Pakistan J. Zool., vol. 54(6), pp 2977-2980, 2022 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20211010071052

Short Communication

Association of rs 9340799 and rs 224693 SNPs in Estrogen Receptor Gene with Breast Cancer

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ABSTRACT

It was attempted to molecularly characterize breast cancer patients of district Bannu, KP, Pakistan for any possible polymorphism in the estrogen receptor (*ESR1*) gene. Blood samples in this regard were collected from clinically diagnosed eighteen breast cancer patients, all possessing invasive ductal carcinoma. DNA was amplified through PCR for 2 different sequences of *ESR1* gene. Twelve normal individuals of different ages were also characterized. Amplified products were digested with *Xbal* and *Pvull* restriction enzymes and were electrophoresed to visualize single nucleotide polymorphisms (SNPs); rs9340799 (G>A) and rs2234693 (C>T) in *ESR1* gene. The amplified product of 524bp of rs9340799 sequence of ESR1 gene was treated with *Xbal* restriction enzyme while amplified product of 451bp of rs2234693 sequence was treated with *PvuII* restriction enzyme. Nine patients were found homozygous and 8 were heterozygous for minor allele while 1 patient was carrying major allele. For rs2234693, all the patients were carrying major allele. All the normal individuals were lacking the above stated 2 SNPs. There is thus strong correlation between SNPs of *ESR1* gene and breast cancer and hence, can be used as a significant marker in the determination of breast cancer.

Article Information
Received 10 October 2021
Revised 15 November 2021
Accepted 28 November 2021
Available online 22 February 2022 (early access)
Published 23 September 2022

Authors' Contribution
MI and MR collected the samples. MI compliled the data. JK supervised the research and wrote the manuscript. ZR peformed statistical analysis. MI, MR and KK presented methodology.

Key words
Breast cancer, Polymorphism, Estrogen receptor, *ESR1* gene, SNPs, Single nucleotide polymorphism, Consanguineous marriages, high penetrance genes

Breast cancer is the most widely recognized type of cancer and the second most common cause of death in women (Ferlay *et al*., 2010; Lalloo and Evans, 2012). Breast cancer causes about 458,000 deaths every year, making it the most common cause of female deaths from cancer (Siegel *et al*., 2013). It is expanding at a quicker rate in Asia than in western nations, because of changes in the lifestyle and diet (Maddams *et al*., 2010). Since in majority of Asian countries, there is no availability of the population-based screening programs, great numbers of women have life-threatening disease of breast cancer. Screening and in-time detection of breast cancer is not possible in such under-privileged societies like Pakistan, mainly due to lack of awareness, non-availability of funds, absence of infrastructure and mismanaged public health programs (Shin *et al*., 2012). In Pakistan, Breast cancer is the most well- known threat, accounting for 34.6% of all women cancers (Agarwal *et al*., 2007; Bhurgri, 2004; Hashmi, 1997). The age of affected women usually are below 40 years (Usmani *et al*., 1996). Among Pakistani women, the occurrence rate of the breast cancer seems to be equal to the west (Usmani *et al*., 1996; Ahmed *et al*., 1997; Malik *et al*., 1992; Hashmi, 1997). Two important factors, life style and reproductive factors have their key roles in the high rates of incidence, yet the specific elements have not been identified (Maddams *et al*., 2010). It is likewise conceivable that hereditary components, for example, mutations occurring in BRCA1 and BRCA2, may add to a greater extent in breast and ovarian cancers susceptibility. Pakistan has a very high ratio of consanguineous marriages in the world (Easton *et al*., 2009) and this consanguinity enhances the risk of breast cancer due to homozygosity of harmful recessive genes (Stratton and Rahman, 2008). Pakistan occupies seventh position in the world in breast cancer mortality rate (Stratton and Rahman, 2008). The main reasons behind the failure of eradication of this disease are the lack of identification of a particular etiologic agent, the exact initiation time, and the molecular mechanisms responsible for malignancy initiation and progression. Nulliparity, early onset of menstruation, delayed menopause, and short duration of breast-feeding, extensive use of oral contraceptives, extended estrogen replacement therapy and postmenopausal obesity are some of the main risk

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0030-9923/2022/0006-2977 $ 9.00/0

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factors which actually come under the heading of breast cancer (Boral *et al*., 2017; Kanchan *et al*., 2020). Among the hormonal impacts, the main role has been credited to the unopposed exposure to raised levels of estrogens (Yager and Davidson, 2006; Miyoshi and Noguchi, 2003), as has been shown for a majority of women malignancies. For normal functions to perform, estrogen must first ties to estrogen receptor to form complexes in order to exert their physiological impacts, which further recognizes and attaches to particular sequences of the promoter in estrogen-responsive genes (Yager, 2000). Thus, all the estrogen-linked genes are concerned to control the synthesis or degradation of estrogens and thus are grouped as breast cancer susceptibility genes.

The high penetrance genes such as BRCA1, BRCA2, TP53, CDHI, STK1 and moderate penetrant genes like CHEK2, BRIP1, ATM, PLB2, play their roles in the progression of hereditary and non-hereditary breast cancer in the majority of cases respectively (Figueroa and Brinton, 2012; Miyoshi and Noguchi, 2003). We address the issue of a possible relationship between breast cancer and single nucleotide polymorphism (SNPs) in estrogen receptor gene (ESR1) in patients of district Bannu, KP, Pakistan.

Materials and methods

Blood samples of clinically diagnosed 18 breast cancer patients and 12 normal individuals were collected from district Bannu. All the individuals belong to Pashtun ethnic group (Table I) and were molecularly characterized for SNPs in *ESR1* gene. Detailed information of each patient was recorded on study-designed pro forma. The study was approved by ethical committee of Gomal University, D. I. Khan, KP, Pakistan.

DNA was extracted by salting out method (Miller *et al*., 1998). Four ml of blood was washed with T.E (10 mM Tris HCl pH 8.0, 2 mM EDTA) after thawing. The pellet was resuspended in 4.5 ml buffer containing 10 mM Tris HCl of pH 8.2, 2mM EDTA and 400 mM NaCl. 240µg proteinase K and 80 µl of 10% SDS were added for digesting protein. It was then incubated overnight at 37°C. Proteins were precipitated with 0.4 ml of 6M NaCl by shaking vigorously for 45 sec and centrifuged at 3000 rpm for 10 min. The supernatant was transferred to another sterilized tube and DNA was precipitated with 4ml of isopropanol (Miller *et al*., 1998). DNA was dissolved in 0.35 ml TE after washing with 70% ethanol, and then heated at 70 ºC for 2 h. DNA was quantitatively measured with NanoDrop spectrophotometer (Thermo Scientific NanoDrop, 2000).

For PCR reaction to perform, 150 ng genomic DNA, 25 mM of each dNTPs, 1 unit of *Taq* DNA polymerase, 10 mM each of the forward and reverse primers, 1.5 mM $MgCl_2$ and 1x *Taq* reaction buffer were used in 20µl reaction volume in 2 different reactions. The reaction was carried out through 30 cycles that consisted of 3 min denaturation at 95°C, 30 sec annealing at 65°C and one minute and 30 sec extension at 72°C. During the first cycle, denaturation was done at 95°C for 5 min while the final extension was done at 72°C for 10 min. Gel electrophoresis of the PCR product along with 100bp ladder was done on 2% agarose gel containing ethidium bromide for visualization.

Ten µl of amplified products were digested with *Xba*l and *Pvu*ll restriction enzymes under the conditions recommended by manufacturer (New England Bio labs, USA). The digested products were electrophoresed on 3% agarose gel.

Table I. Information of corresponding patient regarding age, type of breast cancer, grade, and date of diagnosis.

S. No	Breast cancer type	Grade	Patient age (Yr)	Breast involved	rs9340799 (G>A)	rs 2234693 (C>T)
1	IDC	III	43	Left	Homozygous	Normal
2	IDC	III	41	Left	Heterozygous	Normal
3	IDC	III	38	Left	Homozygous	Normal
4	IDC	III	44	Left	Normal	Normal
5	IDC	III	35	Left	Heterozygous	Normal
6	IDC	III	40	Right	Homozygous	Normal
7	IDC	III	30	Right	Heterozygous	Normal
8	IDC	III	45	Left	Homozygous	Normal
9	IDC	III	30	Right	Homozygous	Normal
10	IDC	III	26	Left	Homozygous	Normal
11	IDC	II	60	Right	Heterozygous	Normal
12	IDC	II	34	Right	Heterozygous	Normal
13	IDC	II	35	Left	Heterozygous	Normal
14	IDC	II	32	Right	Homozygous	Normal
15	IDC	II	24	Left	Homozygous	Normal
16	IDC	II	23	Right	Heterozygous	Normal
17	IDC	II	36	Left	Heterozygous	Normal
18	IDC	II	47	Right	Homozygous	Normal

IDC, invasive ductal carcinoma.

Results

Clinically diagnosed 18 breast cancer patients and 12 normal individuals, all belonging to Pashtun ethnic group were molecularly characterized for polymorphisms in *ESR1* gene (Table I). *ESR1* gene has two important single nucleotide polymorphisms (SNPs); rs9340799 (G>A) and rs2234693 (C>T). Such polymorphism if present has an impact on breast cancer predisposition. Two restriction enzymes i-e *Xba*l and *Pvu*II were used for identification

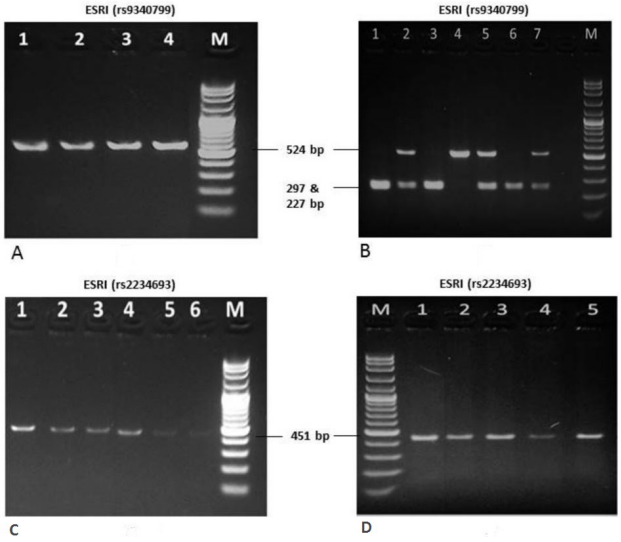

Fig. 1. Amplified product of *ESR1* gene (rs 9340799) of clinically diagnosed breast cancer patients of Pashtun ethnic group. A, Lanes 1,2,3,4 show amplified product of 524bp. Lane M is 100bp ladder as a marker. B, Digestion of amplified PCR product (524bp) with *Xba*l restriction enzyme. Lanes 1, 3 and 6 show the samples completely digested *Xba*l enzyme, producing 2 bands of 297 and 227bp (pretty close to each other that appears as single band). This confirms the presence of G>A polymorphisms and are homozygous for minor allele "A". Lanes 2, 5 and 7 show the samples incompletely digested by the *Xba*l restriction enzyme and are thus heterozygous for minor allele "A", giving 3 bands of 524, 297 and 227bp. Lanes 4 show sample not digested by *Xba*l enzyme and thus showing a single band of 524bp. This sample is homozygous for major allele "G". M is a 100bp ladder. Lane 8 is blank. C, amplified product of *ESR1* gene (rs2234693) of clinically diagnosed breast cancer patients of Pashtun ethnic group. D, Digestion of amplified PCR product using *Pvu*II restriction enzyme. Lanes 1 to 8, samples are not digested by *Pvu*II restriction enzyme, showing a single band of 451b. Thus all the patients lack rs2234693 (C>T) polymorphism. M is a 100bp ladder used as a marker.

of SNPs sequences in *ESR1* gene. The amplified product of 524bp (Fig. 1A) (rs9340799 sequence) of *ESR1* gene was treated with *Xba*l restriction enzyme. Samples 1, 3, 6, (Table I, Fig. 1B) and 8, 9, 10, 14, 15, 18 (not shown in the Fig. 1) were completely digested on treatment with *Xba*l that resulted in homozygous condition for minor allele "A". Moreover, the samples No. 2, 5, 7 (Table I, Fig. 1) and 11, 12, 13, 16, 17 (not shown in the Fig. 1) were incompletely digested on treatment with the same enzyme that resulted in heterozygous condition for minor allele "A" while, sample No 4 (Fig. 1) was homozygous for major allele "G" after treatment with *Xba*l restriction. Thus, sample No 4 was wild (normal) type for rs9340799 sequence. Similarly, the amplified product of 451bp (rs2234693 sequence) of *ESR1* (Fig. 1C) gene from all the breast cancer patients was also treated with *Pvu*II restriction enzyme. In this regard, all the 18 samples were not digested even for longer exposure (Fig. 1D) by *Pvu*II enzyme, meaning that the samples were wild (normal) type for rs2234693 sequence. Likewise, all the 12 normal individuals possessed neither rs9340799 (G>A) nor rs2234693 (C>T) polymorphism.

Discussion

Breast cancer is one of the most familiar malignancies that cause severe health problems in women globally (Ferlay *et al*., 2010; Siegel *et al*., 2013). Inheritance, postmenopausal use of hormones, early age of menarche, delayed first pregnancy, breastfeeding for brief period, low parity, and a long gap between births are some of the factors concerned with the breast cancer predisposition (Maddams *et al*., 2010). Additionally, hormones are one of the key risk factors (Easton *et al*., 2009). Hormones control growth and function of epithelial cells of the breast. Estrogen plays a major role in breast cancer predisposition as its over-expression may induce the cells to build mutations (Maddams *et al*., 2010; Ferlay *et al*., 2013). Single-nucleotide polymorphisms (SNPs) are the most common type of genetic variations in the human genome. Large numbers of estrogen-related genes have been reported with SNPs as the probable risk factors of breast cancer in the women already have gone through menopause (Yager, 2000). Estrogen receptor alpha gene (*ESR1*), encoding ERα protein is more important in initiation, development and therapeutics of breast cancer. Specific SNPs in *ESR1* gene are involved directly or indirectly in the change of its function, that have an impact on the risk of breast cancer (Yager, 2000; Easton *et al*., 2009). The two important SNPs, (rs2234693 and rs9340799), detected with *Pvu*II and *Xba*I, respectively have been shown as important markers in certain types of cancer (Sereno *et al*., 2020). We, here for the first time in Pakistan attempted to molecularly characterize breast cancer patients for possible polymorphism in two SNPs sequences, the rs9340799 (G>A) and rs2234693 (C>T) in *ESR1* gene. No polymorphism was found for rs2234693 (C>T) of *ESR1* gene of all the collected blood samples, when treated with Pvull restriction enzyme. All the 18 patients were found homozygous (normal) for major allele "G". For rs9340799 (G>A) SNP, of the eighteen patients, 9 patients were found homozygous and 8 patients were heterozygous for minor allele "A" of *ESR1* gene, while 1 patient was found normal for the said polymorphism. Interestingly, neither rs9340799 (G>A) nor rs2234693 (C>T) mutation was found in normal individuals of

the same locality. This was an important finding for the patients of breast cancer in Bannu district of KP, Pakistan. Except for one patient having the age of 60 years, all the patients were below 50 years of age. Observing the molecular characterization of breast cancer with emphasis on breast involved, stage, age status, and status of ESR1 gene (homozygous or heterozygous condition), it is worth-mentioning that classification characteristics were found insignificant except age groups (p>5%). Empirically, the researchers came with findings when comparing breast cancer tissues involved and breast-cancer stage that classification criteria did appear independently at 5% level of significance. The Pearsonian coefficient for statistical comparison appeared with value 0.17 that was far greater than 5%. Similarly, on comparing breast involved and gene status, once again, very large coefficient appeared with value 0.64, and, in the same picture, cancer grade and gene status were found statistically independent (p>5%). Hence one can visibly decide that breast cancer is statistically independent of breast side and *ESR1* gene status. Independent and collective effects of these different polymorphisms in *ESR1* gene potentially reveal exposure to estrogen and as a result, an increase in breast cancer susceptibility. Hence, *ESR1* gene polymorphism has a significant role in breast cancer predisposition.

Conclusion

SNPs in *ESR1* gene have been linked with breast cancer development and can be used as one of the significant markers to determination of breast cancer

Statement of conflict of interest

The authors have declared no conflict of interest.

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