Tumor Suppressor PTEN in Breast Cancer: Heterozygosity, Mutations and Protein Expression

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Abstract. *Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is one of the most frequently mutated human tumor suppressor genes, implicated in cell growth and survival and suppressing tumor formation. Loss of PTEN activity, either at the protein or genomic level, has been related to many primary and metastatic malignancies including breast cancer. The present study investigates the heterozygosity, mutation spectrum and protein expression of PTEN in 43 patients with breast cancer or precursor lesions of the breast and 10 healthy individuals. Microsatellite analysis at the PTEN locus using D10S215, D10S541 and D10S579 markers indicated that the observed heterozygosity (Ho) is lower than the expected heterozygosity (Hs) in benign and malignant breast disease. Mutational analysis in exons 1, 5, 7 and 9 of the PTEN gene revealed several mutations, most of which cause truncation of the PTEN protein and consequently loss of activity. Increased circulating levels of PTEN and phosphorylated PTEN protein were also observed by immunostaining in patients with breast cancer and precursor breast lesions. In support, increased PTEN protein expression was detected in corresponding tissue specimens. Our data suggest an association between breast cancer and PTEN mutations, resulting in the production of truncated forms of the corresponding protein, thus indicating that breast carcinogenesis is potentially related to PTEN loss of activity rather than loss of expression. Peripheral blood sampling may provide an advantageous application for the determination of PTEN gene mutations and its protein expression in human cancer.*

Abbreviations: PTEN, phosphatase and tensin homolog; MMAC1, mutated in multiple advanced cancers-1; PTEN-P, phosphorylated PTEN; TEP1, tensin-like phosphatase-1; PIP3, 3-phosphatidylinositol-3,4,5-triphosphate; PI3K, phospho- inositide-3 lipid kinase; Akt, serine-threonine kinase; INN, International Non-proprietary Name; HER2, human epidermal growth factor receptor 2; PDZ, post synaptic density protein, drosophila disc large tumor suppressor and zonula occludens-1 protein; PEST, peptide sequence rich in P, E, S and T; LOH, loss of heterozygosity; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ; ADH, atypical ductal hyperplasia; LN, lobular neoplasia; VABB, vacuum-assisted breast biopsy.

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Chromosome 10 has been the subject of numerous studies on cancer since it has been suggested that it may contain at least one important tumor suppressor gene. In 1997, *PTEN* (phosphatase and tensin homolog deleted on chromosome ten), also known as *MMAC1* (mutated in multiple advanced cancers-1) or *TEP1* (tensin-like phosphatase-1), was identified by three independent research groups as a tumor suppressor gene located at the 10q23 region (1-3). *PTEN* is one of the most frequently mutated tumor suppressor genes in human cancer, with a frequency approaching the one of *p53*. Mutations in both alleles of the *PTEN* gene arise during cancer progression in various tumor types, such as brain, prostate, breast, endometrial cancer and melanoma, with approximately 50% frequency (4). Monoallelic mutations, accompanied by loss of heterozygosity (LOH), are also frequently observed in certain tumors, including glioblastoma and endometrial carcinoma, but to a lesser

extent in breast, colon and lung tumors (4). Mutations in the *PTEN* gene are also observed in inherited cancer syndromes such as the Cowden syndrome (5).

The protein encoded by *PTEN* is a 403-amino-acid phosphatase (EC 3.1.3.67), member of the large protein tyrosine phosphatase family. It is a non-redundant, plasma membrane phosphatase with a relative molecular mass of 55 kDa (6). PTEN has been characterized as a dual-specificity phosphatase. Although it can de-phosphorylate proteins, its primary targets are highly specialized membrane lipids, mainly facilitating removal of the phosphate group from the inositol rings. More specifically, PTEN acts on the phosphatidylinositol-3,4,5-triphosphate (PIP3) that is formed through the action of phosphoinositide-3 lipid kinase (PI3K). PIP3 is an important lipid second messenger in tumorigenesis that activates Akt and other signalling molecules involved in a variety of cellular events, such as survival, proliferation, cell motility and invasion (7). PTEN reduces the pool of PIP3, inhibiting growth and survival signals and suppressing tumor formation (8, 9). This way, an "on-off" switch is evolved that appears to regulate a critical signaling pathway for oncogenesis (6).

The N-terminal domain of the PTEN protein consists of 179 residues and contains the phosphatase domain of the protein, where most mutations arise. The C-terminal domain of PTEN, connected to the N-terminal *via* a flexible loop, consists of 166 residues and contains some basic sub-domains of the protein, also common to other signaling molecules (10). The C-terminal domain also contains a PDZ motif that is responsible for protein-protein interactions and two PEST sequences (degradation signals) in the tail region of the molecule (11).

During the last years, there has been great progress in understanding the mechanisms underlying the enzymic regulation of PTEN. Among the several covalent modifications that regulate the protein's action and sub-cellular distribution is phosphorylation, ubiquitination and acetylation (12). The model that has been proposed to describe PTEN regulation through phosphorylation, suggests that phosphorylation of PTEN in specific residues converts the molecule into an inactive form of closed conformation, while de-phosphorylation converts the enzyme to its active form (13).

PTEN has been associated with initiation and progression of breast cancer through a number of mechanisms, including loss of heterozygosity at the *PTEN* gene locus, germline and somatic mutations in the *PTEN* gene, epigenetic silencing by methylation of the *PTEN* promoter, protein interactions down-regulating PTEN transcription, PTEN protein degradation and post-translational modifications of the PTEN protein (12, 14-16).

Recent studies have demonstrated the significant role of PTEN expression in HER2-overexpressing carcinomas of the breast, for which treatment with trastuzumab has shown significant efficacy (17-20). Human epidermal growth factor receptor-2 (c-erbB-2) is a cell membrane surface-bound receptor tyrosine kinase, while HER2/neu, its extracellular domain is normally involved in the signal transduction pathways leading to cell growth and differentiation. HER2-overexpression is observed in approximately 15-20% of breast cancer cases (17). HER2-positive tumors have aggressive clinical behaviour and are associated with poor prognosis (21-22). It has recently been found that patients with PTEN-positive tumors have a response rate to targeted-therapy with trastuzumab of approximately 70%, compared to less than 20% for patients with negative PTEN expression (23). These results were further supported by Fujita *et al*., who reported a response rate of 89% for PTEN-positive tumors *versus* 12.5% for PTEN-negative tumors following trastuzumab treatment (24). Dave *et al*. indicated that administration of trastuzumab alone was ineffective in decreasing p-MAPK and p-AKT levels *in vitro* and they clinically confirmed that low PTEN confers resistance to trastuzumab (20). In addition, it has been suggested that loss of at least 1 copy of the *PTEN* gene in carcinomas of the breast is associated with poor prognosis (15-16). These data render PTEN a potential tool to predict for clinical outcome and response to targeted-therapy in patients with breast cancer.

Considering the significance of PTEN in breast cancer development, prognosis and treatment, we investigated the heterozygosity at the 10q23 region and the presence of mutations in exons 1, 5, 7 and 9. We also evaluated PTEN protein expression in the systemic circulation and corresponding tissue specimens of 43 patients with carcinoma or precursor lesions of the breast.

Materials and Methods

Patient population and sampling. The study population consisted of 43 patients with breast disease; 22 women were diagnosed with invasive or *in situ* ductal breast cancer (IDC/DCIS) and 21 women with precursor lesions of the breast, namely atypical ductal hyperplasia (ADH) or lobular neoplasia (LN) of the breast. Ten healthy women served as controls. Peripheral blood samples and tissue specimens were obtained from patients during preoperative diagnosis of non-palpable mammographically-detected lesions by vacuum-assisted breast biopsy (VABB) at the First Department of Propaedeutic Surgery, Hippokratio Hospital, School of Medicine, University of Athens.

DNA extraction. Peripheral blood samples were collected in EDTA-containing tubes and were left at room temperature for 30 min. Subsequently, they were centrifuged at 3,300 × *g* for 15 min at 8˚C. The middle layer (buffy coat) was collected, mixed with 1 ml PBS and stored at –80˚C in aliquots. Samples were de-frosted once. Genomic DNA was extracted from approximately 200 μl buffy coat using the Blood Mini Kit (QIAGEN GmbH, Hilden, DE).

Microsatellite analysis at the 10q23 interval. Detection of heterozygosity at the PTEN locus was performed using three

microsatellite markers, known to map to the 10q23 interval, *D10S215, D10S541* and *D10S579*. The order of these markers from centromere to telomere was *D10S579, D10S215, D10S541* based on published maps (25-27). The primers used for the amplification of the three loci were as follows: 5'-TGGCATCATTCTGGGGA-3' forward and 5'-GCTTTACGTTTCTTCACATGGT-3' reverse primer for *D10S215*, 5'-AAGCAAGTGAAGTCTTAGAACCACC-3' forward and 5'-CCACAAGTAA CAGAAAGCCTG TCTC-3' reverse primer for *D10S541*, and 5'-CCGATCAA TGAGGAGTGCC-3' forward and 5'-ATACACCCAGCCAATGCTGC-3' reverse primer for *D10S579*. PCR reactions using genomic DNA were performed on a MJ Research P200 thermal cycler (MJ Research Inc., MA, USA).

The PCR reactions were performed in a mix containing 2.5 mM $MgCl_2$, 0.2 units per reaction of *Taq* polymerase in Gibco PCR buffer (Gibco BRL, NY, USA), 0.4 mM of each primer, 0.2 mM of each dNTP and approximately 30 pg of template DNA in a total reaction volume of 10 μl. After an initial denaturing step for 4 min at 95˚C, amplification comprised of 35 cycles of 30 s at 94˚C, 45 s at either 45˚C (*D10S215*) or 51˚C (*D10S541* and *D10S579*) and 1 min at 72˚C. Final elongation was performed for 5 min at 72˚C. The forward sequence of each primer pair was labelled with a fluorescent dye (LI-COR, IR-Dyes) at its 5' end; the IR-Dye700 was used for *D10S579* and the IR-Dye800 for *D10S215* and *D10S541* primers. Electrophoresis and detection of PCR products were carried out on denaturing polyacrylamide gels (6.5% acrylamide-bisacrylamide 38:2, 25 cm) using a LI-COR DNA analyzer. Gels were run for 1.5h at 1500V in TBE buffer. The resulting electrophoregrams were analysed with the SAGA software (LI-COR, NE, USA).

Estimation of heterozygosity. Statistical analysis of the genetic data was performed with the FSTAT v2.9.3.2 software (28). The total number of detected alleles (N_A), the average observed and expected heterozygosity within population (H_O and H_S), the unbiased estimator of Nei (29) and the total gene diversity (H_T) were calculated for all loci in each patient. Data obtained from each group of patients were processed as sub-populations, while healthy controls were excluded form this analysis.

Sequencing analysis. Sequencing at the *PTEN* exons was performed using specific primers. The primers used for the amplification of *PTEN* exons were as follows: 5'-CAGAAGCCCCGCCACCAG-3' forward and 5'-AGAGGAGCAGCCGCAGAAA TG-3' reverse primer for exon 1, 5'-TATTCTGAGGTTATCTTTTA-3' forward and 5'-CCTTTCCAGCTTTACAGTGAA-3' reverse primer for exon 5A, 5'-GCTAAGT GAAGATGACAATCA-3' forward and 5'-AGGA AAAACATCAAAAAATAA-3' reverse primer for exon 5B, 5'-CCTGTGAAATAATACTGGTAT-3' forward and 5'-CTCCCAATG AAAGTAAAGTAC-3' reverse primer for exon 7, and 5'-TTCATT TTAAATTTTCTTTCT-3' forward and 5'-TGGTGTTTTATCC CTCTTG AT-3' reverse primer for exon 9. PCR reactions using genomic DNA were performed on a MJ Research P200 thermal cycler (MJ Research Inc., MA, USA).

The PCR reactions were performed in a mix containing of 2.5 mM $MgCl_2$, 0.5 units per reaction of *KAPA2G* Robust DNA polymerase, 0.25 mM of each primer, 0.2 mM of each dNTP and approximately 30 pg of template DNA in a total reaction volume of 25 μl. After an initial denaturing step for 3 min at 98˚C, amplification comprised of 35 cycles of 30 s at 98˚C, 45 s at either 50˚C (exon 5) or 60˚C (exon 1, exon 7 and exon 9) and 1 min at 72˚C. Final elongation was performed for 7 min at 72˚C. The PCR products were purified with the PCR Purification Kit (NoucleoTrap). The sequencing analysis was performed by the VBC-BIOTECH Service. Sequencing results were translated and aligned against the native sequence using the proteomic tools of ExPASy Bioinformatics Resource Portal. All detected mutations were searched in the COSMIC catalogue of somatic mutations in cancer (available at: http://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=PTEN&ss=all&src=tissue&in=t&sh=all&sn=breast&hn=all).

Electrophoresis and immunoblotting. Proteins were isolated from Buffy Coat using TRI Reagent as previously described (30) and were subsequently analyzed by western blotting. Total protein concentration was determined by the Bradford method (31) using bovine albumin as standard. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 8.5% (w/v) polyacrylamide gels, as described by Laemmli (32). Proteins were transferred to nitrocellulose membranes following the method of Towbin *et al*. (33) and immunostained with the rabbit polyclonal antibodies against PTEN and PTEN-P. Antibodies were purchased from R&D Systems (R&D Systems, MN, USA) and were used according to the manufacturer's instructions in 1 μg/ml for anti-PTEN-P and 0.1 mg/ml for anti-PTEN. The anti-PTEN-P was raised against a phosphopeptide containing S380 at the C-terminal regulatory domain of human PTEN.

Considering that immunoblotting is a semi-quantitative technique, a standard procedure was applied to ensure for detection of differences in protein expression levels between different groups. A standard sample was used as an internal control in all electrophoresis and subsequent blots. The duration of transfer as well as the incubation and chromogen development times were kept constant. Electrophoresis and staining with silver nitrate was performed for all samples prior to immunoblotting to ensure that equal amounts of proteins were added in each lane. The intensity of each band was estimated using Gel-Pro Analyzer (version 3, Media Cybemetics, 1993-97). Based on their staining intensity, PTEN and PTEN-P protein expression levels were scored into four groups as follows: (–) negative; (+) weak; (++) moderate; and (+++) strong (34).

Immunohistochemistry. Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded tissue sections from 34 specimens with breast cancer and precursor lesions of the breast. PTEN tissue expression was determined using the corresponding mouse monoclonal antibody [NCL-PTEN, Clone 28H6] (Novocastra Labs Ltd, UK) at a dilution of 1/100, after 60 min incubation at room temperature. The staining was visualized with 3,3'-diaminobenzinide tetrahydrochloride followed by counterstaining with hematoxylin. Antigen retrieval was achieved in Dako PTLink [DAKO (3 in 1)] buffer, pH 9.0, for 15 min.

Immunohistochemical assessment was performed both in the lesion and the adjacent normal breast tissue independently by two observers,. Both PTEN intensity and percentage of positively-stained cells were examined for the scoring of PTEN expression as: (–) negative; (+) weakly-positive; (++) moderately-positive and (+++) strongly-positive.

Results

Microsatellite analysis and heterozygosity estimation in the 10q23 region. Microsatellite analysis was performed for the detection of heterozygosity in the 10q23 region using

genomic DNA, isolated from peripheral blood samples of patients and healthy individuals. Three microsatellite markers (*D10S215, D10S541* and *D10S579*), known to map to the 10q23 interval, were investigated and the PCR-detected genetic variants were used for the characterization of each sample as homozygous or heterozygous (Table I). Even though the majority of both patients and controls were heterozygous, a markedly higher percentage of homozygous cases was observed in breast disease patients, compared to healthy controls (31.7% and 10.0%, respectively) (Table II). Further analysis was performed in order to estimate the observed heterozygosity (H_O), expected heterozygosity (H_S) and total gene diversity (H_T) according to Nei's estimation, in breast disease samples. Typically, values range between 0 (total homozygosity) and 1 (total heterozygosity), and the difference between the observed and the expected heterozygosity represents the loss of heterozygosity (LOH). Our data indicate that the observed heterozygosity (H_O) is lower than the corresponding expected value (H_S) for all three loci investigated as well as overall, suggesting the presence of LOH in breast carcinoma and precursor breast lesions (Table III). Among the investigated loci, the *D10S215* locus was shown to be the less polymorphic.

Mutation analysis in exons 1, 5, 7 and 9. Exon 5, encoding the catalytic region of PTEN was amplified, as previously described. In total, 34 different mutations were detected in this exon, which were all single-base alterations. Amongst them, 1 was a non-sense mutation leading to an immediate stop, 8 were missense point mutations, 8 were insertions, 15 were deletions and 2 were silent mutations (Table IV). It is worthy to note that 14 of the 34 mutations (41.2%) in exon 5 are predicted to truncate the PTEN protein downstream the mutation point (Figure 1).

A T deletion on base 324 of exon 5 was the single-nucleotide polymorphism (SNP) with the highest rate (32/43) among all the detected alterations, which however has no effect on the protein's amino acid sequence (L108L). The other two more frequent alterations were a G deletion on base 477 and a T deletion on base 461 (11/43 and 8/43, respectively). The G deletion also results in no alteration of the amino acid sequence (R159R). On the contrary, the T deletion has a significant effect on amino acid 154 (F154S), resulting in the production of a truncated form of the PTEN protein downstream of the mutation point (Figure 1). Surprisingly, no mutations in the protein tyrosine phosphatase (PTPase) core motif were detected.

It has been reported that the combination of specific variations in exons 1, 7 and 9 reduces the membrane affinity of the PTEN protein, thereby affecting its activity (34). To further investigate this, a total of 14 breast disease samples with high expression levels of PTEN and/or PTEN-P were selected and were subjected to sequencing analysis of exons 1, 7 and 9. In total, 21 different mutations were detected in exon 1 (Table V), exon 7 (Table VI) and exon 9 (Table VII). Eight of them were missense point-mutations, 2 were insertions, 10 were deletions and one was a silent mutation. Out of the 21 mutations identified, 9 (42.9%) are predicted to truncate the PTEN protein downstream of the mutation point.

Exon 1 encodes the PIP2 binding motif at residues 1-15, which intrinsically binds to the catalytic site to shield it from substrate in the membrane (34-35). A T deletion on base 68 was the most frequent mutation (12/14), leading to a stop codon (L23STOP) (Table V) (Figure 2). A T deletion on base 62 and a G deletion on base 58 also showed high mutation rates (10/14 and 9/14, respectively) (Table V). Their predicted effects are exerted on amino acids 21 and 20 respectively (F21S and G20D), and both result in truncated forms of the PTEN protein downstream of the mutation points (Figure 2). It is noteworthy that we identified two mutations in codon F21, the F21S and F21Y (Table V). This codon and in particular the mutation F21S has been reported to be crucial for the cellular localization of the PTEN protein (35). We also identified the K13E mutation, which has also been suggested as a human cancer mutation (36). Interestingly, the PTEN protein is modified by ubiquitin in this particular codon (37).

Exon 7 encodes for calcium binding region 3 (CBR3) loop at residues 160-169, which is critical for the association of the C-terminal fragment with the rest of the protein. Mutations in the CBR3 loop have been shown to completely disrupt this association (34). Among the detected mutations, three were characterized by high mutation rates (13/14). These are a G insertion on base 635, a T deletion on base 770 and a T deletion on base 773, with predicted effects on amino acids 212, 257 and 258, respectively (N212R, F257S and F258S, respectively) (Table VI). All three mutations truncate the PTEN protein downstream of the mutation points (Figure 3). It is important to note that no mutations were detected within the CBR3 region.

Exon 9 encodes the PDZ domain at residues 401-403, and is necessary for PTEN protein interactions (34). The most frequently detected mutation was a T deletion on base 1200 (8/14), with no effect on amino acid 400 (I400I). We also observed an A to G substitution on base 1201, exerting a significant effect on amino acid 401 (T401R) (Table VII, Figure 4). This latter mutation may be of major importance, since this residue is involved in PTEN protein interactions (34).

Determination of circulating PTEN and phosphorylated PTEN protein levels. Circulating levels of the PTEN and phosphorylated PTEN (PTEN-P) protein were analyzed by western blotting in samples from 43 patients with breast disease and 10 healthy individuals. Figure 5 shows a set of samples subjected to silver staining and immunoblotting for

Table I. *PCR products (bp) representing alleles for each loci*.

Sample number	Diagnosis	*D10S215*		*D10S541*		*D10S579*		Homozygous/ Heterozygous
		Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	
1	BrCa	153	153	247	249	265	265	Heterozygous
2	BrCa	NA	NA	NA	NA	NA	NA	-
3	BrCa	157	157	255	255	267	267	Homozygous
4	BrCa	153	161	245	271	267	269	Heterozygous
5	BrCa	153	161	245	259	267	269	Heterozygous
6	BrCa	157	157	249	251	267	269	Heterozygous
7	BrCa	153	161	245	249	267	269	Heterozygous
8	BrCa	157	157	245	245	265	267	Heterozygous
9	BrCa	155	157	255	265	267	267	Homozygous
10	BrCa	153	153	257	257	NA	NA	Homozygous
11	BrCa	NA	NA	255	267	265	267	Heterozygous
12	BrCa	157	157	247	247	265	267	Heterozygous
13	BrCa	153	153	247	247	265	265	Homozygous
14	BrCa	155	155	245	245	265	265	Homozygous
15	BrCa	155	159	247	273	267	273	Heterozygous
16	BrCa	153	153	259	259	267	269	Heterozygous
17	BrCa	153	153	247	257	267	267	Heterozygous
18	BrCa	153	153	245	245	267	267	Homozygous
19	BrCa	153	161	255	271	269	269	Heterozygous
20	BrCa	157	157	249	269	267	269	Heterozygous
21	BrCa	153	153	245	271	267	269	Heterozygous
22	BrCa	151	155	249	267	269	269	Heterozygous
1	Precursor	153	159	245	269	265	267	Heterozygous
2	Precursor	153	161	249	257	265	267	Heterozygous
3	Precursor	155	155	245	245	265	265	Homozygous
4	Precursor	157	157	247	247	267	267	Homozygous
5	Precursor	153	153	259	259	267	267	Homozygous
6	Precursor	153	153	245	245	267	267	Homozygous
7	Precursor	153	153	249	275	267	267	Heterozygous
8	Precursor	147	147	245	249	267	269	Heterozygous
9	Precursor	151	153	245	255	265	273	Heterozygous
10	Precursor	155	155	245	245	265	265	Homozygous
11	Precursor	155	155	269	269	267	267	Homozygous
12	Precursor	153	153	245	245	267	267	Homozygous
13	Precursor	157	157	245	249	265	267	Heterozygous
14	Precursor	153	153	245	267	265	267	Heterozygous
15	Precursor	NA	NA	NA	NA	NA	NA	-
16	Precursor	NA	NA	245	249	265	273	Heterozygous
17	Precursor	153	153	259	259	267	269	Heterozygous
18	Precursor	155	155	245	247	265	267	Heterozygous
19	Precursor	155	157	245	267	269	269	Heterozygous
20	Precursor	153	153	245	249	269	271	Heterozygous
21	Precursor	155	155	245	267	267	267	Heterozygous
1	Healthy	157	157	245	245	267	269	Heterozygous
2	Healthy	147	147	245	245	267	269	Heterozygous
3	Healthy	147	147	245	255	267	267	Heterozygous
4	Healthy	155	155	245	245	267	267	Homozygous
5	Healthy	153	153	245	249	269	269	Heterozygous
6	Healthy	153	153	247	269	265	273	Heterozygous
7	Healthy	153	155	245	255	265	267	Heterozygous
8	Healthy	153	153	245	259	265	267	Heterozygous
9	Healthy	151	153	247	247	265	273	Heterozygous
10	Healthy	153	153	247	247	265	267	Heterozygous

Table II. *Percentage of homozygous and heterozygous samples among patients with breast disease (breast cancer or precursor breast lesions) and healthy controls.*

	Breast disease (n=41)	Healthy (n=10)
Homozygous	31.7%	10.0%
Heterozygous	68.3%	90.0%

Table III. *Nei's estimation of observed heterozygosity (H_O), expected heterozygosity (H_S), total gene diversity (H_T) and loss of heterozygosity (LOH) for patients with breast disease (breast cancer and precursor lesions of the breast).*

Locus	H_O	H_S	H_T	LOH
D10S215	0.283	0.717	0.724	0.434
D10S541	0.601	0.855	0.842	0.254
D10S579	0.515	0.649	0.646	0.134
Total	0.466	0.740	0.737	0.274

PTEN and PTEN-P. Our immunostaining data demonstrate a more pronounced expression of both PTEN and PTEN-P in patients with breast disease, compared to healthy controls. More specifically, high expression of PTEN was observed in 58.1% (25 of 43) of patients with breast disease, while in only 10.0% (1 of 10) of healthy individuals (Figure 6A). Similarly, expression of phosphorylated PTEN was higher in patients compared to controls, even thought in a smaller percentage of participants (34.9%, (15/43) and 20.0%, (2/10), respectively) (Figure 6B).

Further analysis indicates that both the de-phosphorylated and phosphorylated forms of the PTEN protein exhibit slightly higher expression in patients with precursor lesions of the breast, compared to those with breast cancer (Table VIII). More specifically, moderate and strong expression of PTEN was detected in 54.5% (12 of 22) of patients with breast cancer and 61.9% (13 of 21) of patients with precursor breast lesions. Moderate and strong PTEN-P expression was observed in 31.8% (7 of 22) of breast cancer patients and 38.1% (8 of 21) of women with precursor lesions.

Determination of tissue PTEN protein expression. Immunohistochemistry was applied in 34 breast-disease and adjacent normal tissue specimens for the determination of tissue expression of the PTEN protein. Markedly increased expression of PTEN was observed in both breast disease and normal tissue, with the vast majority of specimens exhibiting moderate and strong expression levels (97.1%; 33 of 34 and 96.9%; 31 of 32, respectively). Increased PTEN expression was detected in 95.2% (20 of 21) of breast cancer specimens and in 94.7% (18 of 19) of adjacent normal tissue samples. As far as precursor lesions of the breast are concerned, moderate and strong PTEN expression was detected in all lesion (100%, 13 of 13) and normal tissue (100%, 13 of 13) specimens (Table IX).

Table IV. *Summary of mutations in the 5th exon of pten.*

Number of samples	Position	Mutations	Predicted effect
32/43	324	T deletion	L108L
1/43	333	G deletion	W111C
1/43	334	C to T	L112L
4/43	339	T deletion	S113R
3/43	340	G deletion	E114aK
1/43	340	G to A	E114bK
1/43	342	T insertion	E114D
3/43	346	G deletion	D116T
1/43	358	G to T	A120Q
1/43	411	G insertion	A137A
1/43	420	A to C	L140F
2/43	437	T deletion	L146STOP
1/43	440	A insertion	K147K
1/43	440	A deletion	K147R
1/43	443	C deletion	A148D
1/43	454	C to G	L152V
1/43	456	A to G	L152L
1/43	457	C insertion	D153A
1/43	457	G to A	D153N
1/43	459	T to A	D153E
8/43	461	T deletion	F154S
5/43	462	C deletion	F154F
1/43	463	T insertion	Y155L
1/43	465	T to A	Y155STOP
2/43	468	G deletion	G156G
2/43	469	G deletion	E157K
3/43	469	G to A	E157K
2/43	471	A insertion	E157E
1/43	475	A insertion	R159R
1/43	476	G to A	R159K
11/43	477	G deletion	R159R
1/43	483	A deletion	R161R
1/43	484	G deletion	D162T
1/43	486	C or A insertion	D162D OR E

*Mutations are indicated in the sense orientation.

Discussion

It is well-known that regulation of the PI3K/Akt pathway is crucial for the initiation and progression of tumorigenesis. PTEN plays an important role in tumor suppression through de-phosphorylation of PIP3 (38), thereby inhibiting the PI3K-dependent activation of Akt. The PI3K signalling pathway is frequently de-regulated in human solid tumors,

Table V. *Summary of mutations in the 1th exon of PTEN.*

Number of samples	Position	Mutations	Predicted effect
2/14	37	A to G	K13E
1/14	51	A insertion	Q18E
9/14	58	G deletion	G20D
1/14	60	A deletion	G20G
10/14	62	T deletion	F21S
1/14	62	T to A	F21Y
1/14	66	C to A	D22E
1/14	67	T to G	L23V
12/14	68	T deletion	L23STOP

*Mutations are indicated in the sense orientation.

Table VI. *Summary of mutations in the 7th exon of PTEN.*

Number of samples	Position	Mutations	Predicted effect
13/14	635	G insertion	N212R
13/14	770	T deletion	F257S
13/14	773	T deletion	F258S
2/14	778	A to G	K260E
2/14	788	A deletion	K263R
4/14	792	G deletion	M264I

*Mutations are indicated in the sense orientation.

Table VII. *Summary of mutations in the 9th exon of PTEN.*

Number of samples	Position	Mutations	Predicted effect
1/14	1134	A to T	R378S
1/14	1136	A to T	Y379F
8/14	1200	T deletion	I400I
1/14	1200	T to A	I400I
1/14	1201	A to G	T401R
2/14	1206	A deletion	K402K

*Mutations are indicated in the sense orientation.

Table VIII. *Circulating expression of PTEN and phosphorylated PTEN protein in patients with breast cancer or precursor breast lesions, determined by western blotting.*

	Expression	Breast cancer (n=22)	Precursor breast lesions (n=21)
PTEN	Negative/Weak	45.5%	38.1%
	Moderate/Strong	54.5%	61.9%
PTEN-P	Negative/Weak	62.8%	61.9%
	Moderate/Strong	31.8%	38.1%

Table IX. *Tissue expression of PTEN in patients with breast cancer or precursor breast lesions and adjacent normal tissue, determined by immunohistochemistry.*

PTEN tissue expression	Breast cancer (n=21)	Adjacent normal (n=19)	Precursor breast lesions (n=13)	Adjacent normal (n=13)
Negative/Weak	4.8%	5.3%	0	0
Moderate/Strong	95.2%	94.7%	100%	100%

including breast cancer, through Akt1 or PIK3CA (catalytic subunit of PI3K) mutations, HER2 overexpression and PTEN loss or mutation (39-42). In the present study, we investigated the heterozygosity in the 10q23 interval, where the *PTEN* gene is located, as well as the *PTEN* mutation spectrum in patients with breast cancer and benign lesions of the breast. In addition, expression levels of the phosphorylated and de-phosphorylated forms of the PTEN protein were determined in peripheral circulation and breast tissue.

We used microsatellite genetic markers to amplify 3 specific loci of the 10q23 region that have shown considerable allele loss in primary breast carcinoma (25-27). Our data indicate an increased number of homozygous patients with breast disease, compared to healthy controls. Moreover, significant deviation of the observed heterozygosity H_O from its corresponding expected value H_S has been estimated, suggesting an association between decreased heterozygosity in the PTEN region and malignant and benign breast disease. Our data are supported by previous studies, reporting LOH in the 10q23 region in approximately 30-41% of breast cancer cases (15-16, 43-44). Moreover, we present evidence that among the investigated loci, the D10S215 polymorphic marker exhibits the greatest homozygosity. Existing data have correlated loss of heterozygosity in the region near D10S215 with breast carcinomas of poor prognosis (15).

Analysis of LOH typically involves genomic DNA extracted from tumor tissue. In our study, DNA was extracted from peripheral blood samples of breast disease patients and healthy individuals, consisting mainly of germline DNA but also containing small quantities of DNA released by apoptotic and necrotic tumor cells. According to a recent study, there is almost complete concordance between germline and matched somatic DNA in variants of pharmacogenetic genes applied in cancer research (45). Complete concordance between germline and somatic DNA has also been observed in genes involved in angiogenesis and pathogenesis of breast cancer (46-47). Therefore, DNA obtained through the minimally-invasive method of blood sampling can be regarded as a reliable source for the study of LOH.

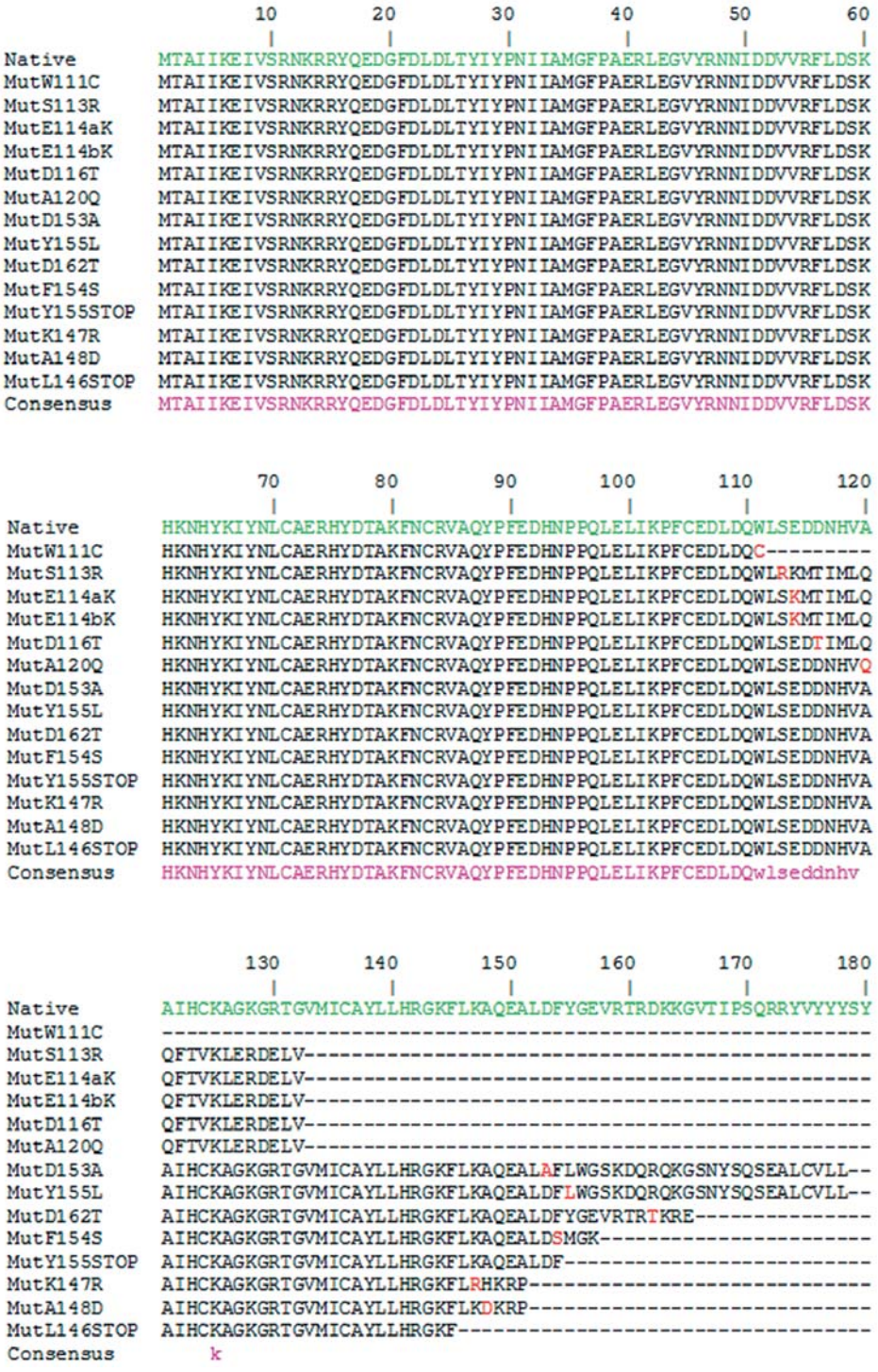

Figure 1. *Alignment of exon 5 mutative sequences.*

Several studies demonstrate that mutations in the *PTEN* gene are closely associated with the development of breast cancer, but the mutation frequency is not necessarily reflected in the expression levels of the PTEN protein (48). It is well-known that mutations in the catalytic domain, located in exon 5, reduce the lipid and protein phosphatase activity of the PTEN protein (35). We attempted to investigate the mutation spectrum in exon 5, the most

Figure 2. *Alignment of exon 1 mutative sequences*.

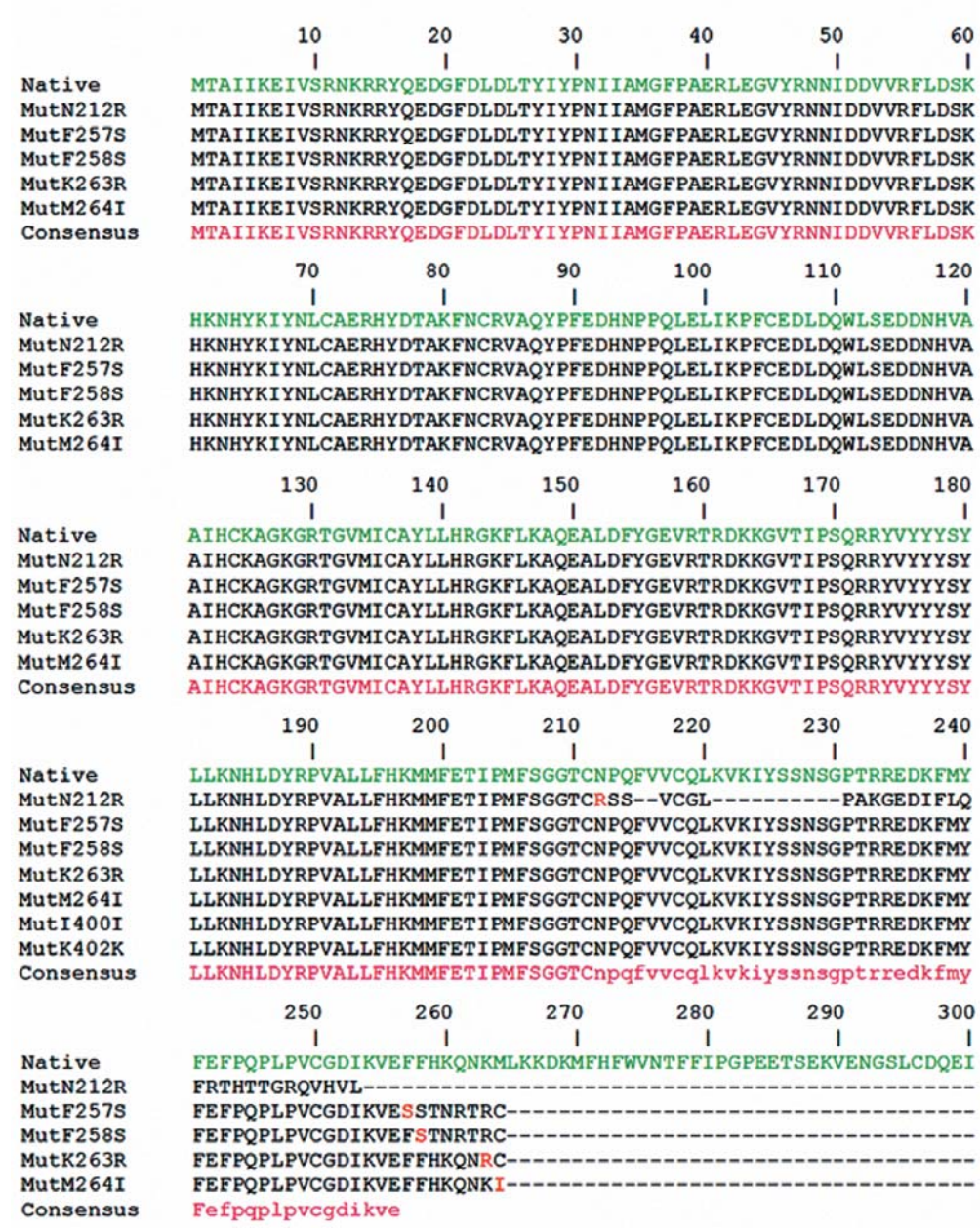

Figure 3. *Alignment of exon 7 mutative sequences*.

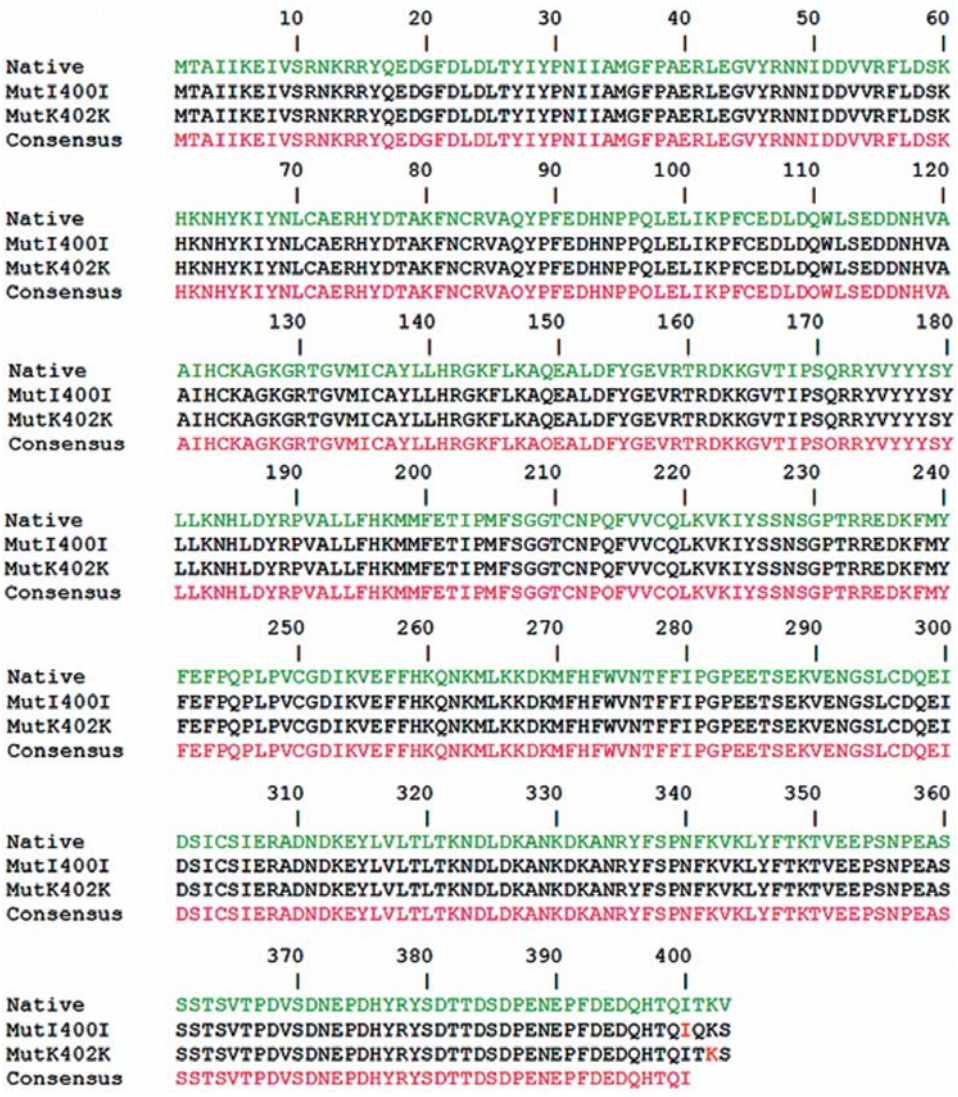

Figure 4. *Alignment of exon 9 mutative sequences.*

frequently mutated exon of the *PTEN* gene, in benign and malignant breast disease. We observed several mutations in exon 5, but none of them in the catalytic core motif. However, several alterations that truncate the PTEN protein downstream of the mutation points were observed in a significant percentage of samples (19/43; 44.2%). The mutation frequency was higher in patients with invasive or *in situ* breast cancer (13/22; 59.1%), compared to those with benign lesions (6/21; 28.6%). These mutations may have deleterious effects on the protein function with serious subsequent implications in its activity. Thus, our data support the presence of mutations in exon 5 of the *PTEN* gene that may potentially provide a mechanism of PTEN loss of activity in breast cancer.

Analysis of mutations in exons 1, 7 and 9 is meaningful considering scientific evidence indicating that the combination of specific domains in these three exons is required for membrane affinity of the PTEN protein (34-35). We observed various crucial mutations in the aforementioned exons in samples characterized by increased expression of PTEN-P or PTEN. Several mutations leading to truncated forms of the protein have been detected, mainly in patients diagnosed with invasive or *in situ* breast cancer. Amongst them, four mutations (G20G, K263R, M264I and K402K) were selectively observed in breast cancer samples characterized by increased levels of PTEN-P. These data suggest that specific mutations in these exons of the *PTEN* gene may be associated with increased phosphorylation of PTEN in breast cancer.

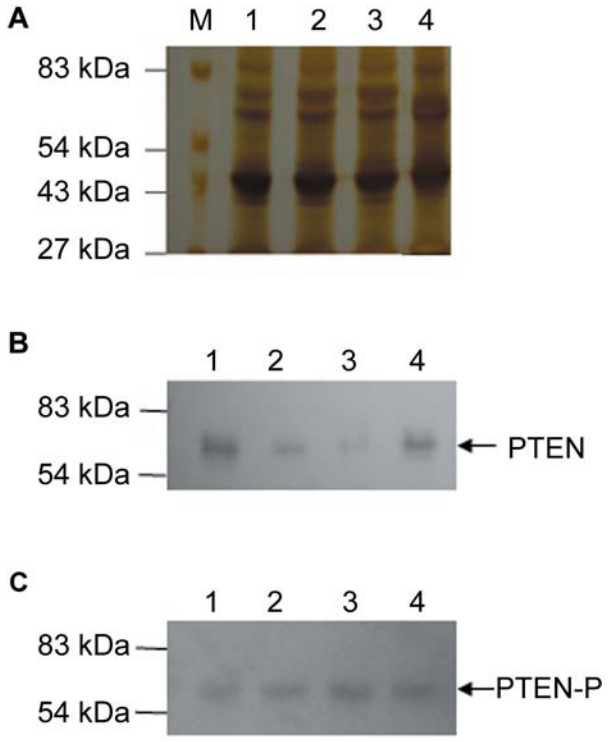

Figure 5. *Circulating PTEN and PTEN-P protein levels. A) Staining with silver nitrate, B) western blotting with anti-PTEN, C) western blotting with anti-PTEN-P. The samples were from patients with precursor breast lesions, atypical ductal hyperplasia (lanes 1, 2 and 4) or lobular neoplasia (lane 3).*

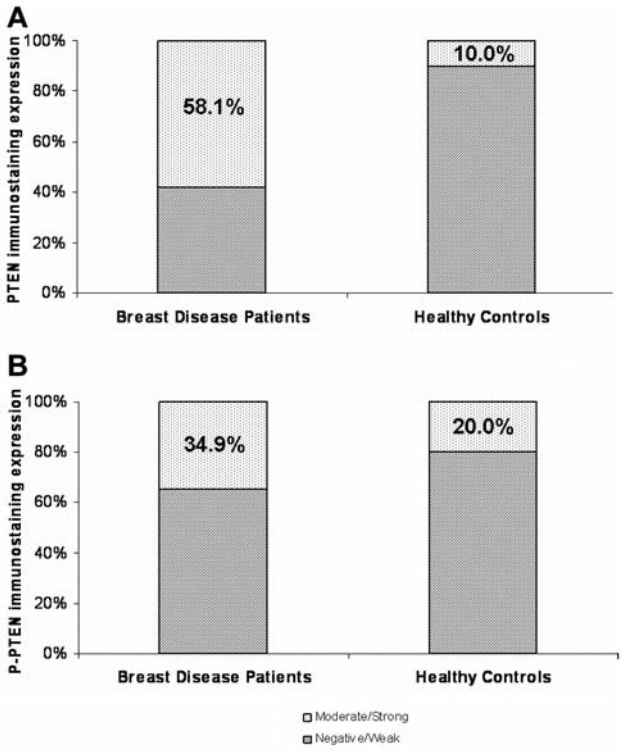

Figure 6. *Circulating expression of (A) PTEN and (B) phosphorylated PTEN protein in patients with breast disease (breast cancer and precursor breast lesions), compared to healthy controls, determined by western blotting.*

Interestingly, many of the mutations we observed in our samples have been previously associated with several types of cancer. More specifically, the mutations p.37A>G (49), p.454C>G (50), p.461Tdeletion (51) and p.476G>C (52) have been observed in glioblastoma. The mutations p.60Adeletion (53), p.67T>G (54), p.773Tdeletion (55) and p.437Tdeletion (56) have been associated with endometrial carcinoma, while the mutations p.457G>A (57) and p.469G>A (58) with colorectal cancer. Thus, we provide evidence that mutations previously observed in other types of cancer are also detected in carcinoma of the breast, potentially suggesting a common mechanism of PTEN action.

To our knowledge, this is the first study attempting to determine circulating levels of the PTEN protein, either in its de-phosphorylated or phosphorylated forms, in breast cancer. Our immunostaining results demonstrate increased expression of the PTEN protein in the systemic circulation of patients with breast cancer and precursor lesions of the breast, compared to healthy controls. In support, moderate and strong PTEN protein expression is observed at the tumor site and adjacent normal tissue in almost all breast disease specimens, suggesting that there is no loss of PTEN expression. Several studies have previously reported reduced PTEN expression in breast cancer (43, 59-62). However, our results are in agreement with those of Qi *et al*., who also failed to detect loss of PTEN expression in invasive and *in situ* ductal breast carcinoma (63). Loss of PTEN function has been attributed to either down-regulation or absence of PTEN expression, or to protein inactivation through post-translational protein modifications (12, 64). As previously reported, phosphorylation of PTEN in specific residues converts the molecule into an inactive form of closed conformation (13). Vazquez *et al*. demonstrated that the C-terminal domain of the PTEN protein is necessary for maintaining protein stability and also acts to inhibit PTEN function. In addition, this domain is capable of regulating PTEN function through phosphorylation of three specific residues (65). Therefore, phosphorylation of PTEN would restrict its activity, while de-phosphorylation would result in an increase in protein activity and in its rapid degradation. Since phosphorylation converts the molecule into its inactive but stable form, it is suggested that normal cells maintain PTEN in the phosphorylated form and activate it when needed.

Within our study, we also determined circulating expression levels of the PTEN protein, phosphorylated at residue S380 of the C-terminal regulatory domain. We were unable to investigate the corresponding tissue expression due

to unavailability of appropriate antibodies. According to our results, moderate and strong expression of PTEN-P seems to be more pronounced in patients with breast disease compared to healthy controls, suggesting that phosphorylation is a potential mechanism of protein inactivation in breast cancer. Nevertheless, increased expression of PTEN appears to be mostly associated with the de-phosphorylated form of the protein. Thus, it is likely that PTEN is inactivated either through phosphorylation at another residue or through other post-translational modifications, like acetylation or ubiquitination.

Our data analyzing the PTEN mutation spectrum, heterozygosity and protein expression in benign and malignant conditions of the breast, give further insight on the role of PTEN in breast carcinogenesis. Altogether, our results indicate that breast disease is associated with decreased heterozygosity and the presence of several mutations at the *PTEN* gene, while no correlation with loss of PTEN expression is revealed. Breast cancer appears to be associated with a high frequency of mutations that truncate the corresponding protein, suggesting a potential mechanism of PTEN loss of activity in breast malignancy. Complementing the current findings with further studies incorporating a larger series of samples will allow for the assessment of statistically significant associations and the investigation of potential mechanisms of protein inactivation associated with the presence of disease. In addition, the data obtained so far provide good evidence that peripheral blood can be effectively used for the investigation of *PTEN* gene mutations and its protein expression, offering the additional advantage of non-invasive sample collection at any time during the course of disease as well as during its management.

Conflicts of Interest

None to declare.

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