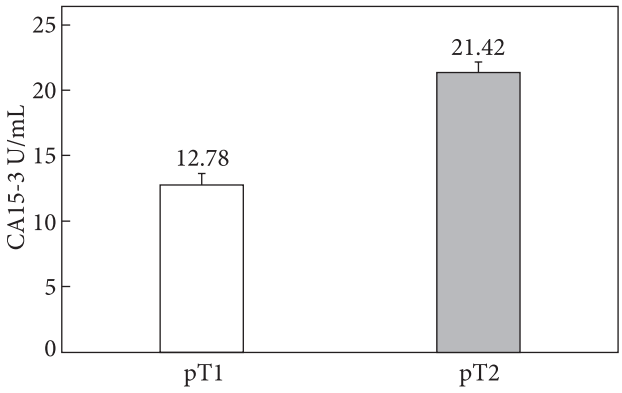

Fig. 3. Variation of CA15-3 serum levels according to disease stage, $p < 0.01$, *t*-test, pT1 represents smaller tumors (≤2 cm) and pT2 represents larger tumors (>2 and ≤5 cm)

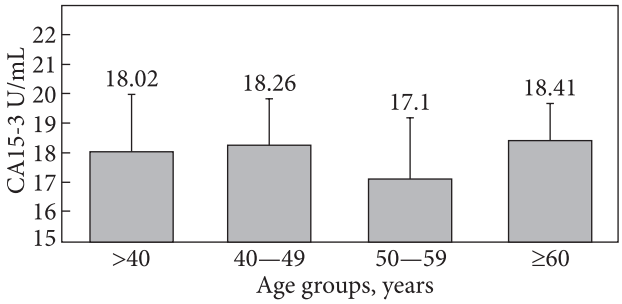

Fig. 4. Difference in serum CA15-3 among patients' age groups, $p > 0.05$, ANOVA.

tential relationship between the 4a/b polymorphism and this marker. The mean CA15-3 serum levels of patients increased from 6.37 U/mL in the b/b allele group to 15.66 U/mL and 22.91 U/mL in patients with b/a and a/a alleles, respectively. Moreover, the overall CA15-3 levels difference among allele categories (homozygous wild-type, heterozygous, and homozygous mutant) in patients was highly significant ($p < 0.01$) (Fig. 2). Accordingly, CA15-3 serum levels increased significantly in pT2 stage cancer patients as compared to pT1 stage (Fig. 3).

The mean CA15-3 levels of patients across different age categories were also analyzed in this study. The data demonstrated that the difference in CA15-3 serum levels was not significant, although elderly patients had the highest mean of the aforementioned marker (Fig. 4).

Discussion

This study provides new insights into the association between the *NOS3* 4a/b polymorphism and BC susceptibility and progression among Iraqi women.

Our findings demonstrate a significantly lower frequency of the wild-type b/b genotype in BC patients compared to healthy controls, suggesting a potential protective effect of this allele in Iraqi women with BC. The significantly increased frequency of the a/b genotype in patients suggests that this heterozygosity may contribute to BC susceptibility. Moreover, the a/a genotype, which was markedly more frequent in patients and strongly associated with BC risk, reinforces the hypothesis that the 4a allele may act as a genetic risk factor for BC among Iraqi women. These findings align with previous research by Ramírez-Patiño et al. [12], who demonstrated that the b/b genotype is less frequent in BC Mexican women, as compared to controls, and there is a relationship between the a/a-a/b genotypes as a risk factor in patients and raised levels of glutamate-oxaloacetate transaminase (SGOT). In the same vein, our finding is consistent with a study of Fard [22], who found that Iranian BC patients tend to have a higher frequency of the *NOS3* 4a and a lower frequency of the *NOS3* 4 b/b genotypes as compared to controls. In addition, Erdem et al. [23] demonstrated a protective role for the *NOS3* b/b genotype in Turkish women with BC, though they did not find a significant association with the a/a genotype. While studies directly investigating the role of this polymorphism in BC remain limited, related associations have been observed in other cancer types. For instance, individuals carrying the «a» allele of the *NOS3* 4a/b polymorphism have been found to be at a higher risk of developing advanced prostate cancer [24]. Similarly, a study conducted in Taiwan indicated that the *NOS3* 4a/b polymorphism may increase

the risk of early-onset colorectal cancer, particularly among individuals with the higher-risk a/b or a/a genotypes, compared to those with the bb genotype [25]. Yet, a study on the Turkish population could not demonstrate a correlation for 4 a/b polymorphism between patients with bladder cancer and control groups [26], which was attributed to ethnic variation as evidenced by a meta-analysis study [27]. Although the exact functional effects of this polymorphism are not fully understood, it has been proposed that it modulates *NOS3* expression through the production of short RNAs (siRNAs), which appear to inhibit *NOS3* expression at the transcriptional level [12, 28].

Angiogenesis, invasion, and metastasis are among the cancer-related processes that NOS3 can regulate. The vascular endothelial growth factor (VEGF) and prostaglandin E2 are two endothelium growth stimulators that are centrally mediated by eNOS. Both *NOS2*+/+ and *NOS2*–/– mice can have their angiogenesis increased by the former (VEGF), but *NOS3*–/– mice cannot. This suggests that NOS3 plays a major role in VEGF-induced angiogenesis. Furthermore, trophoblast cancer cell vascular invasion has been linked to elevated NOS3 expression, according to an in vivo investigation. Strong NOS3-positive tumor cells are consistently seen in lung metastatic locations, indicating that NOS3 expression promotes metastasis [19, 29]. Yet, reduced levels of NO might also contribute to carcinogenesis [30].

Importantly, the observed distribution of genotypes across different tumor stages indicates that *NOS3* polymorphisms may influence not only the risk of developing BC but also its clinical progression. The a/a genotype was predominantly detected in patients with pT2 (advanced) tumors, while the b/b genotype was exclusive to early-stage (pT1) patients. These patterns suggest that the a/a genotype may be associated with a more aggressive tumor phenotype, potentially contributing to worse outcomes. A similar association between *NOS3* polymorphisms and tumor stage was reported in other studies, where certain *NOS3* genotypes were found to be linked with advanced clinical stages in BC [19, 31]. This was further supported by our results regarding the significantly elevated CA15-3 levels in patients with the a/a genotype as a well-established marker of tumor burden and poor prognosis [16].

Overall, these results suggest that the *NOS3* 4a/b polymorphism, especially the 4a allele, is linked not only to a higher risk of developing BC but may also play a role in determining disease progression and prognosis. The consistent trends across genotype, tumor stage, and CA15-3 levels underscore the potential utility of *NOS3* genotyping as a biomarker for risk stratification and clinical management in BC.

This study demonstrates a significant association between the *NOS3* 4a/b polymorphism and BC risk among Iraqi women. The 4a allele, especially in the homozygous a/a genotype, was strongly linked to increased susceptibility, more advanced tumor stages, and elevated levels of CA15-3 in serum, suggesting its potential role in disease progression and prognosis. These findings highlight the value of *NOS3* genotyping as a promising tool for risk assessment and prognosis in BC patients.

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Conflict of interest

The authors have no conflicts of interest to declare.

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АСОЦІАЦІЯ МІЖ ПОЛІМОРФІЗМОМ VNTR (4A/B) ІНТРОНУ 4 ГЕНА ЕНДОТЕЛІАЛЬНОЇ СИНТАЗИ ОКСИДУ АЗОТУ ТА ЗАХВОРЮВАНІСТЮ НА РАК МОЛОЧНОЇ ЗАЛОЗИ У ЖІНОК ІРАКУ

Стан питання. Поліморфізм VNTR (4a/b) інтрону 4 гена *NOS3* є важливим фактором для різних біологічних процесів і пов'язаний зі схильністю до розвитку раку. Цей поліморфізм впливає на експресію гена *NOS3* і може сприяти злоякісному росту у молочній залозі. **Мета** роботи полягала в дослідженні асоціації між поліморфізмом *NOS3* 4a/b та сприйнятливістю до розвитку раку молочної залози у жінок Іраку та оцінити потенційну кореляцію між цими генетичними варіантами та рівнем сироваткового ракового антигену 15-3 (CA15-3) як прогностичним маркером. **Матеріали та методи**. Для визначення ролі поліморфізму 4a/b застосували метод ПЛР з ДНК, виділеною з крові 50 хворих на РМЗ жінок і здорових жінок. Визначали також рівні CA15-3. **Результати**. Гомозиготний генотип b/b дикого типу може мати захисний ефект щодо розвитку раку молочної залози, оскільки він зустрічається значно рідше у хворих (8%) в порівнянні із здоровими жінками (72%) ($p < 0,01$). І навпаки, гетерозиготи a/b та гомозиготи з мутантними a/a зустрічаються частіше у хворих на рак молочної залози (50% та 42%, відповідно) в порівнянні із здоровими жінками (22% та 6%, відповідно) ($p < 0,01$). Генотип a/a вірогідно асоційований зі збільшенням ризику розвитку раку молочної залози (співвідношення ризиків 3,08, 95% ДІ: 1,19—5,47) і переважно зустрічається у випадках розповсюдженого раку стадії pT2. Крім того, середні рівні CA15-3 у сироватці були вірогідно вищими у хворих з генотипами a/a та a/b (15,66 од./мл та 22,91 од./мл, відповідно) в порівнянні з хворими з генотипом b/b (6,37 од./мл) ($p < 0,01$). **Висновок.** Різниця в генотипах та частоті алелей між хворими на рак молочної залози і здоровими жінками та асоціація цих поліморфізмів за рівнями CA15-3 дозволяють вважати, що цей генетичний маркер може бути корисним для оцінки ризику та прогнозування у хворих на рак молочної залози. Необхідні подальші дослідження на більш численних та різноманітних популяціях.

Ключові слова: VNTR 4 a/b, рак молочної залози, поліморфізм.