Research Article

Analysis of P4 receptors polymorphisms in the development of breast cancer: A study of Southern Punjab (Pakistan)

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Abstract

Contrary to reports with conflicting results, progesterone is suspected to play an important role in the development of tumours in the women. A research work was conducted to assess the significance of PGR SNPs (progesterone receptor) polymorphisms in the progression of breast cancer in southern Punjab (Pakistan). Because of the lack of detailed analysis of *PGR* gene variations in the Pakistani community, two variants *PROGINS* and +331G/A were selected. The Radiology and Oncology Department at Nishtar Medical College and Hospital in Multan, Pakistan, assisted researchers for enrolling 100 invasive breast cancer patients and one hundred and fifteen healthy controls. DNA was isolated using a non-organic method, and two SNPs were analysed by (Polymerase Chain Reaction Restriction Fragment length polymorphism) PCR-RFLP. Study participants were examined for their clinical/family history, demographic factors (e.g. age, Menopause, age of menopause, marital status, gestation status, history of memory carcinoma, cigarette consumption, and chemical exposure), and other variables being related to specific symptomatology. The wild type allele of both SNPs was observed with highest prevalence in patients and control individuals followed by heterozygous allele while homozygous mutant allele was present in least numbers. Chi square statistics for *PROGINS* showed ($p=0.448$) an evidence of non-significant association while +331G/A variant ($p=0.004$) showed an inverse association with breast cancer risk. Further genetic studies using progesterone receptors for breast cancer risk can use our results as a database.

Keywords: Breast Cancer; Menopause; Progesterone; Progens

Introduction

It affects more than one million people each year, mostly women, and is the main reason of death in females. Our country is bearing a double load of disease due to substantial incidence of cancer and increasing tendency in risk factors like smoking and alcohol consumption [1]. According to a study, every ninth woman in Pakistan is at

risk of developing breast carcinoma. Age, menopause, alcohol, sex, race, with already affected persons in the family, reproductive condition, living standard, iodine deficient level, high-cholesterol food intake, overweight, smoking, UV rays, hormones disturbance and work load are some of the known risk factors predisposing population towards breast carcinoma [2, 3]. Molecular

analysis can help to generate useful data for public health management since the prevalence and death ratio because of this disease are very alarming in our country and there is no population-based registries data available.

In addition to the environmental factors, genetic causes also have significant contribution in enhancement of chances of cancer development. There are a number of genes associated with breast cancer, including estrogen receptor (*ESR*), progesterone receptor (*PR*), *GSTT1*, *GSTM1*, *GSTO2*, *GSTP1*, and *XPB*.

This steroid is an essential structural constituent of the testosterone. It resides in the cells of the endocrine glands, testicles, brain, and placenta and undergoes a variety of biochemical processes during gestation, characterized with multiple stimulation and inhibition functions. In conjunction with estrogen, it is associated with the progression of pregnancy, adolescence, mammary ductal branching and differentiation of lobuloalveolar branches in a pregnant female [4, 5].

Seven variables along with two promoter variants (+44C/T and +331G/A), four single nucleotide polymorphisms (p.Ser334Thr, p.Gly393Gly, p.Val660Leu & p.His770His), and an insertion in non-coding region were studied previously in different population with conflicting results [6]. Val660Leu, His770His, Ser334Thr and *Alu* insertion in intron 7, these three variables form the *PROGINS* allele complex [7]. Because of linkage disequilibria, all polymorphisms have substantially similar genotype.

PROGINS allele does not tend to be involved with any impacts on the biological activity of progesterone receptors. Nevertheless, the *PROGINS* SNP has an association with an elevated breast, ovarian, and endometrial carcinoma potential. [8, 9], with some studies showing non-significant association between *PROGINS* and cancer susceptibility. Other studies showed that women who have both alleles of the *PROGINS* gene are at a

greater risk of getting ovarian carcinoma with lower breast cancer risk [10-12].

Similar conflicting studies have been stated regarding the relationship of +331G>A variant with breast cancer. Contradictory data exist regarding the involvement of progesterone receptor genes in cancer growth through in vitro research indicating that it may increase and decrease the process of cell proliferation or have no role at all [13, 14]. The *PROGINS* and +331G/A variations were chosen on account of their relation with endometrial, uterine and breast carcinomas showing conflicting findings in various communities.

Objectives

It was the purpose of this research work to ascertain whether progesterone polymorphisms were present among Pakistani females who progressed breast cancer, and to compare the frequency of polymorphisms identified in Pakistan with that observed in other communities.

Materials and Methods

Sample collection

A research work was approved by the Institutional Review Boards (IRB) at Nishtar Medical College and Hospital in Multan, Pakistan and the Institute of Molecular Biology and Biotechnology at Bahauddin Zakariya University, Multan, Pakistan. Before taking blood samples from the control subjects and each case, there was a written informed agreement. All medical and demographical information was collected with the help of a special questionnaire (menstrual/breast feeding histories, menarche, menopause, use of birth control pills, family history, chemical exposure and tobacco history). The research identified and enrolled over 100 women aged 15 to 65 who had invasive breast cancer. We arbitrarily selected 115 healthy persons without any record of cancer in family from Southern Punjab (Multan, Dera Ghazi Khan, Rajan Pur, Layyah and Lodhran districts) Pakistan.

Molecular exploration

A blood sample was collected for DNA analysis by using a non-organic protocol

[15]. Analysis of Val660Leu and +331G>A, SNPs was performed by using PCR restriction fragment length polymorphism (PCR-RFLP) method using forward and reverse primers. Amplification of Val660Leu and +331G>A variants of *PROGINS* gene was carried out by using a nested PCR approach. Amplified products of *PROGINS* (138bp) and +331G>A (206 bp) polymorphisms were restricted with BsrI and NlaIV (Fermentas, USA) respectively and were studied on 1.5% agarose gel. Results for polymorphisms of *PROGINS* were determined by observing the restriction pattern i.e. homozygous wild type (+/+) has a fragment of 138bp, heterozygous (+/-) has three fragments 115, 95 and 23 bp and homozygous mutant (-/-) has two fragments of 115 and 23 bp.

Results

One hundred females diagnosed with breast cancer from Nishtar Medical College and Hospital and one hundred and fifteen control individuals enrolled from different areas of Punjab were included in the study. Each case and control subject was between 15 and 65 years old. Several other factors were also used to analyze what effect they had on the phenotype, such as the occurrence of menopause and the age of menopause, marriage, pregnancy, history of breast carcinoma, the influence of smoking, and chemicals.

In the study, there were about half menopausal women and at least half premenopausal women with breast cancer. Menopausal age ranged between 28-58 years. About 97 percent of patients were married and breast cancer was diagnosed mostly between 36-45 years of age. In patients, only 16 had a family history, whereas all the others had an isolated growth. Only 4 of the breast cancer patients had smoked cigarettes, and 16 of them had been exposed to pesticides (Table 1). *PROGINS* and +331G/A SNPs were analyzed with PCR-RFLP technique. A fragment of the progesterone receptor gene of 138 bp was amplified by primers designed for the *PROGINS* SNP (Fig. 1),

The amplification of +331G>A allele was achieved by exploiting a fragment of 206 base pairs. Wild type allele (+331GG) produces two segments of 149 and 57bp and heterozygous alleles of 206 and 149 bp; mutated allele (+331AA) produces only one fragment of 206 bp due to absence of any NlaIV restriction site.

Statistical analysis

For the purpose of determining the relationships between all variables, the frequencies were calculated. Hardy-Weinberg equilibrium in control samples was checked by applying Chi-square test. The development of breast tumor due to *PROGINS* and +331G/A variants was checked by Pearson's Chi-square applying in SPSS. Based on a probability of greater than 0.05, an association was established. whereas primers for +135G/A result in 206 bp of a gene fragment, as shown by ethidium bromide stained gel (Fig. 2). Majority of the patients (22.3%) were between 36-45 years of age, with maximum frequency of breast cancer ($\chi^2 = 50.26$; $df = 4$; $P < 0.0001$). Based on statistical analysis, it was found that menopause made a significant impact on breast cancer incidences ($P < 0.0001$; Table 1).

It is depicted by the results that menopausal women have 5 to 6 times greater risk of carcinoma development (22.3%; $\chi^2 = 27.72$; $d.f. = 1$; $P < 0.0001$) when matched with the women of premenopausal condition (8.4%). Moreover, there is a great effect of age of menopause ($\chi^2 = 29.41$; $d.f. = 3$; $P < 0.0001$) on breast carcinoma development in local community. The menopause age was observed between 36 - 45 years for 15.8% of the cancer patients. The findings furthermore showed that the maximum cancer rate in most of our patients (20.5%) is between 36-45 years of age. This study suggests that cancer predisposition is also related to the patient's age, menopausal status, and the age at cancer diagnosis. and Interesting to note, these risk factors show almost the same correlation over the course of the same time period. Furthermore, breast cancer was

significantly more prevalent in those with a family history of cancer as compared to control samples ($\chi^2 = 19.87$; d.f. = 1; $P < 0.0001$). But other factors such as marital status, the age at the first pregnancy, the total number of pregnancies, breastfeeding, maternal smoking, and pesticide prior exposure did not indicate a significant association with breast cancer susceptibility (Table 1).

Logistic regression analysis of causative agents like pre-menopause/menopause with breast cancer was implemented. There appeared to be a surprising correlation between the middle-aged women (36-45 years) and the likelihood of breast cancer ($t = 0$; $B = -5.14$; d.f. = 213; $P < 0.0001$). Breast feeding enhances the chances of developing cancer in premenopausal females by a significant value ($t = 0$; $B = 2.31$; d.f. = 213; $P = 0.021$).

Premenopausal women who feed their kids have a higher risk of developing cancer. Although, no significant association was found between *PROGINS* and premenopausal women ($t = 0$; $B = 3.32$; d.f. = 213; $P = 0.60$), significant associations for cancer susceptibility were observed with +331G/A during pre-menopause but not with +331G/A during menopause ($t = 0$; $B = -6.24$; d.f. = 213; $P = 0.045$). So, it can be concluded that pre-menopause has an impact on polymorphism +331G/A which is associated with cancer development (Table 2).

There were no significant associations between *PROGINS* genotypes and phenotypes of breast cancer for either SNP ($\chi^2 = 1.6$; df = 2; $P > 0.05$; Table 3), as homozygous wild type was observed in 69% of the patients with very nearly same frequency in healthy group (72.2%). The percentage of heterozygous allele of the *PROGINS* polymorphism was 29% and 27% in patients and normal individuals of total samples. Homozygous mutant allele of *PROGINS* was observed in only 2% of the case samples. Likewise, it is more frequent to have wild-type alleles ($F = 0.84$) in *PROGINS* than mutant alleles ($F = 0.16$).

With the wild-type genotype being more prevalent than other genotypes both in healthy individuals and in those affected with breast cancer showing non-significant association with breast cancer development, so it can be demonstrated that *PROGINS* variation may have no important function in cancer predisposition. On the other hand, Breast cancer risk was inversely related to the polymorphism +331G/A ($\chi^2 = 9.13$; d.f. = 2; $P = 0.004$). The frequency of wild type allele of +331G/A (93%) was higher than heterozygous allele (3.3%). The mutant allele was not noticed in any of the affected persons enrolled for the study. Same allele ratio was observed in case and control individuals for +331G/A SNP (Case: $F = 0.97$; Control: $F = 0.99$; Table 3). Therefore, a negative association of genotype and phenotype was observed for +331G/A mutation and cancer occurrence in our community.

We correlated the *PROGINS* polymorphisms and pre-menopause/menopause with a probability of getting breast cancer. No significant association with breast cancer development was observed between *PROGINS* SNP and pre-menopause/menopause (pre-menopause: $\chi^2 = 1.015$; df = 2; $P > 0.05$, menopause: $\chi^2 = 2.821$; df = 2; $P > 0.05$; Table 4). Women with or without menopause and *PROGINS* polymorphisms are at similar risk for developing cancer. Cross tabulation studies to check the inference between +331G/A SNP and pre-menopause/menopause with breast cancer was also studied. The study showed that the *PROGINS* polymorphism did not have a significant association with disease susceptibility in the context of menopause. ($\chi^2 = 1.563$; df = 1; $P = 0.219$). The case-control study to check the association of breast cancer with +331G/A SNP in premenopausal females reported an inverse association ($\chi^2 = 6.313$; df = 2; $P > 0.05$; Table 4). The incidence ratio of alleles of *PROGINS* studied were 70.7% (+/+),

26.0% (+/-) and 3.3% (-/-) in local community.

By making comparison of the allele incidence ratios of *PROGINS* SNP in global communities like Australia, England, Netherland, Brazil, Italy, and USA [11, 16-20], it was noticed that the percentage of *PROGINS* in Australia,

England, Netherlands and Italy is same as observed in present study, while high incidence rate was observed in case of Brazil (80.5%). The percentages of three alleles of +331G/A polymorphisms studied in local community of Pakistan were 96.3% (+/+), 3.3% (+/-) and 0.5% (-/-). (Table 5).

Table 1. Frequencies of demographic factors in Pakistani population

Characteristics	Category	Control N=115	Case N=100	P value	d.f.
Age	15-25	34(15.8%)	1(0.46%)	0.000***	4
	26-35	39(18.1%)	19(8.8%)		
	36-45	24(11.2%)	48(22.3%)		
	46-60	4(1.9%)	12(5.60%)		
	60-70	34(15.8%)	1(0.46%)		
Menopause	Post	18(8.4%)	49(22.8%)	0.000***	1
	Pre-	97(43.7%)	51(23.8%)		
Menopause Age	28-38	1(0.5%)	6(2.8%)	0.000***	3
	39-48	10(4.7%)	34(15.8%)		
	49-58	7(3.3%)	8(3.7%)		
	N/A	97(43.7%)	52(24.1%)		
Marriage	Yes	101(46.9%)	97(43.7%)	0.013*	1
	No	14(6.5%)	3(1.4%)		
Marriage Age	15-25	91(42.3%)	89(41.4%)	0.060 ^{ns}	2
	26-35	9(4.2%)	7(3.3%)		
	N/A	15(6.9%)	4(1.9%)		
Breast Cancer Age	26-35	0	26(12.1%)	0.000***	4
	36-45	0	44(20.5%)		
	46-55	0	22(10.2%)		
	56-65	0	8(3.7%)		
	N/A	0	0		
History of Breast Cancer	Yes	0	16(7.4%)	0.000***	1
	No	100	84(39.1%)		
Age at 1st Pregnancy	15-25	79(36.7%)	71(33.0%)	0.522 ^{ns}	2
	26-35	15(6.9%)	16(7.4%)		
	N/A	21(9.8%)	13(6.0%)		
No of pregnancies	1-5	66(30.7%)	53(24.7%)	0.252 ^{ns}	3
	6-10	24(11.2%)	32(14.9%)		
	11-15	4(1.9%)	2(0.9%)		
	N/A	21(9.8%)	13(6%)		
Breast feeding	Yes	78(36.3%)	82(38.1%)	0.019*	1
	No	37(17.2%)	37(17.2%)		
Breast feeding Age	15-25	21(9.8%)	8(3.7%)	0.071 ^{ns}	3
	26-35	55(25.6%)	52(24.1%)		
	36-45	17(7.9%)	24(11.2%)		
	N/A	22(10.2%)	16(7.4%)		
Smoking	Yes	13(6.0%)	4(1.9%)	0.074 ^{ns}	1
	No	102(47.4%)	96(96%)		
Pesticide Exposure	Yes	34(15.8%)	16(44.7%)	0.023*	1
	No	81(37.7%)	84(39.1%)

Figure 1. Gel electrophoresis for RFLP product of *PROGINS*. *PROGINS* wild type is shown in lanes 3, 5, 7, 10, 11 & 12; homozygous mutant in lanes 1 & 2; heterozygous in lanes 4, 8 & 9 respectively. Uncut is given in lane 6 and 100bp ladder (lane M)

Figure 2. Gel electrophoresis for restriction product of +331G/A. +331G/A wild type is shown in lanes 2, 3 & 4; homozygous mutant in lanes 5, 6, 7 & 9; heterozygous in lanes 8 & 10 respectively. Uncut is given in last lane and 100bp ladder in lane M

Table 2. Logistic regression analysis of menopause and other variables

	Parameters	Categories	B-test	T-test	d.f.	P value
Menopause	Present age	15-25	-21.82	-11.3	213	0.000***
		26-35	-7.28	0	213	
		36-45	-5.14	0	213	
		46-60	-7.47	0	213	
		60-70	-	-	-	
Pre-menopause	B. feeding	Yes	2.31	0	213	0.021**
		No	0	-	-	
	PROGINS	(+/+)	3.32	0	213	0.60 ^{ns}
		(+/-)	3.186	0	213	
		(-/-)	0	0	-	
	+331G/A	(GG)	-6.24	0	213	0.045*
		(GA)	0	0	-	
		(AA)	-	0	213	

Table 3. Genotype and allelic frequency of progins and +331 G/A

PROGINS					+331 G/A			
Control	(+/+)	(+/-)	(-/-)	Sig. Test	GG	GA	AA	Sig. Test
	83(72.2%)	27(23.5%)	5(4.3%)	$X^2=1.6$ d.f.=2 $P=0.4^{ns}$	114(99%)	0	1 (0.9%)	$X^2=9.1$ d.f=2 $P=0.004^{**}$
Case	69(69%)	29(29%)	2(2%)		93(93%)	7(7%)	0	

Table 4. Significance test of progins and +331G/A and menopause with breast carcinoma

Genotype	Pre-Menopause			Menopause		
	Control	Case	Sig. Test	Control	Case	Sig. Test
Progin (+/+)	70(32.56%)	33(15.35%)	$X^2=1.015$	13(6.05%)	36(16.74%)	$X^2=2.821$
Progin (+/-)	23(10.7%)	16(7.44%)	df=2	4(1.86%)	13(6.05%)	df=2
Progin (-/-)	4(1.86%)	2(0.93%)	$P=0.605^{ns}$	1(0.47%)	0	$P=0.253^{ns}$
+331G/A (+/+)	96(44.65%)	48(22.32%)	$X^2=6.313$	18(8.37%)	45(20.93%)	$X^2=1.563$
+331G/A (+/-)	0	3(1.39%)	df=2	0	4(1.86%)	df=1
+331G/A (-/-)	1(0.5%)	0	$P=0.045^*$	0	0	$P=0.219^{ns}$

Table 5. Global distribution of frequencies of progesterone receptor

Population	No.	PROGINS			+331G/A			Reference
		(+/+)	(+/-)	(-/-)	(+/+)	(+/-)	(-/-)	
Pakistan	215	70.7	26.0	3.3	96.3	3.3	0.5	Present study
Australia	175	70.2	26.95	2.8	89.1	10.9	10.9	[16]
Brazil	303	80.5	18.25	2.5	-	-	-	[17]
Italy	258	73.3	25.15	1.5				[18]
Netherland	546	73.1	24.4	2.6	89.0	9.0	2.1	[19]
USA	987	-	-	-	89.0	10.9	0.7	[20]

Discussion

Different factors contribute to a female's chances to develop breast tumor, including factors related to her hormonal status. Not only is estrogen essential for breast and ovarian tissues to develop, but progesterone is also important for differentiation and proliferation. As the menstrual cycle nears its end, progesterone is released from the ovaries to prepare the uterus for receiving the egg. When the egg has been fertilized, the progesterone hormone works to prevent further egg release. Because of this ability, it is also called the "pregnancy-supporting" hormone. Anyhow its clear function in the progression of a disease is not yet known. Currently, no authentic data is present for the function of these SNPs in local

Various studies of healthy and affected mammary tissues have produced contradictory findings, depicting that progesterone may either lead to enhancement, decline or no variation at all. PR receptor-bound hormones act as nuclear transcription factors, with multiple effects in addition to binding to PR receptors. There is no doubt that progesterone receptor SNPs cause PR expression, thereby disrupting hormonal homeostasis in the cells. Also described are the effects of these SNPs on the risk for benign and malignant gynecological anomalies among different populations throughout the world [21].

community. This study is one of the first to investigate the function of progesterone

polymorphisms in breast cancer risk in the southern Punjab region. It was shown that progesterone did not affect cancer phenotype in a significant way and an inverse correlation was observed in Pakistani women when analyzing the relative susceptibility to breast cancer with +331G/A polymorphism. The new results concur with those found in previous studies performed in populations in Germany and Ireland that indicated that the *PROGINS* polymorphism did not affect the chance of breast cancer progression. The *PROGINS* polymorphism was not found to be associated with breast cancer in other case-control studies [16, 22].

Evaluation of *PROGINS* mutations on the Nurses' also indicated that *PROGINS* alleles have no significant association with memory tumor development. The function of *PROGINS* polymorphism have contrasting findings about breast cancer development. There has been research suggesting a positive relationship between the 2nd progesterone variant +331G/A and either mammary, endometrial, or ovarian tumors in multiple populations. Some studies did not support the hypothesis of role of +331G/A variant in cancer development. One study reported that two functional PGR polymorphisms in the Australian population were non-significantly associated with the development of breast cancer [20]. In the same way, four variants of this gene (+44C/T, +331G/A, Gly393Gly, Val660Leu) were analyzed showing no significant link between the SNPs and the development of ovarian and endometrial tumor [23, 24].

According to our study results, cross-tabulation test between +331G/A and the *PROGINS* genotypes revealed that some demographic factors had a strong influence on breast cancer incidences among the Pakistani population, such as family history of cancer and menopause. Various reports are present to support the association of family history with disease development. It has been shown that in women who had

their mother or sister diagnosed with breast cancer when they were young, their risk of developing breast cancer remains elevated. A systematic review about the risk factors of breast cancer particularly family history was published. The results were consistent for various study results showing more chances of evolving breast cancer with family history [25].

Female reproductive factors are responsible for about 40- 50% of breast cancer risks among women. Hormonal conditions including early menarche and late menopause, have high chance of cancer and according to the studies; women with natural menopause had greater the risk of breast cancer. We can explain how cancer susceptibility is related to these risk factors by considering hormone levels, menstrual periods, irregular cycles and induction of cell differentiation [11].

Conclusion

It can be concluded from all of the foregoing that *PROGINS* polymorphism does not have any relation to breast tumor occurrences in our population., while +331G/A mutation is conversely related to breast tumor and The situation is exacerbated even further by hormonal changes during menopause, resulting in an increased risk of breast cancer with the *PROGINS* and +331G/A gene mutations. But there was lack of any link with other variables like marital status, conception age, number of gravidities, breastfeeding, age at last breastfeeding, and smoking.

The study was performed with small number of samples consisting of one-year data due to shortage of funds. Increasing the sample size will assist in analyzing these polymorphisms more effectively. All data were based on self-reports of women who did not have a confirmed history of breast cancer and whose memory of the exact age of menopause was not reliable. Further studies are obviously needed, including age, gender, and known polymorphisms of the progesterone gene, along with other hormones in breast cancer pathways, to pinpoint the role of the

progesterone gene in the development of breast cancer in the Pakistani population.

Authors' contributions

Conceived and designed the experiments: RS Shaikh, Performed the experiments: S Sadia, Analyzed the data: N Tariq, Contributed materials/ analysis/ tools: RS Shaikh, Wrote the paper: N Tariq & T Kausar.

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