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AN INTRONIC VARIANT IN *PTEN* GENE IS PROBABLY INVOLVED IN SPLICING ALTERATION EVENTS.

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ABSTRACT

PTEN is a gate-keeper tumor-suppressor gene involved in various cell cycle pathways. Germline mutations of one allele of PTEN were found associated with Cowden syndrome, which is an inherited disease characterized by multiple hamartomas. PTEN is frequently inactivated also by somatic mutations in various array of human tumors. In this study,a cohort of 50 Sicilian breast cancer patients, negative to screening for pathogenic mutations in BRCA1/2 genes, was analyzed. Variant c.492+46 G>A (rs775537003) in intron 5 of *PTEN* gene was identified. The possible role of this variant in splicing mechanisms was evaluated by in silico analysis. Alteration of an ESE and ESS site was found. This is a preliminary study with the aim to investigate about the involvement of *PTEN* genein the breast cancer and the possible role of splicing alteration events into the risk of the disease.

1. Introduction

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a tumor-suppressor gene which encodes a major lipid 3-phosphatase. This protein signals down the PI3 kinase/AKT proapoptotic pathway and various cell-survival pathways, such as the mitogen-activated kinase (MAPK) pathway. The role of *PTEN* mainly consists in the ability to dephosphorylate and degrade PIP3 phospholipid of membrane, and consequently inhibit serine/threonine kinase oncogenic AKT [1].Furthermore, *PTEN* coordinates G1 arrest through up-regulation of p27, an inhibitor of cyclin-dependent kinases, and acts as a negative regulator of the cell cycle and concomitant down-regulation of cyclin D1[2].Originally germline mutations of one allele of *PTEN* were found associated with Cowden syndrome (CS), which is an inherited disease characterized also by hamartomatous gastrointestinal polyposis that can evolve into early-onset esophagogastric cancer. [3-5].

PTEN is frequently inactivated by somatic mutations in a wide array of human tumors. Selective inactivation of *PTEN* in the developing brain causes defects in the neuronal migration, synapses formation and stabilization, and progression of glioblastoma multiforme (GBM), one the most malignant astrocytic tumor [6]. The astrocytes play an important role in several different biologic activities and the *PTEN* loss can increase the proliferation of astrocytes in vivo [7].

Previous studies have shown that glial fibrillary acidic protein (GFAP) co-localizes with a wide range of proteins expressed in astrocytes, as sarcoglicans and distrophin complex**.** The loss of some elements of these complex could be cause of the loss of structural plasticity of the synapses resulting in a tumor progression.

Other neoplastic diseases in which PTEN takes part is prostate cancer; however, in this case, mechanism involves the glyoxalases (GLOs) [8]**,** a class of enzymes involved in cellular detoxification from Reactive Oxygen Species (ROS); moreover, polymorphisms in GLO genes were associated to several pathological conditions affecting Central Nervous System and retina [9-11]. Their role in breast cancer is, instead, controversial [12-13]**.** Importantly, the reduced expression of *PTEN* is not only due to loss of heterozygosity (LOH) but also to epigenetic mechanisms, as methylation of promoter [14-15]. This phenomenon was also observed in endothelial cells derived from Cerebral Cavernous Malformations (CCMs) sporadic lesions [16]. CCMs are benign neo-formations derived from angiogenetic impairment due to mutations at three loci [17-19]. PTEN/VEGF signaling modulates the three CCM proteins activity [20].

In present study on a cohort of 50 Sicilian breast cancer patients, negative to screening for pathogenic mutations in BRCA1/2 genes, the entire coding sequence of *PTEN* gene was analyzed in order to evaluate the role of this gene in the disease.

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2. Methods

Patients

The peripheral blood samples were collected from 50 women (over 50 years of age) living in Sicily. Before recruitment, each patient was asked to sign an informed consent form. No adjuvant chemotherapy was administered. We have no information on treatment with radiation and or endocrine therapy.

The study followed the tenets of the Declaration of Helsinki and was approved by the Scientific Ethics Committee of the Azienda Ospedaliera Universitaria-Policlinico "G. Martino" Messina.

Molecular analysis

Primers and polymerase chain reaction (PCR). The heparinized peripheral blood samples were collected from breast cancer patients. Genomic DNA was extracted from lymphocytes by kit QI Amp DNA Blood Mini Kit (QIAGEN®).

The nucleotide sequence of primers employed for PCR reaction isshown in Table 1.

Gene	Exon	Sequence primer	Amplicon size (bp)	Ta
PTEN	1	PF: 5'-AAGTCCAGAGCCATTTCCATC-3' PR: 5'-AACTACGGACATTTTCGCATC-3'	261	59.5°C
PTEN	2	PF: 5'-AGTTTGATTGCTGCATATTTC-3' PR: 5'-GAAGTCCATTAGGTACGGTAA-3'	300	56,5°C
PTEN	3	PF: 5'-TGTAATTTCAAATGTTAGCTC-3' PR: 5'-TCTTGGACTTCTTGACTTAAT-3'	258	53,5°C
PTEN	4	PF: 5'-GTGATAACAGTATCTACTTAAT-3' PR: 5'-TGACAGTAAGATACAGTCTAT-3'	284	54,5°C
PTEN	5	PF: 5'-CTGTTAAGTTTGTATGCAACA-3' PR: 5'-ACTTGTCAATTACACCTCAAT-3'	451	54,5°C
PTEN	6	PF: 5'-CCAGTTACCATAGCAATTTAGT-3' PR:5'- CTGTTCCAATACATGGAAGGA-3'	266	58,5°C
PTEN	7	PF: 5'-ATTGCAGATACAGAATCCATA-3' PR: 5'-AAA ACA CCT GCA GAT CTA ATA-3'	410	55,5°C
PTEN	8	PF: 5'-AATGTTTAACATAGGTGACAG-3' PR: 5'-TTGTCAAGCAAGTTCTTCAT-3'	426	53,5°C
PTEN	9	PF: 5'-AAGATCATGTTTGTTACAGTG-3' PR: 5'-ATCTGACACAATGTCCTATTG-3'	428	55,5°C

PF= forward; PR=reverse; Ta=annealing temperature.

Table 1. Primer sequences used for PCR and sequencing reaction

PCR reaction was carried out adding 0.8 µg of genomic DNA to 50µl reaction mixture containing a 0.3µm concentration of each primer and 1.5 U MyTaq polymerase (Bioline). It was carried out in the thermal cycler (Gene Amp PCR System 2700; PE Applied Biosystems, Foster City, CA) under following conditions: denaturation at 95°C for 15 sec, annealing for 15 sec and extension at 72°C for 15 sec for 35 cycles, after an initial 1 min denaturation at 95 °C. The final extension step at 72°C was extended to 10 minutes. Annealing temperature was optimized for each primer set as described in Table 1.

The PCR products were resolved on 1% agarose gel and visualized by ethidium bromide staining.

Sequencing was carried out on the primary amplicons purified by kit "ISOLATE II PCR and Gel Kit (BIOLINE)", using the DNA direct sequencing with the ABI PRISM® Big Dye® Terminator v3.1 Cycle Sequencing Kit on the capillary electrophoresis analyzer 3500 Dx System (Applied Biosystems).

The specificity for the detected variants was tested by NCBI GeneBank,http://www.ncbi.nlm.nih.gov/genbank/and NCBI Blast data base,http://blast.ncbi.nlm.nih.gov/Blast.cgi.

In silico analysis

Intron variant c.492+46 G>A was found andits possible role in splicing mechanisms was evaluated. The possible creation of exonic splicing silencer (ESS) and the abolition of exonic splicing enhancer (ESE) sites,as well as the abolition and creation of canonical sites and cryptic splicesites respectively, were analyzed through an insilico analysis using Human Splicing Finder software (http://www.umd.be/HSF3/HSF.html) [21].

3. Results

The mutation analysis was performed in the patients negative for pathogenic mutations in BRCA1/2 genes. The analysis of the entire coding sequence of *PTEN* gene, performed on 9 exons and partial intron sequences upstream and downstream of each exon (+/- nucleotides) identified the variant c.492+46 G>A (rs775537003) (http://www.ensembl.org/Homo_sapiens/Gene/Sequence?db=core;g=ENSG00000171862;r=10:87863113-87971930) in intron 5, a few dozen nucleotides upstream of exon 6.

Although this variant does not alter the structure of the protein, it could be involved in splicing mechanism.To evaluate the possible role of the variant in the splicing mechanism, in silicoanalysis was performed by Human Splicing Finder. The software revealed the alteration of an exonic splicing enhancer (ESE) site and an exonic splicing silencer (ESS) site. (Figure 1).

Sequence Position	cDNA Position	Enhancer motif reference sequence	Enhancer motif mutant sequence	Variation
402	+402		ctgaaa	New Site
403	+403	tgagaa		Site broken
404	+404		gaaaat	New Site

Sequence Position	cDNA Position	Silencer motif reference sequence	Silencer motif mutant sequence	Variation
406	+406	gaattt		Site broken

Figure 1. Possible alteration of the splicing process due to intronic c.492+46 G>A variant.

In addition, a reduction in SF2/ASF pattern score may be correlated with an exon-skipping phenotype associated with various disease-causing mutations, such as breast cancer (doi: 10.1093 / hmg / ddl171) (Figure 2).

Sequence Position	cDNA Position	Linked SR protein	Reference Motif (value 0-100)	Linked SR protein	Mutant Motif (value 0-100)	Variation
399	+399	SC35	gatctgag (77.75)			Site broken -100
402	+402	SF2/ASF (IgM-BRCA1)	ctgagaa (76.23)			Site broken -100
402	+402	SF2/ASF	ctgagaa (73.50)			Site broken -100

Figure 2 - Reduction in SF2/ASF pattern score due to c.492+46 G>A variant.

4. Discussion

More than 50 genes are involved in the progression of breast cancer. Over 1500 variants in two principal genes (BRCA1 and BRCA2) were listed in the Breast Cancer Information Core (BIC) [22-23].

In this study, the choice about *PTEN* is first linked to the important pathway largely controlled by this gene, to its role as gate-keeper tumor-suppressor gene, but above all because it is an haploinsufficient tumor-suppressor gene. Therefore, a single mutation can determine the production of a dysfunctional phosphatase.

Furthermore, the variant identified in *PTEN* gene is a polymorphism. To establish potential pathogenic or protective role of polymorphisms in diseases' development is very complex; while, for rare disease, few cases may be sufficient [24-27], for breast cancer, having a wide incidence, a very large cohort of patients is required. Moreover, this study was limited to *PTEN* coding regions and intron/exon boundaries and did not consider possibility of presence of variants in the promoter region that was shown may be interfere with normal gene expression [28-30]**.**

Certainly, further functional studies will be necessary to confirm the lack of activity of the aberrant protein form. Other unidentified genes should be screened in order to complete the framework in the field of the breast cancer.

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