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RESEARCH ARTICLE
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EXOME SEQUENCING COUPLED WITH *IN SILICO* FUNCTIONAL ANALYSIS IN CONSANGUINEOUS PAKISTANI FAMILIES INHERITING BREAST CANCER DETERMINED THE FOUNDER EFFECT OF THE MUTATION C.1961DUPA (P.TYR655VALFS18*) IN THE *BRCA1* GENE

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Abstract:
Mutations in several genes are now known to cause susceptibility to breast cancer. In Pakistan, very little is known about the genetic spectrum of Breast cancer. So, the main aim of the current genetic study was to investigate the four unrelated Saraiki origin consanguineous Pakistani families segregating breast cancer. Methodology for genetic analysis includes Whole Exome Sequencing (WES) and Sanger sequencing; however, *in silico* functional analysis was done using I-TASSER (for 3D structure modeling and Cluspro tool (for Protein-Protein interaction). Analysis of exome data identified previously reported frameshift heterozygous duplication mutation c.1961dupA (p.Tyr655Valfs18*) in the 10th exon of the *BRCA1* gene inherited in the autosomal dominant pattern in all the families. No symptoms were found in the male individuals of the families. *In silico* functional analysis also revealed the mutation's adverse effect on protein structure and interaction. We hypothesized on the evolutionary importance of this mutation and suggest that it has a substantial founder impact in the Saraiki-derived Jhakar tribe of Pakistan. Based on these findings, we propose creating a molecular diagnostic tool for premarital and prenatal screening of breast cancer-risk families.

Keywords: Breast Cancer, Pakistani, Mutation, Dominant, BRCA1, Exon

1. Introduction:

Breast cancer is a hereditary type of neoplastic disorder, which is actually brought about by an accumulation of molecular gene mutations in hereditary materials- the explanation being that ultimately the cumulative events lead to dysregulation of cell proliferation. [1]. This kind of malignant

disease has the highest visibility among all cancers and happens to be the second most common cause of death-from-an-equality-cause for the same sex, that is, women. [2–4]. It causes around 0.458 million fatalities per year, making it the most common cause of female mortality worldwide [5,6]. This type of cancer is the main neoplastic disorder in Asian females [7]. The majority of Asian countries lack population-based programs for screening breast cancer, and as a result, a large proportion of women suffer from this potentially fatal disease. Breast cancer screening and detection could not be viable in these underprivileged communities due to a lack of awareness, a lack of funding, a lack of infrastructure, and a low priority placed on public health programs. Breast cancer is the most common malignancy among Pakistani women, accounting for 34.6% of all types of women's cancers [8–10]. According to previous reports, the annual rate of breast cancer in Pakistan was 69.1 per 100,000 [11]. Below 40 years, the majority of the affected women were found. Young women usually suffer from breast cancer and live with it in an advanced stage of the neoplastic illness. The incidence rate of breast malignancy falls below 40 per 100,000 per year in all other registries of Asia, while in India, it ranked about 8.7 per 100,000 every year, except Manila in the Philippines, where it was 47.7 per 100,000 every year [2]. The reasons behind the high rates and failure in the eradication of breast as well as ovarian malignancies in Pakistan are probably the lifestyle as well as reproductive factors, and the lack of documenting a particular etiologic agent. Furthermore, the genetic constituents, such as mutations affecting the *BRCA1* and *BRCA2* genes, could greatly amplify the risk for breast and ovarian cancer in Pakistan [2]. Breast cancer mortality rates rank Pakistan seventh in the world, with 26.76 deaths per 100,000 population. Among the many risk factors for breast cancer, we may include nulliparity or late first childbirth, early onset of menstruation, late menopause, and less prolonged breastfeeding, heavy use of contraceptive pills, prolonged estrogen replacement therapy, and postmenopausal obesity [2,7,12,13]. Among the hormonal impacts, the major contribution has been attributed to the unopposed exposure to raised levels of estrogens, as this has been documented for a majority of women's malignancies, specifically liver, vagina, and cancer of cervix [10,11,14–17]. The purpose of this study was to investigate the genetics of breast cancer characteristics in four seemingly unrelated consanguineous families of Saraiki descent recruited from Pakistan. The exome sequence analysis of all families revealed a previously known single-base deletion mutation in *BRCA1* [c.1961dupA (p.Tyr655Valfs18*)], suggesting the founder effect in the Jhakar tribe, attributing its origin to Balochi tribal ancestry.

2. Materials and Methods:

2.1. Family recruitment and Sample collection.

The families with a clinical diagnosis and a family history of breast cancer were located in Pakistan and included in the current study. Intravenous blood samples were collected from patients as well as regular family members (involving paternity) of impacted families in accordance with customary procedures. EDTA tubes containing blood samples were kept at 4^{o}C until processing. DNA was then extracted using the salting-out method [18].

2.2. Genetic analysis.

Whole exome sequencing was done following the protocol previously described by [19–24]
To know about the pathogenicity and their functional consequences, bioinformatics tools like SIFT (Sorting Intolerant from Tolerant) (http://SIFT.jcvi.org/), Mutation Taster (http://www.mutationtaster. org/), Pantherdb.org (https://www.pantherdb.org/) and I-Mutant (https://folding.biofold.org/i-mutant/i-mutant2.0.html) were used

2.3. Sanger Sequencing.

On exome sequencing of one of the patients of each family, targeted Sanger Sequencing of *BRCA1* gene was carried out to know about the mutation and its segregation pattern in the whole family. Following Primers were used for the study: Forward 3-TTCAAAACGAAAGCTGAACCT-5 and reverse 5-TGTTAACTTCAGCTCTGGGAAA-3. For sequence alignment, the online tool BLAST

(Basic Local Alignment Search Tool) [25] was used. Alongside, BioEdit v7.0.5 was used to scrutinize the genetic variation capturing sequence chromatogram.

2.4. Protein 3D Modelling and Docking.

For designing 3D protein representations of the unaffected and mutated BRCA1 protein with its close interactor, the I-TASSER online [26] tool was used. 3D protein model having a maximum confidence score (C-Score) was chosen for additional exploration [27]. Designed 3D models were visualized by Chimera 1.13.1 [28]. After designing and visualizing 3D protein models, Protein-protein docking was carried out using the online Cluspro server tool with its close functional interactors [29]. Close functional interactor was predicted via the String Database [30–33]

3. Results.

It was aimed to molecularly characterize those families of breast cancer having at least two clinically diagnosed breast cancer patients. Blood samples of four families were collected and characterized using exome/Sanger sequencing. All four families were of the Saraiki ethnic group. The detailed information of each family is as follows;

3.1. Family 1.

Family 1 was a Consanguinity family, with a family history of the disease (breast cancer). Family pedigree consists of three generations with one affected female in each generation, confirming its dominant mode of disease inheritance. All the males were normal and no sign of any cancer or related disease was noted in the male individuals of the family. Affected Female (III:2) was ~ 38 years old, while her grandmother (I:1) died because of breast cancer at the age of 60 (figure 1a). Her mother (II:2) was also suffering from the disease, but she was not willing to the blood. Blood was obtained from two individuals, including a normal male sibling (III:1) and an affected individual (III:1). No additional clinical data were available because the family was financially so weak, nor were they willing to undergo the additional tests due to cultural hardens. Analysis of exome data of the patient (III:1) identified previously reported heterozygous frameshift duplication mutation [c.1961dupA (p.Tyr655Valfs18*)] in the *BRCA1* gene inherited in the autosomal dominant pattern. Her normal sibling (III:1) was a wild-type of the mutation (Figure 2).

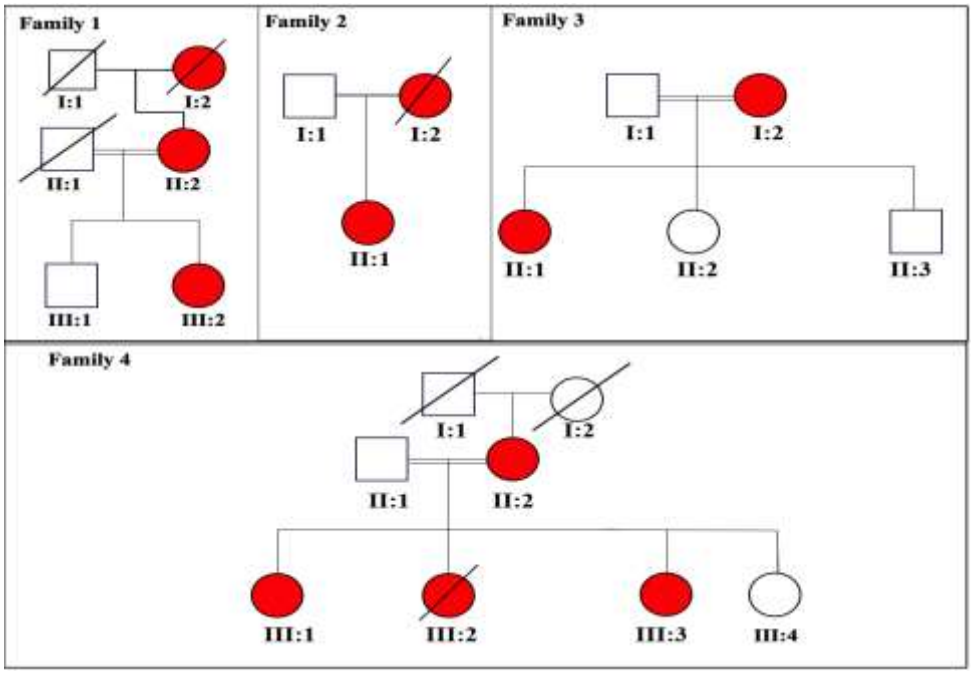

Figure 1: Family pedigrees showing the autosomal dominant mode of disease inheritance in the families

3.2. Family 2.

Family number two comprised dual dual-generation pedigree with one diseased female in each generation, confirming its dominant mode of disease inheritance. Her father (I:1) was normal, and no sign of any cancer or related disease was noted. Affected Female (II:2) was ~ 43 years old and unmarried due to the disease, while her mother (I:2) died because of breast cancer at the age of ~55 (Figure 1b). Blood samples were collected from two individuals, including a normal father (I:1) and an affected individual (II:1). No additional clinical data were available because of financial and social problems.

Analysis of exome data of the patient (II:1) identified previously reported heterozygous frameshift duplication mutation [c.1961dupA (p.Tyr655Valfs18*)] in the *BRCA1* gene inherited in the autosomal dominant pattern. Her father (I:1) was a wild-type of the mutation, confirming that the mutation was inherited from her deceased mother (II:2) (Figure 2).

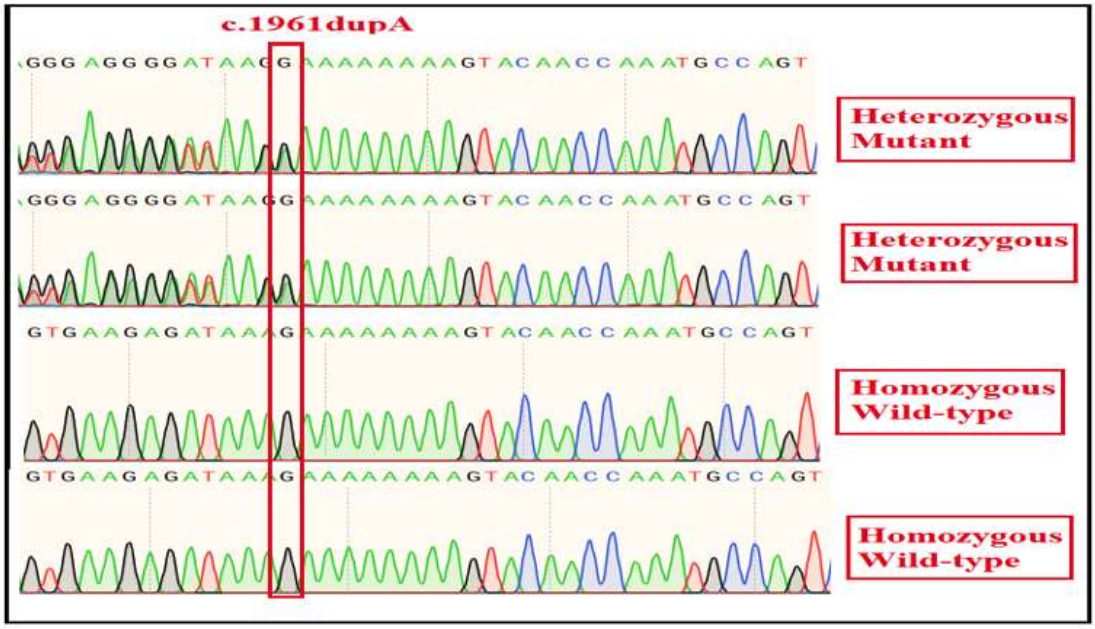

Figure 2: Sanger sequencing chromatograms showing the inheritance of c.1961dupA variant in the families, all the patients were heterozygous for the mutation while the normal individuals were showing wild-type status.

3.3. Family 3.

Family 3, a consanguineous family, involving 2 generations of lineage, having one affected female (I:2) plus (II:1) in each cohort. Her father (I:1) and normal siblings (II:2 and II:3) were normal and no sign of any cancer or related disease was noted in them. Affected Females (I:2 and II:1) were ~ 63 and ~40 years old, respectively, and the affected female (II:1) was also unmarried due to the disease (figure 1c). Blood samples from his four individuals, including a normal father (I:1) and a normal sister (II:2), and both affected individuals (I:2 and II:2) were collected. No additional clinical data were available due to financial and cultural issues.

Analysis of exome data of the patients (I:2 and II:1) identified previously reported heterozygous frameshift duplication mutation [c.1961dupA (p.Tyr655Valfs18*)] in the *BRCA1* gene inherited in the autosomal dominant pattern. Her father (I:1) and normal sister (II:2) were wild-type normal, confirming that the mutation was inherited from her affected mother (II:2) in an autosomal dominant pattern (Figure 2).

3.4. Family 4.

Family 4 was also a Saraiki-origin consanguineous family with a family history of the disease (breast cancer). Family pedigree consists of three generations with one affected female (II:2) in the first generation and three affected individuals (III:1, III:2, and III:3) in the third generation. Her father

(II:1) and normal sibling (III:4) were normal, and no sign of any cancer or related disease was noted in them. Affected Females (II:2, III:1, III:2 and III:3) were ~ 70, ~50, ~49 and ~48-year-old respectively. Affected females (III:1 and III:3) were also unmarried due to the disease, while the affected female (III:2) was deceased (Figure 1d). Blood was obtained from four individuals, including a normal father (II:1) and a normal sister (III:4), and two affected individuals (II:2 and III:2). In this family also no additional tests were available because the family was financially weak, nor were they willing to undergo the additional tests due to cultural hardens.

Analysis of exome data of the patients (I:2 and II:1) identified previously reported heterozygous frameshift duplication mutation [c.1961dupA (p.Tyr655Valfs18*)] in the *BRCA1* gene inherited in the autosomal dominant pattern. Her father (II:1) and normal sister (III:4) were wild-type of the mutation, confirming that the mutation was inherited from her affected mother (II:2) in a dominant pattern (Figure 2). In this family, grandparents from the mother's side were deceased, but no disease was observed by the family members, showing that the variant was inherited from the grandmother. Complete clinical details of all the families are summarized in Table 1.

Table 1: Clinical Details of Patients

Family ID	Patient ID	Age	Consanguinity	Language	Cast	Family History	Diabetes	BMI
Family 1	II:2	~38	Yes	Saraiki	Jakhar	Yes	No	Normal
Family 2	II:1	~43	Yes	Saraiki	Jakhar	Yes	No	Normal
Family 3	I:2	~63	Yes	Saraiki	Jakhar	Yes	No	Normal
	II:1	~40					No	Normal
Family 4	II:2	~70	Yes	Saraiki	Jakhar	Yes	No	Normal
	III:1	~50					No	Normal
	III:2	~49					No	Normal
	III:3	~48					No	Normal

3.5. Structural findings.

Protein 3D models of wild-type and mutant BRCA1 proteins were created and stacked, revealing just 1.79% similarity. Frameshift mutation disrupts the protein sequence, resulting in a significant structural alteration. This confirms the adverse effect of the mutation. 3D structure of the wild-type, mutant, and superimposed structure is shown in Figure 3.

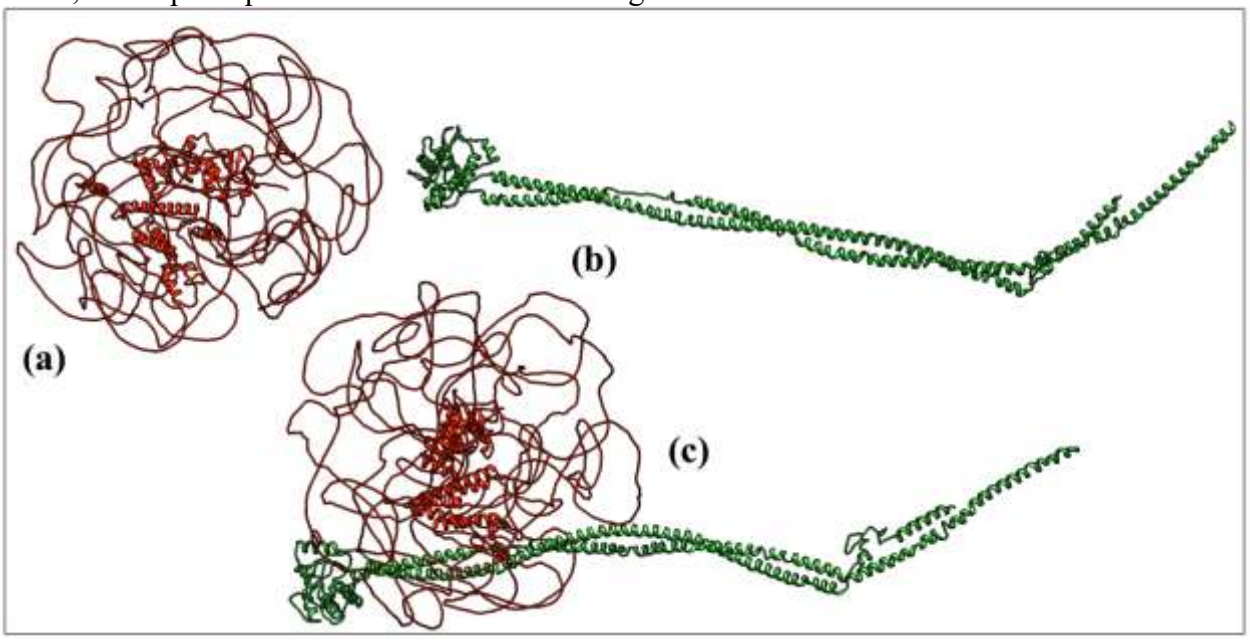

Figure 3: 3D models of wild-type (b), mutant (c) superimposed structure of the BRCA1 protein

Protein-protein docking also illustrates the adverse influence of the genetic alteration. Wild-type and mutant BRCA1 proteins were docked through their close interactor, BARD1 proteins (Figure 4), and found huge differences in the docking site and docking residues. Wild-type BRCA1 protein was docking with close interactor BADR1 Protein through 29 residues via 37 hydrogen and 6 salt bridges, while in the case of mutant BRCA1, and close interaction BARD1 protein interaction through 14 residues via 21 hydrogen bonds (Figure 4). This change in the docking sites and bonding confirms the adverse effect of the mutation, which exposes the hydrophobic residues in the mutant protein due to mutation.

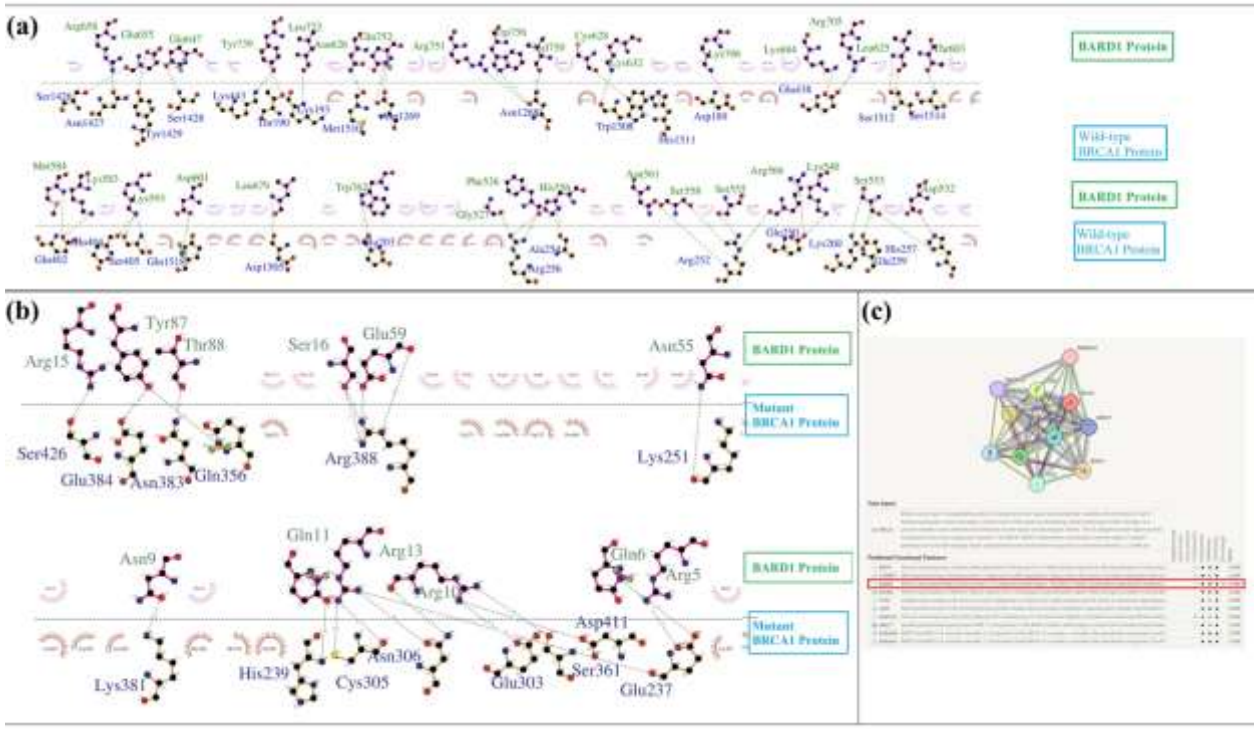

Figure 4: (a) Protein-protein interaction of wild-type and close interactor BARD1 protein (b) Protein-protein interaction of mutant and close interactor BARD1 protein (c) Screenshot of string database showing BARD1 as a close functional interactor of BRCA1 protein

4. Discussion.

During this study, very interestingly, a heterozygous frameshift duplication mutation [c.1961dupA (p.Tyr655Valfs18*)] in *BRCA1* gene inheriting in the autosomal dominant pattern was found in the affected female patients of all the four families. In all the cases, the grandmother had died of breast cancer, showing that the said frameshift mutation was inherited from their grandmothers in an autosomal fashion [34–39]. This mutation was not reported previously in the Saraiki ethnicity. 3D model representation of wild-type and mutant BRCA1 proteins showed that the Frameshift mutation resulted in the disturbance of the protein sequence that led to huge alterations of the structure. Similarly, protein-protein docking also showed the adverse effect of the mutation. The wild-type as well as mutant BRCA1 proteins had been docked with their close interactor, BARD1. Wild-type BRCA1 protein was docking with close interactor BADR1 Protein through 29 residues via 37 hydrogen bonds and 6 salt bridges, while in the case of mutant BRCA1, and close interaction BARD1 protein interaction through 14 residues via 21 hydrogen bonds, showing a significant change in the docking sites and an adverse effect of the mutation.

Mutations in several genes are now known to increase the risk of developing breast cancer. The *BRCA1* and *BRCA2* genes are particularly important in high-risk families [40–43]. Hereditary breast cancer affects approximately 10% of all instances and is caused by a loss of a tumor suppressor gene, rather than the acquisition of an oncogene. BRCA1 and BRCA2 account for 80-90% of genetically determined tumors. Rarely, these genes mutate in sporadic forms, implying that they tend to play

some pathogenetic role in oncogenesis. The BRCA genes are also in male breast cancer. Male carriers of germline mutations in the BRCA2 gene have a significantly higher risk for developing breast carcinoma than men from the general population, whereas men who carry germline mutations in the BRCA1 gene might also be at increased risk, although this is a less established association [44–52].
In Pakistan, all five ethnic groups prefer to have consanguineous marriages in most instances, especially the Saraiki, Pashtun, and Balochi. They very rarely get married outside their own ethnicity. Hence, the genetic diseases are transmitted within the same ethnic group from generation to generation. During this study, Blood samples, clinically diagnosed individuals with breast cancer were collected from four consanguineous families in the Saraiki ethnic group, along with normal individuals of Pakistan. Such families were selected that had at least two affected females. The ages of patients were in the range of 35-41 years. Previous studies reported the age of breast cancer patients as 40 years in most cases [45,50,53,54].
Previous studies reported 30 deleterious germ-line mutations in both *BRCA1* and *BRCA2* genes in Pakistani families [1,10,14,17,55,56]. Of these mutations, 23 were found in *BRCA1* and *7 in BRCA2* [10,14,57–60].
Over half of breast cancer incidences from BRCA1/2-associated families had been affected by age 40, and 90% by age 50 [44,45].
Carriers of the c.185delAG Mutations were of Pashtun origin, while carriers of the 185insA, Ser1503*, and Arg1835* were of Punjabi heritage. The 2nd predominant mutation was observed in three Punjabi families, with 185insA being the most prevalent. This mutation had previously been found in one 40-year-old Punjabi ovarian cancer patient as well as numerous additional individuals of other ethnicities [10,60–63]. Regarding the breast cancer patients of Pashtun ethnicity, a previous report showed two important SNPs, (rs2234693 and rs9340799), either homozygous or heterozygous conditions in the estrogen receptor alpha gene (*ESR1*) in unrelated breast cancer patients [14].
Furthermore, a similar duplication mutation (c.1961dupA) was previously identified in a consanguineous Pakistani family with Pakistani ancestry. According to unverified historical data, all of the families appear to be descended from the Jhakar tribe of Saraiki. However, a subsequent mutation study confirms that each of the families is remotely connected.

Conclusion.
In current genetic analysis, we screened four unrelated Saraiki-origin Pakistani families segregating breast cancer and identified previously reported c.1961dupA (p.Tyr655Valfs18*) in the 10th exon of the *BRCA1* gene. This paper describes a founder mutation in the Jhakar tribe of Pakistan. All of the families' parents received genetic counselling to reduce the risk of the disease in future pregnancies. Moreover, patients with breast cancer must be molecularly characterized at the beginning of the onset of disease to determine the course of disease and proper management.

Statements & Declarations
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Conflict of Interest
The authors disclose that they have no financial or competing interests.

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Data Availability Statement: Data will be made available upon request.

Declaration of generative AI in scientific writing
No AI tools were used in the current study.

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