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S. Tabassum¹, **F. Munir**¹, **A.A. Al Awadh**², **Z. Anwar**^{3,*}

¹ Department of Biological Sciences, International Islamic University, Islamabad 1243, Pakistan

² Department of Clinical Laboratory Sciences, Najran University, Najran 61441, Saudi Arabia

³ Department of Biochemistry, Medical University of Warsaw, Warsaw 02-097, Poland

* Correspondence: Email: zagenes@gmail.com

zubair.awan@wum.edu.pl

PATHOLOGICAL SIGNIFICANCE OF CDH1/E-CADHERIN GERMLINE SEQUENCE VARIANTS IN BREAST CANCER PATIENTS

Background. Germline alterations of the *CDH1* (E-cadherin) tumor suppressor gene have been reported in several epithelial malignancies like hereditary diffuse gastric cancer and lobular breast cancer. E-cadherin plays a central role in proliferation, maintenance of cell-to-cell adhesion, polarity, and epithelial-mesenchymal transition of tissue cells. It is necessary to analyze the impact of the *CDH1* germline sequence variants on protein and predict its clinical significance in breast cancer (BC) progression. The **aim** of the current study was to evaluate the impact and association of *CDH1* gene potentially pathogenic variants/likely pathogenic variants (PVs/LPVs) with the initiation and progression of BC. **Materials and Methods.** In this study, the clinical data of 200 BC patients have been analyzed based on the type of BC, age, grade, stage, hormonal status, and risk factors. Blood samples from 50 healthy donors were used as a control. Furthermore, *CDH1* gene molecular analysis, along with *in silico* analysis, was provided to assess the invasiveness and progression of BC caused by the E-cadherin protein. **Results.** Four variants were identified by genetic screening within the *CDH1* gene that included variations in exons 7, 8, 10, 11, and 13. Exon 10 had splice site mutation at position c.1337C>A, affecting the protein structure. In exon 11, there was an insertion of T base at position 1669, resulting in truncated protein compared to a normal one that can lead to the disease-causing nonsense-mediated decay and exon 13 variant c.2076T>C has already known polymorphism. *In silico* analysis of *CDH1* showed the presence of the different variants that indicated the overall disruption of protein structure and function. **Conclusions.** The further functional analysis of these variants and their association with BC can be ensured by increasing the sample size and *in vivo* studies using mouse models.

Keywords: breast cancer, *CDH1*, E-cadherin, SSCP, sequencing, variant analysis.

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Breast cancer (BC) is one of the most common malignant diseases among females with 11.7% of patients worldwide, making it a major public health concern [1, 2]. The BC prevalence in Pakistani women has been reported to be the highest among the Asian population and to affect young women with an advanced disease stage [3].

While the most common BC type is an invasive ductal carcinoma (IDC), about 5%–15% of cases are invasive lobular carcinomas (ILC). ILC risk is more strongly associated with exposure to female hormones, the early menarche, the late menopause, and the late age at the first birth [4].

Various genetic factors are known to be involved in the hereditary BC cases, where the most common are pathogenic variants/likely pathogenic variants (PVs/LPVs) of the high-susceptibility *BRCA1* and *BRCA2* genes, accounting for a risk of 15%–45% of developing BC in lifetime [5]. The hereditary cases of BC are involved in the hereditary breast and ovarian cancer (HBOC) syndrome, an autosomal dominant inherited disorder that includes 5%–7% of all BC [6]. Although 75%–80% of BC are sporadic, ~15%–20% are considered of the familial type and 5%–10% are hereditary [5, 7].

Even if *BRCA1* and *BRCA2* are the major genes involved, the PVs/LPVs in other high- and moderate-risk susceptibility genes are known to be disease-causing with various penetrances, in particular *CDH1*, *PALB2*, *PTEN*, *STK11*, *TP53*, *ATM*, *CHEK2*, *BARD1*, *BRIP1*, *RAD51C*, *RAD51D*, *TRAIL*, *RECQL1*, and *BRIP1* [8, 9].

Among the different BC types, the altered *BRCA1* and *TP53* genes are predominantly associated with IDC, while the *BRCA2* genes are associated with both types [4].

Researchers have demonstrated that the germline PVs/LPVs of the *CDH1* gene can be associated with the hereditary lobular breast cancer (HLBC) syndrome, without clear correlation with the known hereditary diffuse gastric cancer (HDGC) [10, 11].

The *CDH1* gene (chromosome 16q22.1) translated into E-cadherin protein, which functions in cell-to-cell adhesion [12], is responsible for tumor progression and metastasis. The downregulation of E-cadherin in BC is mediated by several mechanisms that include the epigenetic and structural genetic changes [13]. Therefore, E-cadherin acts as a classic tumor suppressor gene, and the E-cadherin expression loss is mostly present in the infiltrating lobular tumor types accounting for almost 85% of all BC. The *CDH1* gene alterations are associated with ILC, but never with IDC [4], whereas the ductal histology shows a varying level of expression, which is related to epigenetic transcriptional downregulation [14].

The *CDH1* gene was known as the main susceptibility gene of HDGC, but the familial anamnesis of patients with BC of the lobular type in *CDH1* families led researchers to consider and investigate it as a gene for ILC susceptibility. It has been observed that the risk of ILC is high in the female carriers, as about 50% of them are expected to develop BC [4].

Recently, Kurian et al. [15] have shown an increased BC risk in association with a lot of genes, such as *BRCA1*, *BRCA2*, *ATM*, *BARD1*, *CHEK2*, *PALB2*, *PTEN*, and *TP53*. However, there was no significant association between BC risk and *CDH1* alterations, unlike a strong association of *CDH1* with lobular BC.

Germani et al. [8] carried out a multigene panel testing on 113 *BRCA*-negative patients with BC, OC, or pancreatic cancer (PC). They identified a PV/LPV in other genes beyond *BRCA1/2* genes, namely *CHEK2*, *RAD51C*, *ATM*, *MLH1*, *MSH2*, and *RECQL*, in 14 patients and found a *CDH1* variant of uncertain significance (VUS) in an early onset BC patient (36 y.o.).

Recently, Fanale et al. [9] and Bono et al. [16] have shown that ~15% of PVs/LPVs in high- and moderate-penetrance susceptibility genes, such as *PTEN*, *PALB2*, *CHEK2*, *ATM*, and *RAD51C*, could have been lost in the absence of a specific

analysis on BC, OC and PC patients, and identified a *CDH1* altered BC case harboring the PV c.2164+2T>C in this gene.

With this evidence, it has become necessary to study the *CDH1* gene to offer potential preventive and therapeutic applications for carriers of these sequence alterations [17].

Based on the database harvested at the Nuclear Oncology & Radiotherapy Institute (NORI) Hospital, Islamabad, Pakistan, the current study was aimed to investigate the typology and gene location of germline PVs/LPVs detected in the *CDH1* susceptibility gene in BC patients, in order to investigate the prevalence of the different inherited genetic variants using the single-stranded conformational polymorphism (SSCP) analysis. The identified novel mutations were further explored using different *in silico* tools for the prediction of their structural and functional effects on the E-cadherin protein.

Materials and Methods

Patients. An observational and a molecular population-based prospective study has been carried out from August 2019 to August 2020 at the NORI Hospital, Islamabad, Pakistan. The clinical information regarding 200 BC patients was collected and analyzed, while 50 blood samples from healthy donors were used as a control. All patients provided an informed consent, and the information regarding personal and familial history of cancer, family geographical origin, age at cancer diagnosis time, histological tumor subtype, molecular phenotype, and disease stage (I–IV) was recorded. Clinical information, which include gender, age, cancer type, grade, stage, hormonal status, and different risk factors for each enrolled patient was anonymously recorded and coded. Patients were grouped in the age range from >20 and <100 y.o.

According to the current ESMO guidelines, the genetic testing for the *CDH1* gene is recommended for patients with familial pedigree with

suspected HDGC and bilateral or multiple cases of LBC, especially the onset at an early age (< 50 years) [18].

Blood sampling. The peripheral blood samples of 200 BC patients and 50 healthy donors were collected through a vacutainer syringe containing EDTA. The standard phenol-chloroform method was used to extract DNA from the collected blood samples.

Molecular analysis. The *CDH1* gene primers were designed using the Primer 3 online available tool. The exonic sequences of the *CDH1* gene including exons 7, 8, 10, 11, and 13 were analyzed using SSCP method. The samples with deviant movement on SSCP gels were analyzed by Sanger sequencing using an ABI 3130 Genetic analyzer (Applied Biosciences, USA). The chromatograms were analyzed in Sequencing Analysis 5.2 software and aligned with reference to the *CDH1* gene sequence to identify any sequence variant.

In silico analysis. Different bioinformatics tools were used to study the identified sequence variants. Sequence alignment was done using Mega 7 Software (<https://www.megasoftware.net/>) with the characteristic of Clustal W option to compare the normal and variant sequence separately for each exon. BLAST was used to analyze the genetic variations identified along with the phylogenetic analysis to evaluate the conserved sequence of the specific changing variants in other species.

PredictSNV. PredictSNV (version 1.0) (<http://loschmidt.chemi.muni.cz/predictSNV1/>) online tool was used to predict the functional aspect of the amino acid change combining various other software, such as SIFT, PolyPhen-1 PolyPhen-2 MAPP, PhD-SNV, SNAP, PANTHER, PredictSNV, and nsSNVAnalyzer.

Sequence conservation analysis. ConSurf web-based tool (<http://consurf.tau.ac.il/>) was used to identify the amino acid conservation within the protein through the multiple sequence alignment algorithm. The conservation was determined on the basis of the score color that ranges within 1–9.

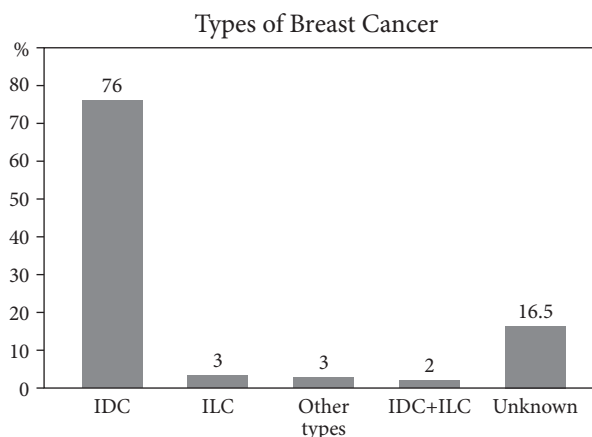


Fig. 1. Percentage distribution of BC types (n = 200)

Prediction of change in protein stability.

The protein stability change was identified using I-Mutant2.0 (<https://folding.biofold.org/i-mutant/i-mutant2.0.html>) that works on the basis of Support Vector Machine and automatically predicts the protein stability on the basis of amino acid change. The results are given in DDG values, which represent a free energy change in kcal/mol and a sign of DDG. Furthermore, to validate the results of I-Mutant2.0 the MUpro tool (<http://mupro.proteomics.ics.uci.edu/>) was also utilized, based on SVM and also on the neural network.

Structural analysis using SWISS model and UCSF chimera. Swiss Model (<http://swissmodel.expasy.org/>) was used for the structural analysis of protein through the sequence homology modeling for a 3D model generation [19]. The Uniprot ID P12830 was used for homology modeling with the Swiss model platform (<https://swissmodel.expasy.org>). The model has a QMEAN of -2.57 and a sequence identity of 81.73%. UCSF Chimera was used to validate the subsequent positions of the SNVs and to construct two mutant models.

RMSD and total hydrogen bond prediction.

RMSD value for both PVs/LPVs in carriers and non-carriers was calculated after superimposition by using UCSF Chimera. Furthermore,

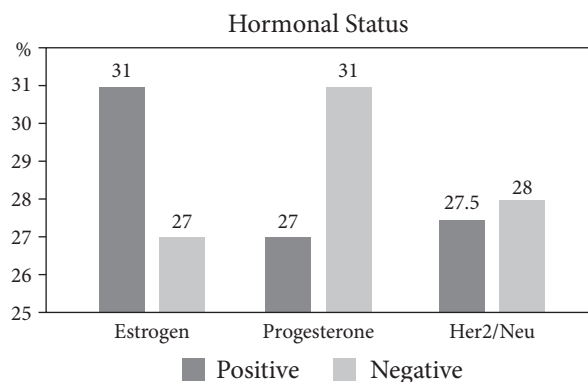


Fig. 2. Percentage distribution of estrogen, progesterone and Her-2 hormonal status in BC patients (n = 200)

H-bond values for each structure were also calculated.

Prediction of protein-protein interactions.

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, available at <http://string-db.org>) (<https://string-db.org/>) online available database was used to identify and predict the interactions of proteins, providing information based on the direct and indirect contact as well as the experimental evidence.

Results

Clinicopathological data assessment. The clinical data of patients enrolled in the present study showed that BC was diagnosed mainly in females (99.5%) rather than males (0.5%). The highest BC occurrence (47.5%) was found in women < 50 y.o. The IDC subtype was present in 76% of our patients, while only 3% of patients exhibited ILC (Fig. 1). The primary tumor was located in the left breast in 33% of cases. 30% of patients were diagnosed at stage II and 27% at stage III. In this study, 6 cases showed recurrence, and triple-negative hormonal status was found in only 3 patients. The distribution of patients according to the status of estrogen, progesterone, and human epidermal growth factor (Her-2) receptors of the tumor cells is presented in Fig. 2.

Our data depicted that 8% of the patients used contraceptives before the development of BC that may indicate contraceptive medicine being a low-risk factor in the BC development (Supplementary Table S1)¹. The hormonal treatment for premenopausal and postmenopausal problems is very common nowadays, and 18% of our patients had been going through this treatment. An accident or any type of injury to the breast can be a cause of cancer: 4% of our patients developed BC due to the injury to the breast. 20% of patients had a family history of cancer that includes blood relatives and immediate family members that were closely related in the previous generation. Nicotine can be a risk factor for cancer, 8% of patients agreed that they are addicted to nicotine intake in different forms like naswar intake, smoking cigarette, and tobacco intake through huqqa. Most of the patients have stopped nicotine intake after the diagnosis of cancer. Statistical analysis of the IDC stage and risk factors assessed using Statistical Package for the Social Sciences are shown in Supplementary Table S1.

Mutation screening of CDH1 gene through SSCP analysis and sequencing. The analysis of the SSCP results shows the band variations that clearly indicate the absence of variations particularly in exon 7. The confirmation of any change in the samples with mobility shift bands was assessed through sequence analysis (Supplementa-

ry Fig. S1, a). The sequencing was repeated with a primer that shows non-conformational results at the intron site demonstrating either intronic deletion that may or may not influence the protein or insertion of some other sequence at the intron. The results of the study of exon 8 revealed no significant band separation through SSCP. Further confirmation for any presence of variance was obtained through the sequencing (Supplementary Fig. S1, b). The data on exon 10 SSCP showed the absence of significant band variation. However, some mutation was observed in sequencing at position c.1337C>A leading to protein change from non-polar alanine to the negatively charged aspartate (acidic amino acid) due to the codon variation GCC > GAC (Supplementary Fig. S1, c). Exon 11 sequence analysis showed an insertion of T base at position 1669 (c.insT1669) (Supplementary Fig. S1, d), which results in the premature termination and truncated protein at amino acid 556 instead of normal 882 amino acids. Exon 13 displayed nucleotide change at c.2076T>C (GCT > GCC), however, both codons represented alanine, and no changes were observed at the level of protein structure and function (Supplementary Fig. S1, e).

Sequence alignment. Nucleotide sequence of the gene inserted into Mega 7 software tool and sequence was aligned with Clustal W. Translated sequence was detected by inserting normal and mutated change sequence of each exon to analyze the translational changes in the resultant protein sequence of the BC patients (Supplementary Fig. S2). A non-synonymous missense mutation was observed in exon 10 at position

¹ Supplementary materials are posted at https://www.researchgate.net/publication/372953113_Pathological_Significance_of_CDH1E-Cadherin_Germ-line_sequence_variants_in_Breast_Cancer_patients#fullTextFileContent

Table 1. PredictSNV and integrated tools results of selected SNVs of CDH1

Mutation	Effect	Predict SNV	MAPP	PHDSNV	Polyphen-1	Polyphen-2	SIFT	SNAP	PANTHER
A446D	Neutral	83%	74%	72%	67%	74%	68%	83%	47%
V556	Not calculated	—	—	—	—	—	—	—	—
A692A	Neutral	—	—	—	—	—	—	—	—

446 resulting in the change of amino acid code GCC>GAC, with an amino acid change from alanine > aspartate in the conserved domain. Furthermore, in exon 11, there is a nonsense alteration at position 1669 due to insertion of T base causing premature termination, and the resultant protein is composed of 556 amino acids compared to 882 amino acids in normal protein. Exon 13, with amino acid at position 692, is also part of the conserved region, however, a silent mutation was observed with no alanine replacement at the level of protein structure.

Phylogenetic analysis. The exon regions of CDH1 gene were analyzed using the BLAST tool to identify the changes in the amino acid sequence in different species (Supplementary Fig. S3). The BLAST results showed the presence of conserved domains across different species.

SNV analysis. The prediction of SNV using PredictSNV resulted in a percentage score. Exons 7 and 8 were excluded from *in silico* analysis because of the absence of variations. The SNV with A446D substitution results in the replacement of alanine with aspartate, that is, a neutral result, and according to all the parameters, there is no effect on the protein functioning (Table 1). Alteration V556 cannot be calculated because there was a termination of sequence, not an addition. Moreover, the A692A variant was neutral because of alanine replacement with alanine.

Mutation Taster in silico online tool was used to study the structural and functional effects on the proteins with the acquired variants. The re-

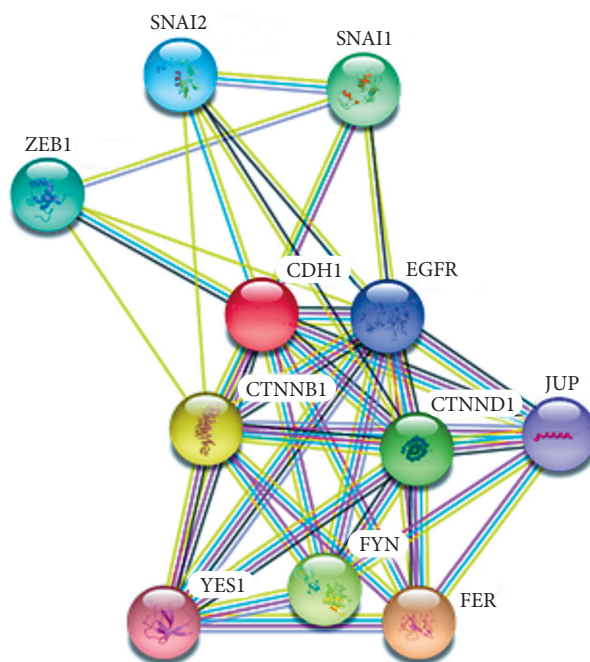


Fig. 3. Protein-protein interaction of CDH1 gene using STRING

sults indicate (Table 2) that the change mediated by exon 10 is due to polymorphism, which can cause structural changes in the protein. This may indicate the presence of splice site alterations, which need further study. The nonsense-mediated decay is observed due to the change in amino acid in exon 11, which may affect the protein structure and can cause the disease. Exon 13 shows the reported disease-causing alteration which is homozygous in TGP or ExAC reported as rs180155.

Table 2. CDH1 sequence alterations in BC (n = 200)

Codon	Exon number	Nucleotide change	Amino acid change	Putative effect
446	Exon 10	1337C>A	GCC>GAC Alanine>Aspartate	Premature termination, truncated protein
556	Exon 11	1669insT	Premature termination	Premature termination, truncated protein
692	Exon 3	2076T>C	GCT>GCC Alanine> Alanine	Single nucleotide polymorphism

Sequence conservation analysis. The ConSurf analysis resulted in the identification of conserved amino acids within the E-cadherin protein. The amino acids at positions 446 and 692 were not conserved. However, the amino acid at position 556 was conserved with a score of 7 representing the importance of amino acid for the conservation of protein structure (Supplementary Table S2).

The impact of predicted mutations on E-cadherin protein stability. Two online tools I-Mutant2.0 and MUPro were utilized to view the effects of mutations on protein stability. The DDG value for the variant at position 446 was -1.31 kcal/mol, which represented decreased stability, and similarly, for position 556 alteration, there were -0.91 to -3.22 kcal/mol DDG values, which also represented a decrease in stability. Both analyses highlight that the variations at either position 446 or 556 resulted in the reduced stability of E-cadherin protein, however, at position 692, amino acid change was not identified: there was neither increase nor decrease in the stability effect (Supplementary Table S3).

Protein structure prediction using SWISS model and UCSF chimera. Genetic variants of E-cadherin protein showed a differential change in the protein domains, which prompted us to predict the structural features of the protein. Protein 3D models were predicted through swiss-model.expasy.org. The 3D structure for normal E-cadherin protein is shown in Supplementary Fig. S4, whereas Supplementary Fig. S5 shows a mutated prematurely truncated protein due to insertional alteration at position 1699 of exon 11. The RMSD between 397 pruned atom pairs is 0.039 angstroms; (across all 397 pairs: 0.039).

The UCSF Chimera results showed the presence of a new hydrogen bond due to a variant at position 446 with the 2.737 length of a hydrogen bond with amino acid 443, predicting structural changes within the protein (Supplementary Fig. S6). Furthermore, RMSD between 536 pruned atom pairs was 0.005 angstroms (across all 536

pairs: 0.005) (Supplementary Fig. S7). However, an increase in the RMSD was associated with the altered function of the protein.

Protein-protein interaction analysis for CDH1. The study of the protein-protein interaction allowed us to clarify various pathways that will be disrupted due to the CDH1 sequence variants. String data showed the first ten interactions where high interaction takes place with the catenin delta-1 EGFR, JUP junction plakoglobin, CTNNB1 catenin beta-1 FER tyrosine-protein kinase, SNAI1 zinc finger protein, SNAI2 zinc finger protein 2 and ZEB1 zinc finger E-box-binding homeobox. Furthermore, an interaction was also observed that included FYN tyrosine-protein kinase and YES1 tyrosine-protein kinase (Fig. 3).

Discussion

BC exists in many heterogeneous forms, which show different subtypes with discrete features, treatment response, and patient outcomes. The precise classification of BC for treatment and prognosis is very crucial. The genetic alterations play a critical role in the disease prognosis making the genetic profile of each breast tumor and going toward personalized medicine [20]. The positive ER in BC individuals shows better prognosis approaches as compared to patients with the negative ER status. In contrast, the positive status of HER-2 is related to the late diagnosis [21]. The reduced expression of the *CDH1* gene causes aberrations in the system of a cell-to-cell adhesion, which results in cancer progression and metastasis [22]. ER and PR do not contribute to the methylation of the *CDH1* gene, but HER-2 negative status causes favorable conditions for methylation increasing BC risk immensely, which makes it a marker for prognosis along with ER [23].

The epidemiological risk factors of BC patients are diet, family history, early puberty, high ER exposure, socio-economic status, oral

contraceptives, and environmental factors. The molecular genetic analysis gives us better understanding of the risk of BC development and the therapy response. We can find the prime-risk factors by studying the correlation of various BC risk factors with SNPs of various genes. The frequency of *CDH1* gene mutation in BC patients is 42.8%, and numerous molecular changes are also reported in ILC patients [3].

SSCP analysis indicated that the variant in exon 10, c.1337C>A leads to change in amino acid GCC>GAC with Alanine>Aspartate, leading to structural modifications. Similarly, missense c.1774G>A (A592T) was observed in association with IDC [24]. Likewise, insertion of 669insT in exon 11 can also cause premature termination of protein in E-cadherin protein analysis, whereas variants in exon 13 at position c.2076T>C have no putative effect because of the single nucleotide polymorphism with the same amino acid codes. The same result with exon 13 silent substitution c.2076T>C was identified in gastric cancer [25]. Furthermore, our *in silico* analysis also confirmed the effect of these variants on the stability of protein. The A446D variant predicted instability of protein, however, insertion at position V556 leads to termination and the resultant protein is short causing loss of E-cadherin protein. To control the quality of protein within the cytoplasm, the proteasome ubiquitin system (UPS) takes part, where San1 E3 ligase is known to maintain the quality of proteins by degrading truncated and misfolded protein [26]. Similarly, *in silico* analysis of the *CDH1* gene was also employed in gastric cancer to predict missense variations effects [27].

The *CDH1* gene acts as a tumor suppressor gene as it functions in cell-cell adhesion and maintains a proper epithelial environment [28]. Due to its aggressive nature of expression, the *CDH1* gene is a potential predictive and prognostic biomarker for BC clinical management. The loss of the function or the deleterious sequence alterations in E-cadherin affect the cellular integrity and architecture [29]. By describing a link amongst genetic alterations and all high peril liability genes along with the family history of cancer, the age of the onset, and the epidemiological risk factors can be used to develop a model structure of different criteria for identifying BC accurately.

Overall, the current study evaluated the effect of non-synonymous missense, nonsense, and silent *CDH1* potentially PVs/LPVs in BC patients in a selected Pakistani population. We have found a novel alteration at position A446D, which leads to the reduction of stability in the protein and the insertion of T at position 556, resulting in a truncated and non-functional protein. Furthermore, *in silico* results also suggest the damaging effect of both alterations on the structure and function of E-cadherin protein. These abnormalities within the *CDH1* gene may play a crucial role in probing the defective signaling pathways according to which targeted BC therapy can be designed. Hence, our study can be extended further by increasing the sample size to develop a deep ethnic base study on the Pakistani population.

Conflicts of interest

The authors declare no conflict of interest.

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C. Табассум¹, Ф. Мунір^{1,3}, А.А. Аль Авад², З. Анвар^{3,*}

¹ Відділ біологічних наук, Міжнародний ісламський університет, Ісламабад, Пакистан

² Відділ клінічних лабораторних наук, Наджранський університет, Наджран, Саудівська Аравія

³ Відділ біохімії, Варшавський медичний університет, Варшава, Польща

ПАТОЛОГІЧНЕ ЗНАЧЕННЯ СПАДКОВИХ ВАРІАНТІВ ГЕНА CDH1/Е-КАДГЕРИНУ У ХВОРИХ НА РАК МОЛОЧНОЇ ЗАЛОЗИ

Стан питання. Спадкові зміни гена-супресора пухлинного росту CDH1 (Е-кадгерину) виявлено при кількох типах епітеліальних злоякісних новоутворень, таких як спадковий дифузний рак шлунка та лобулярний рак молочної залози. Е-кадгерин відіграє центральну роль у проліферації, підтримці міжклітинної адгезії, полярності та епітеліально-мезенхімальному переході. Це свідчить про необхідність аналізу впливу варіантів гена *CDH1* на структуру відповідного білка для прогнозування його клінічного значення в прогресії раку молочної залози (РМЗ). **Метою** дослідження було оцінити вплив та зв'язок потенційно патогенних/імовірно патогенних варіантів (PV/LPV) гена *CDH1* з ініціацією та прогресуванням РМЗ. **Матеріали та методи.** Дослідження ґрунтується на результатах обстеження та лікування 200 хворих на РМЗ. В якості контролю використовували зразки крові 50 здорових донорів. Проведено молекулярний аналіз гена *CDH1*, а також дослідження *in silico* з метою оцінки інвазивності та прогресування РМЗ, спричиненого білком Е-кадгерину. **Результати.** За допомогою генетичного скринінгу ідентифіковано чотири варіанти послідовності гена *CDH1*, які включали варіації в екзонах 7 8 10, 11 і 13. Екзон 10 характеризувався наявністю мутації сайту сплайсингу в положенні с.1337C>А що впливало на структуру білка. В екзоні 11 відзначалася вставка Т-основи в позиції 1669, що призводило до вкорочення білка порівняно з нормальним та може призводити до асоційованого із захворюванням нонсенс-осередкованого розпаду mРНК. Зауважимо, що у екзоні 13 детектувався уже відомий поліморфізм у позиції с.2076Т>С. Аналіз *in silico* показав наявність різних варіантів послідовності гена *CDH1*, що свідчить про загальне порушення структури та функцій білка. **Висновки.** Подальший функціональний аналіз цих варіантів та їх асоціацій з РМЗ можна забезпечити шляхом збільшення розміру вибірки та досліджень *in vivo* з використанням мишачих моделей.

Ключові слова: рак молочної залози, *CDH1*, Е-кадгерин, одноланцюговий конформаційний поліморфізм, секвенування, варіантний аналіз.