Short Communication

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

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A B S T R A C T

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 XbaI and PvuII 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 XbaI 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.

Background

Breast cancer is the most widely recognized type of cancer and the second most common cause of death in women (Ferlay et al., 2010; Laloo 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, lifestyle 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 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 tie 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 1) 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 °C 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 MgCl₂, 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 Xbal and PvuII 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 type | Grade | Patient age (Yr) | Breast involved | rs9340799 (G>A) | rs2234693 (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    | Right            | heterozygous   | Normal          |                  |
| 6 IDC  | III         | 40    | Right            | normal         | 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            | heterozygous   | Normal          |                  |
| 15 IDC | II          | 24    | Left             | heterozygous   | 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 1). 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 Xbal and PvuII were used for identification
Breast Cancer and ESRI Gene

of SNPs sequences in ESRI gene. The amplified product of 524bp (Fig. 1A) (rs9340799) of ESRI gene was treated with XbaI 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 XbaI 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 XbaI restriction. Thus, sample No 4 was wild (normal) type for rs9340799 sequence. Similarly, the amplified product of 41bp (rs2234693 sequence) of ESRI gene from all the breast cancer patients was also treated with PvuII restriction enzyme. In this regard, all the 18 samples were not digested even for longer exposure (Fig. 1D) by PvuII 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 PvuII and XbaI, 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 PvuII 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 worthwhile 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 and gene status were found statistically independent with value 0.64, and, in the same picture, cancer grade and gene status, once again, very large coefficient appeared than 5%. Similarly, on comparing breast involved and cancer grade, a comparison appeared with value 0.17 that was far greater than 5%.

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.

References
Agarwal, G., Pradeep, P.V., Aggarwal, V., Yip, C.H., and Cheung, P.S., 2007. World. J. Surg., 1: 1031-1040. https://doi.org/10.1007/s00268-005-0585-9
Ahmed, R., Shaikh, H., and Hasan, S.H., 1997. J. Pak. med. Assoc., 47: 114–116.
Bhurgri, Y., 2004. Asia. Pac. J. Cancer, 5: 77–82.
Boral, D., Vishnoi, M., Liu, N., Yin, W., Sprouse, M.L., Hong, D.S., Tan, T.Z., Thiery, J.P., Chang, J.C., and Marchetti, D., 2017. Nat. Commun., 8: 196. https://doi.org/10.1038/s41467-017-00196-1
Easton, D.F., Pooley, K.A., Dunning, A.M., Pharoah, P.D., Thompson, D., and Ballinger, D.G., 2009. J. natl. Cancer Inst., 101: 1012–1018.
Ferlay, J., Parkin, D.M., and Steliarova-Foucher, E., 2010. Eur. J. Cancer, 46: 765–781. https://doi.org/10.1016/j.ejca.2009.12.014
Ferlay, J., Steliarova-Foucher, E., Lortet-Tieulent, J., Rosso, S., Coebergh, J.W., Comber, H., Forman, D., and Bray, F., 2013. Eur. J. Cancer, 49: 1374–1403. https://doi.org/10.1016/j.ejca.2012.12.027
Figueras, J.D., and Brinton, L.A., 2012. J. natl. Cancer Inst., 104: 641–642. https://doi.org/10.1093/jnci/djs193
Hashmi, M., 1997. J. Pak. med. Assoc., 47: 75–78. https://doi.org/10.1093/ocmedc47/2.75
Kanchan, R.K., Siddiqui, J.A., Mahapatra, S., Batra, S.K., and Nasser, M.W., 2020. Adv. Ther. Mol. Cancer, 19: 29. https://doi.org/10.1186/s12943-020-1140-x
Lalloo, F., and Evans, D.G., 2012. Clin. Genet., 82: 105–114. https://doi.org/10.1111/j.1399-0004.2012.01859.x
Maddams, J.B., Utley, M., and Moller, H., 2010. Br. J. Cancer, 107: 1195-1202. https://doi.org/10.1038/bjc.2012.366
Malik, I.A., Mubarak, A., Luqman, M., Ullah, K., Ahmad, M., Alam, S.M., and Mughal, T., 1992. J. environ. Pathol. Toxicol. Oncol., 11: 353.
Miller, S.A., Dykes, D.D., and Polesky, H.F., 1998. Nucl. Acids Res., 16: 1215. https://doi.org/10.1093/nuclards/16.3.1215
Miyoshi, Y., and Noguchi, S., 2003. Biomed. Pharmacother., 57: 471–481. https://doi.org/10.1016/j.biopharma.2003.09.008
Sereno, M., Hasko, J., Molnar, K., Media, S.J., Reisz, Z., Malho, R., Videiro, M., Tiszalvicz, L., Booth, S.A., Wilhelm, I., Krizbai, I.A., and Brito, M.A., 2020. Mol. Oncol., 14: 520-538. https://doi.org/10.1002/1878-0261.12632
Shin, H.R., Carlos, M.C., and Varghese, C., 2012. Jpn. J. Clin. Oncol., 42: 867-881. https://doi.org/10.1093/jjc/jys077
Siegel, R., Naishadham, D., and Jemal, A., 2013. Cancer J. Clin., 63: 11–30. https://doi.org/10.3322/caac.21166
Stratton, M.R., and Rahman, N., 2008. Nat. Genet., 40: 17–22. https://doi.org/10.1038/ng.2007.53
Usmani, K., Khanum, A., Afzal, H., and Ahmad, N., 1996. J. environ. Pathol. Toxicol. Oncol., 15: 251–253.
Yager, J.D., 2000. J. natl. Cancer Inst., 92: 1040. https://doi.org/10.1093/jnci/104.20-1140-x
Yager, J.D., and Davidson, N.E., 2006. N. Engl. J. Med., 354: 270–282. https://doi.org/10.1056/NEJMra050776