RESEARCH ARTICLE

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First Screening For Germline Variations In Exon 5 *PTEN* Gene and Their Contribution to Triple Negative Breast Cancer in Eastern Algeria

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Abstract

Objective: Triple negative breast cancer (TNBC) is the most aggressive breast cancer subtype associated with younger age, bigger tumor size, high grade tumor, high risk of tumor recurrence and death. PTEN is one of the tumor suppressor genes that have been analyzed to provide its role in predisposition to TNBC. The aim of this study was to screen germline variants in exon 5 of the *PTEN* gene in Algerian TNBC patients and to assess their association with the clinical characteristics of TNBC. **Methods:** 69 TNBC patients coming from different regions of eastern Algeria were analyzed for germline variants in exon 5 of the *PTEN* gene, among them 6 patients (8.69%) had a family history of breast cancer. Peripheral blood samples were obtained and genomic DNA was extracted from leukocytes using the salt-saturation method. Exon 5 was amplified by PCR and then sequenced. The resulted sequences were aligned against the reference sequence available in GenBank. All detected variants were annotated using the Ensembl database and their pathogenicity was predicted according to their REVEL scores. **Results:** 30 different variants in 27 (39.13%) of the 69 patients were identified. 6 missense variants were predicted to be likely benign and 24 variants were predicted to be pathogenic. Among them, 19 were missense variants, 2 were nonsense variants and 3 were frameshift variants, including 1 deletion and 2 novel insertions. The pathogenic variants occurred in 17 patients, who harbored between 1 and 4 pathogenic variants. No pathogenic variants were found in patients with a family history of breast cancer. The correlation between pathogenic variants and the clinical characteristics of TNBC patients was statistically insignificant. **Conclusion:** The frequency of pathogenic variants identified in the Algerian population is higher than that in other populations; however, they are not associated with susceptibility to TNBC.

Keywords: Eastern Algeria- TNBC- exon 5 of the *PTEN* gene- pathogenic germline variants- DNA sequencing

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Introduction

Phosphatase and tensin homolog (*PTEN*) is the most frequently mutated tumor suppressor gene in human cancer after p53 [1]. Initially identified as a protein tyrosine phosphatase [2], it was later determined to have phosphatidylinositol phosphatase activity involved in the regulation of phosphatidylinositol 3kinase (PI3K) signaling pathway [3].

PTEN antagonizes the PI3K cascade by dephosphorylating the substrate phosphatidylinositol 3,4,5 triphosphate (PIP3), thereby inactivating downstream signaling pathways such as cellular proliferation and cell survival [4]. *PTEN* also inhibits cell migration through its protein phosphatase activity that targets focal adhesion kinase (FAK) [5].

Function alteration of *PTEN* has been observed in several types of cancer through a number of mechanisms such as mutations in the *PTEN* coding sequence, epigenetic silencing by methylation of the *PTEN* promoter, loss of heterozygosity and loss of expression of the *PTEN* protein [6]. However, *PTEN* protein loss is more implicated in cancer than *PTEN* alterations, especially in breast cancer [7].

Somatic *PTEN* mutations have been found predominantly in endometrial carcinomas and

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glioblastomas. They have also been observed in ovarian carcinomas, prostate carcinomas, melanoma and breast cancer [8]. Biallelic mutations in the *PTEN* gene were observed in various sporadic cancers such as breast cancer, endometrial cancer and melanoma. Monoallelic mutations were also identified in several tumors, including glioblastomas, endometrial carcinomas and breast tumors [9].

Germline *PTEN* mutations were mainly found in patients affected by the autosomal dominant cancer predisposition syndrome, Cowden disease (CD) associated with a high risk for several types of cancers, including breast cancer [10]. Germline *PTEN* mutations have also been detected in hereditary breast cancer [11, 12] and sporadic breast cancer [13] without clinical features of CD. Several studies [8, 9, 14] have shown that exon 5, which encodes the catalytic domain of *PTEN*, has the highest rate of somatic and germline mutations as compared to other exons.

Triple negative breast cancer (TNBC) is a heterogeneous group characterized by the absence of expression of hormone receptors to estrogen, progesterone and human epidermal growth factor receptor-2 (HER-2) [15]. TNBC is the most aggressive breast cancer subtype, characterized by a high risk of tumor recurrence and death [16]. In eastern Algeria, it represents 21.61% of breast cancers with high tumor size, high tumor grade and lymph node infiltration [17]. TNBC shows a frequent loss of *PTEN* expression compared to the other molecular subtypes of breast cancer, associated with poor prognosis and significant links with high grade tumor, larger tumor size, lymph node metastasis and tumor recurrence [18-20].

The present study, conducted for the first time in Algeria, aims to screen germline variations in the catalytic domain encoded by exon 5 of the *PTEN* gene in TNBC patients, to predict their pathogenicity and to evaluate their contribution to TNBC.

Materials and Methods

Clinical characteristics of the study population

69 patients with triple negative breast cancer, who were treated and monitored in the oncology service of the Regional Hospital in Constantine (eastern Algeria), were recruited in this study. The ages of the patients varied from 25 to 81 years with a median age of 53 years. The average age was 50.87. Six cases (8.69%) of the 69 patients had a family history of breast cancer with a mother or sister who has already been affected by breast cancer. The average tumor size was 4.1 cm with extremes ranging from 1cm to 11cm (Table 1).

DNA extraction

Peripheral blood was collected in 2 EDTA containing tubes and stored at 4°C. DNA extraction was carried out at the Laboratory of Biology and Molecular Genetic, Constantine 3 University. DNA was isolated from leukocytes using the salt-saturation method as previously described [21]. DNA concentration and purity were determined by the NanoDrop 2000 Thermo scientific spectrophotometer and then stored at -20°C until further analysis at the National Center for Biotechnology Research- CRBt - Constantine.

DNA amplification

PCR was performed to amplify exon 5 of the *PTEN* gene and its flanking intronic regions using the following primers previously published [22]: F(5'TGCAACATTTCTAAAGTTACCTACTTG3'), R(5'GAAACCCAAAATCTGTTTTCCA3').

The PCR reaction was performed in 20µl PCR mixture containing 2 µl PCR buffer, 0.6 µl of MgCl2 (50mM), 4,2 µl of dNTP (2mmol), 2 µl of each primer (100ng/ µl), 0,16 µl of Taq polymerase (5u/ µl) and 1 µl (50 ng/ µl) of extracted DNA. The PCR cycling program comprised an initial denaturation at 95°C for 4 min, followed by 30 cycles: denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 45 sec. Final extension was accomplished at 72°C for 7 min. Amplification products were analyzed on a 2% SYBER Safe stained agarose gel along with 100 bp DNA ladder.

Purification and DNA sequencing

Purification of PCR products was performed using the Invitrogen™ PureLink^R PCR Purification Kit. The purified PCR products were then diluted to 20 ng/µl and sequenced in forward and reverse reactions using the BigDye™ terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequenced products were purified using the Invitrogen™ Big dye X terminator™ purification Kit (Applied Biosystems) and then subjected to capillary electrophoresis on the Applied Biosystems 3500 xL Genetic Analyzer.

Table 1. Clinical Characteristics of the Study Population

Characteristics	Number of patients	Percentage %
Age		
Median age	53 years	-
Average age	50.87 years	-
Range	25-81 years	-
≥50	40	57.9
<50	29	42.1
Tumor size		
Average tumor size	4.1cm	
>2cm	58	84
≤ 2cm	11	16
Histology		
Infiltrating ductal carcinoma	67	97.1
Infiltrating lobular carcinoma	2	2.9
Tumor grade		
II	34	49.3
III	35	50.7
Lymph node status		
Positive	24	34.8
Negative	12	17.4
Missed	33	47.8

Interpretation of variants

Sequencing results were aligned against the reference sequence NG_007466.2 from the human *PTEN* DNA sequence available in GenBank using the Indigo application [23]. All detected variants were annotated using the Ensembl database (version 110) via the Human GRCh38 assembly [24]. The *PTEN* reference sequence used for variation annotation is NM_000314.8. The pathogenicity of the missense variants was predicted in silico according to their REVEL scores calculated by the Ensembl Variant Effect Predictor (VEP). The REVEL scores range from 0 to 1 and variants with higher scores are predicted to be more likely to be pathogenic. The scores > 0.5 indicate likely disease causing variants and scores ≤ 0.5 indicate likely benign variants. Nonsense and frameshift variants were classified as pathogenic.

Statistical analysis

Statistical analysis was performed using the EpiInfo software Version 7 to assess the correlation between *PTEN* exon 5 variants and the clinical characteristics of TNBC patients. A value of $p < 0.05$ was considered statistically significant.

Results

30 different variants were detected in 27 (39.13%) of the 69 patients. 24 variants, which occurred in 17 patients, were predicted to be pathogenic (with REVEL scores > 0.5). Among them, 19 were missense variants, 2 were nonsense variants and 3 were frameshift variants, including 1 deletion and 2 novel insertions. Among the patients harboring pathogenic variants, 8 patients had 1 pathogenic variant and 9 patients had between 2 and 4 pathogenic variants. No pathogenic variants were found in patients with a family history of breast cancer. Only 6 variants were predicted to be likely benign (with REVEL scores ≤ 0.5).

Pathogenic variants

Among the 19 missense pathogenic variants identified in our cohort, 6 of these variants had higher REVEL scores (≥ 0.9), i.e., c.274G>A (p.D92N) in 1case, c.314G>C (p.C105S) in 1 case, c.408T>G (p.C136W) in 1 case, c.463T>A (p.Y155N) in 1 case, c.463T>G (p.Y155D) in 3 cases and c.464A>G (p.Y155C) in 1 case (Table 2; Figure 1). The other missense variants are presented in Table 2 and Figure 2. All the pathogenic variants detected as missense variants were heterozygous, except for the variant c.463T>A which was homozygous.

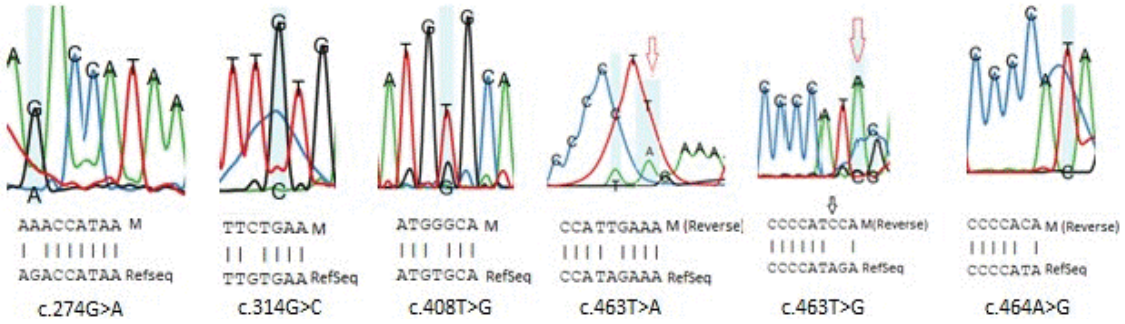

Figure 1. Sequencing Results of Missense variants with higher REVEL scores predicted as pathogenic variants. Arrows and blue rectangles indicate identified variant, M is for mutated sequence, RefSeq is for Reference Sequence

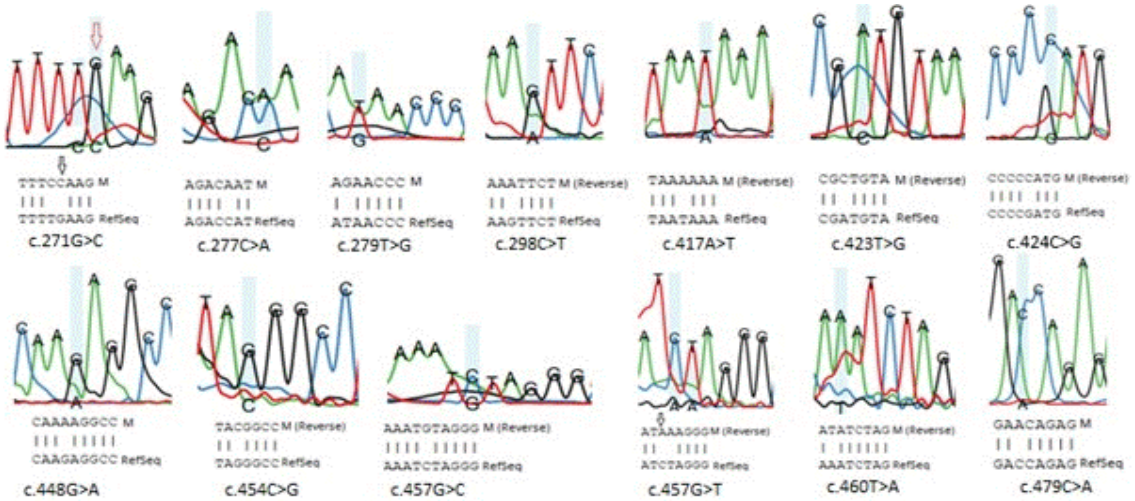

Figure 2. Sequencing Results of Missense Variants Predicted as Pathogenic Variants. Arrows and blue rectangles indicate identified variant, M is for mutated sequence, RefSeq is for Reference Sequence.

Table 2. Pathogenic Variants in *PTEN* Exon 5 Identified in the Study Population

Patients	Nucleotide change	Amino acid change	Variation type	ID rs	Genotype	REVEL score	Clinvar prediction	Reported in	Frequency in breast cancer in other studies
25	c.271G>C	E91Q	Missense	-	Heterozygous	0.733	-	dbSNP	
24	c.274G>A	D92N	Missense	-	Heterozygous	0.924	-	dbSNP	20/350 patients, Pakistan (Baig et al., 2011)
8, 10	c.277C>A	H93N	Missense	rs786204927	Heterozygous	0.891	Likely pathogenic	dbSNP, Clinvar	
21	c.279T>G	H93Q	Missense	-	Heterozygous	0.895	-	RefSeq	
69	c.298C>T	L100F	Missense	-	Heterozygous	0.682	Likely pathogenic	Clinvar	
21	c.314G>C	C105S	Missense	-	Heterozygous	0.939	-	dbSNP	
60	c.408T>G	C136W	Missense	rs869312776	Heterozygous	0.954	Likely pathogenic	dbSNP, Clinvar	
19	c.417A>T	L139F	Missense	-	Heterozygous	0.847	Uncertain significance	Clinvar	
10.21	c.423T>G	H141Q	Missense	-	Heterozygous	0.678	-	-	
19	c.424C>G	R142G	Missense	rs746152219	Heterozygous	0.731	Uncertain significance	dbSNP, Clinvar	
44	c.432del A	Lys144fs	Frameshift	rs1114167657	Heterozygous	-	Pathogenic	dbSNP, Clinvar	
69	c.448G>A	E150K	Missense	-	Heterozygous	0.734	-	dbSNP	
19	c.454C>G	L152V	Missense	-	Heterozygous	0.799	-	dbSNP	1/43 patients, Greece (Kechagioglou et al., 2014)
10.21	c.457G>C	D153H	Missense	rs9651492	Heterozygous	0.640	Uncertain significance	dbSNP, Clinvar	
60	c.457G>T	p.D153Y	Missense	-	Heterozygous	0.873	Uncertain significance	Clinvar	
14, 45	c.460T>A	F154I	Missense	-	Heterozygous	0.792	-	-	
36	c.463T>A	Y155N	Missense	rs398123325	Homozygous	0.911	Likely pathogenic	dbSNP, Clinvar	
14.45.60	c.463T>G	Y155D	Missense	-	Heterozygous	0.960	-	-	
15	c.464A>G	Y155C	Missense	rs1060500126	Heterozygous	0.968	Pathogenic	dbSNP, Clinvar	
55	c.463_464insG	Y155X	Frameshift	-	Homozygous	-		Novel	
10.25.28.36	c.465T>G	Y155X	Nonsense	rs1554898206	Homozygous/ heterozygous	-	Pathogenic	dbSNP, Clinvar	
36	c.465T>A	Y155X	Nonsense	rs1554898206	Heterozygous	-	Pathogenic	dbSNP, Clinvar	1/43 patients, Greece (Kechagioglou et al., 2014)
41	c.479C>A	T160N	Missense	-	Heterozygous	0.861	-	dbSNP	
43	c.491_492insT	K164 X	Frameshift	-	Homozygous	-	-	Novel	

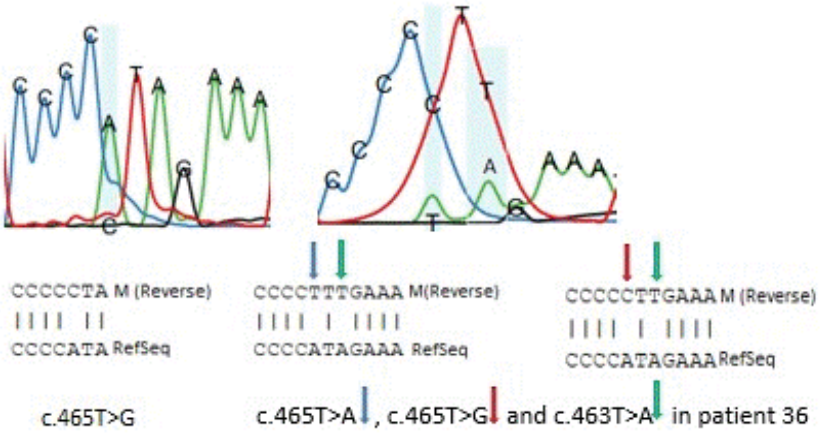

Figure 3. Sequencing Results of Nonsense Variants Predicted as Pathogenic Variants. Arrows and blue rectangles indicate identified variant, M is for mutated sequence, RefSeq is for Reference Sequence.

We classified nonsense and frameshift variants as pathogenic; 2 substitutions were found to be nonsense. They result from a substitution of T at position 465, causing a premature stop codon at amino acid position 155 (p.Y155X). The substitution c.465T>G was observed in 4 cases; among them, 1 case was homozygous and 3 cases were heterozygous. The substitution c.465T>A, observed in 1 case, was heterozygous (Table 2; Figure 3). A heterozygous deletion causing a translational frameshift was found in 1 case, i.e., c.432del A (p.Lys144fs). 2 novel insertions that were homozygous were identified in 1 case for each, i.e., c.463_464ins G (p.Y155X) and c.491_492ins T (p. K164X), resulting in a premature stop codon (Table 2; Figure 4).

Likely benign variants

6 heterozygous missense variants were predicted to be likely benign, i.e., c.319G>A (p.D107N) in 4 cases, c.343G>A (D115N) in 2 cases, c.457G>A (p.D153N) in 3 cases, c.462C>G (p.F154L) in 5 cases, c.462C>A (p.F154L) in 3 cases and c.482G>A (p.R161K) in 8 cases (Table 3; Figure 5).

No statistically significant correlations were observed between exon 5 pathogenic variants and patients' age (p = 0.10), tumor size (p = 0.053), histological grade (p = 0.364), histological type (p = 0.435) and lymph

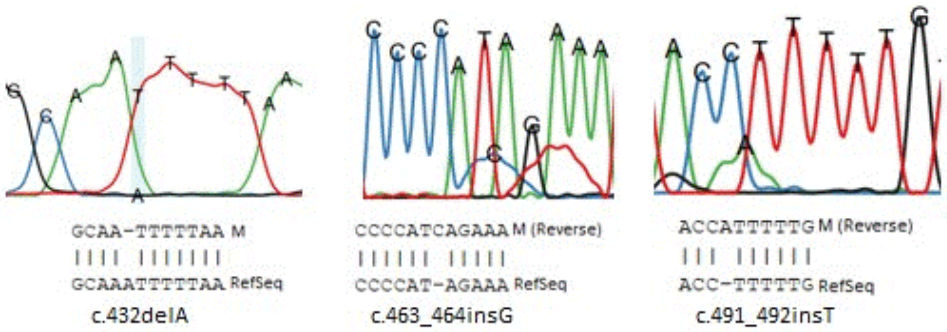

Figure 4. Sequencing Results of Frameshift Variants Predicted as Pathogenic Variants. Arrows and blue rectangles indicate identified variant, M is for mutated sequence, RefSeq is for Reference Sequence.

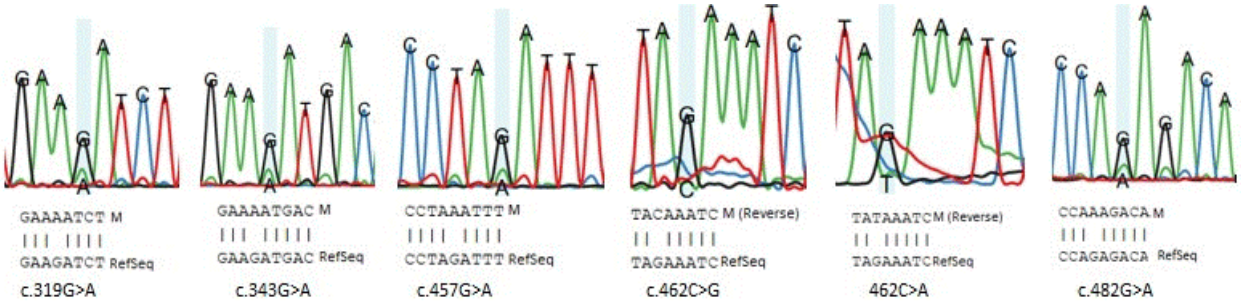

Figure 5. Sequencing Results of Missense Variants Predicted as Likely Benign Variants. Arrows and blue rectangles indicate identified variant, M is for mutated sequence, RefSeq is for Reference Sequence.

node infiltration (p = 0.70). The correlation between the pathogenic variants and clinical characteristics of TNBC patients is presented in Table 4.

Discussion

Exon 5 of the *PTEN* gene is a hotspot for germline mutations, as it encodes the catalytic domain of the *PTEN* protein [8]. Mutations in the catalytic domain of *PTEN* are known to decrease the PIP3 phosphatase activity of the *PTEN* protein [9] and promote the AKT pathway contributing to tumor formation and progression [25].

In our study, we analyzed TNBC patients for germline variations in exon 5 of the *PTEN* gene. Most of the variants found in the study population were predicted to be pathogenic. The frequency of the detected variants was significantly higher than what has been reported in the literature. In a cohort of 267 TNBC patients, only one germline mutation was identified in the *PTEN* gene [26]. Another study [27] indicated that the frequency of germline mutations in the *PTEN* gene was 0.05%. Recently, Wu et al [13] showed that only 1 germline mutation of the *PTEN* gene was identified in one out of 869 TNBC patients.

The pathogenic variants we have identified have previously been reported to reduce the PIP3 phosphatase activity of the *PTEN* protein. The variant p.Y155C had the highest REVEL score (0.968). It has been shown that it inactivates the lipid phosphatase function of *PTEN* which is critical to its tumor suppressor activity [28], it also affected the expression of the *PTEN* protein which was at a low level, suggesting that this variant could also impact the stability of the *PTEN* protein inducing a loss of *PTEN* function [29]. In our cohort, no patient was affected by CD; however, this variant was reported as a pathogenic germline mutation in CD patients who exhibited a significantly elevated risk of breast cancer [30, 31]. Several variants identified in our study, i.e., p.H93N, p.H93Q, p.Y155N, p.Y155D, p.C136W, p.C105S, p.H141Q, p.F154I and p.T160N were reported by Tokheim and Karchin [32] to induce a significant decrease in *PTEN* lipid phosphatase function involved in tumor growth. Other variants found in our study have already been reported to be associated with several types of cancer; the variant p.D92N was identified in breast cancer patients, both as a somatic mutation [33] and as a germline mutation [34]. The variant p.L152V was also reported in patients with breast cancer [9]. The variant p.E91Q was identified in prostate carcinoma, causing complete inactivation of the *PTEN* protein [35]. The variant p.L100F was identified in individuals with papillary thyroid carcinomas [36]. The variant p.E150K was detected in cervical cancer patients, significantly affecting the function of *PTEN* [37]. 4 pathogenic variants identified in our study were classified in the ClinVar database as variants of uncertain significance, i.e., p.L139F, p.R142G, p.D153H and p.D153Y.

2 substitutions at base 465 were found to be nonsense, resulting in a premature truncation of the *PTEN* protein at position 155 due to a change of tyrosine to stop codon. This alteration was classified as pathogenic in

Table 3. Likely Benign Variants in *PTEN* Exon 5 Identified in the Study Population

Patients	Nucleotide change	Amino acid change	Variation type	ID rs	Genotype	REVEL score	Clinvar prediction	Reported in	Frequency in breast cancer in other studies
3. 8 .10 .45	c.319G>A	D107N	Missense	rs57374291	Heterozygous	0.581	-	dbSNP	32/350 patients, Pakistan (Baig et al., 2011), 1/72patients, Iran (Nassiri et al., 2009)
45. 69	c.343G>A	D115N	Missense	rs145124907	Heterozygous	0.390	-	dbSNP	10/350 patients, Pakistan (Baig et al., 2011)
11 . 54. 60	c.457G>A	D153N	Missense	rs9651492	Heterozygous	0.501	Uncertain significance	Clinvar, dbSNP, ClinGen	33/350 patients, Pakistan (Baig et al., 2011), 1/43 patients, Greece (Kechagioglou et al., 2014), 2/51 patients, Egypt (Elwy et al., 2023)
14. 15. 28	c.462C>A	F154L	Missense	-	Heterozygous	0.585	-	-	
17. 45. 53. 60. 67	c.462C>G	F154L	Missense	-	Heterozygous	0.585	Uncertain significance	dbSNP, Clinvar	
3. 11. 47. 52. 54. 57. 60. 65	c.482G>A	R161K	Missense	-	Heterozygous	0.539	-	-	18/350 patients, Pakistan (Baig et al., 2011), 1/50 patients, China (Yang et al., 2010)

Table 4. Correlation between Exon 5 Pathogenic Variants and Clinical Characteristics of TNBC

	Total (n=69)	Patients without pathogenic variants	Patients with pathogenic variants (n=17)	p value
Characteristics				
Age (years)				
≥50	40	33	7	0.10
<50	29	19	10	
Tumor size				
>2cm	58	41	17	0.053
≤2cm	11	11	0	
Tumor grade				
II	34	24	10	0.364
III	35	28	7	
Histology				
Infiltrating ductal carcinoma	67	51	16	0.435
Infiltrating lobular carcinoma	2	1	1	
Lymph node status				
Positive	24	19	5	0.70
Negative	12	8	4	
Missed	33	25	8	

the ClinVar database; it has already been observed in breast carcinomas [9]. In our cohort, 1 deletion and 2 novel insertions were identified. The deletion c.432del A causes a translational frameshift (p.K144fs); however, the insertions c.463_464insG and c.491_492insT create premature stop signals p.Y155X and p. K 164X, respectively. Truncation or frameshift mutations within the phosphatase domain of the *PTEN* gene are known to destroy its phosphatase activity [4]; consequently, *PTEN* loses its tumor suppressor function [14].

Several residues within the catalytic domain of *PTEN* are tolerant to mutations, suggesting that they are not required for *PTEN*'s lipid phosphatase activity [38]. In our study, 5 amino acid changes appear to be likely benign. The variant p.F154L was detected in 8 of the 69 patients; previous studies have predicted this variant to be pathogenic [39] and associated with endometrial cancer [40]. The variant p.R161K was also observed in 8 patients, it was reported in the germline of patients with breast cancer [34] and in breast carcinomas [14]. This variant is located in the TI-loop, which spans residues 160-171 of the *PTEN* active site [41]. It has been demonstrated that the residues in the TI-loop are less important for PIP3 phosphatase activity and more tolerant to mutations [25, 42]. This variant was observed in 1 out of 6 patients who had a family history of breast cancer; no variants were found in the remaining cases. The variant p.D153N was reported as pathogenic in breast cancer; it was identified both as a germline mutation [34] and as a somatic mutation [9, 43]; however, it was classified in the ClinVar database as a variant of uncertain significance. The variant p.D107N was also reported as pathogenic in breast cancer [33, 34]. The variant D115N had the lower REVEL score (0.390); it was reported in the germline of patients with breast cancer [34], but its effect has not been documented in the literature.

It has been reported that the inactivation of only one allele in the *PTEN* gene contributes to tumorigenesis [4, 6, 44]. Our results indicate a high frequency of heterozygous variants, with only 4 homozygous variants observed (Table 2); however, the association between *PTEN* exon 5 variants and the clinical characteristics of the studied population was statistically insignificant (Table 4). Our results align with previous studies suggesting that *PTEN* germline mutations are not associated with the occurrence of TNBC [13, 45]. Macleod [46] reported that germline mutations in one allele of a tumor suppressor gene predispose to tumor initiation and progression, alongside a somatic mutation in the second allele. According to these data and our findings, we suggest that germline mutations in exon 5 of the *PTEN* gene do not predispose to TNBC.

There are several limitations to this study. The small size of the studied population, given that the correlation between exon 5 pathogenic variants and the clinical characteristics of TNBC involved only 17 patients. The pathogenicity thresholds established by in silico prediction tools are different, resulting in a heterogeneous classification of the variants. Consequently, certain variants identified in this study have predictions that differ from those reported in the literature. Other approaches are necessary to refine the mechanisms of *PTEN* inactivation that could contribute to TNBC.

In conclusion, we found that the frequency of the variants identified in our population was higher than that reported in the literature; many of them have been previously reported as germline or somatic mutations in several types of cancer, including breast cancer. Most of the variants we identified were predicted to be pathogenic; however no correlation was found with the clinical characteristics of TNBC. Our study could be extended to somatic variants in the *PTEN* gene and *PTEN* protein expression to provide a comprehensive overview of the

role of *PTEN* alterations in the contribution to TNBC.

Author Contribution Statement

The authors confirm contribution to the paper as follows:

Souad Haddad: Conceptualization, Methodology, Data interpretation, Writing- original draft, Writing-review and editing. Karim Chekroud: Supervision, Resources, Revision, Validation. Housna Zidoune: Data Interpretation. Ali Boumegoura: Methodology. Abdelhak Lakehal: Statistic analysis. Djalila Rezgoune-Chellat: Methodology, Resources. Dalila Satta: Revision, Validation. Nouredine ABADI: Resources.

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Scientific Approval

This study is part of a Ph.D. thesis.

Statement of Ethics

The study procedure was conducted in accordance with the Declaration of Helsinki and the Algerian legislation on the protection of privacy and experimentation on human subjects. Written informed consent to participate in this study was provided by the participants before blood collection.

Availability of data

Data is available upon request.

Conflict of Interest

The authors declare that they have no relevant financial or non-financial conflicts of interest to disclose.

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