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PTEN 32-bp Ins/Del Polymorphism in Breast Cancer: A Case-Control Study in South Indian Population

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ABSTRACT

Phospho tension homolog (PTEN) is a tumor suppressor gene that plays a pivotal role in various cellular processes. Aberrations in the expression and function of PTEN are associated with various diseases including tumor development and progression. The present study is aimed to assess the association of PTEN 32-bp Ins/Del (rs34421660) with breast cancer susceptibility. For this study, 469 breast cancer cases and 391 controls were recruited. PCR method employing a combination of primers was used to detect this polymorphism. We found a significant association between Del/Del genotype and the risk for breast cancer in the recessive model (Del/Del vs.Ins/Ins- Ins/Del: $\chi 2$; p= 0.0051**).** Likewise, the Del allele was found to be significantly elevated in cases (69.09%) as compared to controls (63.55%) ($\chi 2$; p=0.015) and no significant association was observed with epidemiological or clinical variables studied. Further, patients carrying Ins/Ins genotype showed a significant increase in median survival rate as compared to those carrying Ins/Del and Del/Del genotypes. These results indicate Del/Del genotype and Del allele as risk factors for breast cancer development. In summary, this study demonstrated a significant association between PTEN 32-bp Ins/Del polymorphism and breast cancer susceptibility in our population.

INTRODUCTION:

Phospho tensin homolog deleted on chromosome 10 (PTEN), otherwise known as MMAC1 or TEP1 was initially identified in 1997 as a *protein tyrosine phosphatase* due to its sequence homology with the catalytic domain of PTP family members and as a tumor suppressor gene. PTEN is a dual specific, plasma membrane enriched phosphatase that primarily acts on membrane lipids to remove the phosphate group from their inositol rings apart from proteins (Shaw and Cantley, 2006; Nagata *et al*, 2004). It acts as a negative regulator of PI3K-AKT-mTOR signaling pathway that plays a pivotal role in cell growth, survival, differentiation, motility, etc. by dephosphorylating PIP3-a membrane lipid (Maehama and Dixon; 1998; Stambolic *et al*, 1998).

PTEN is located on 10q23.3 chromosomal locus, which is found to be highly susceptible to genetic alterations (Sondka et al, 2018). It is a haploinsufficient tumor suppressor gene which indicates that even 50% loss of PTEN function is enough to promote tumor formation while further loss accelerates tumor progression (Alimonti *et al*, 2010). Bi-allelic mutations in PTEN account for about 50% frequency and commonly occur during the progression of various malignancies including breast (Simpson and Parsons, 2001; Yamada and Araki, 2001).

Gene encoding PTEN contains 9 exons, of which exon 5 encoding phosphatase domain, exon 7 and 8 harbors most of the germline mutations (Bonneau and Longy, 2000). PTEN is a 55kDa protein comprising 403 amino acids. PTEN protein structure comprises five functional domains (Steck *et al*, 1997), which includes a short N- terminal PIP2- binding domain (PBD), a catalytic phosphatase domain, a C2 lipid or membrane-binding domain, C-terminal tail, and class I PDZ domain-binding motif (Lee *et al*, 1999). Translational variants namely PTEN-L, M, N, and O with different N-terminal extensions are generated due to differences in the usage of translation initiation sites that are present upstream to the canonical initiation sequence (Bazzichetto *et al*, 2019).

The implication of PTEN in plethora of cellular processes including tumorigenesis and cancer progression is attributed to its enzymatic and non-enzymatic actions as well as PI3K independent and dependent mechanisms. Its function is regulated at various levels involving transcriptional,

post-transcriptional, post-translational, and genetic events (Bazzichetto *et al*, 2019; Milella et al, 2015).

A combination of genetic and epigenetic mechanisms such as chromosomal deletions, point mutations, hypermethylation of promoter, and post-translational events contribute to loss of PTEN activity which consequently leads to the development of various diseases including cancer (Zhang *et al*, 2010).

PTEN is required for the survival of breast cancer-initiating cells, its subsequent knockdown leads to accelerated growth of normal and malignant mammary stem cells (Korkaya et al, 2009; Razis et al, 2011). A recent study has documented that patients with positive expression of PTEN in breast tumors showed a greater response to trastuzumab treatment. A study by Fujita et al also confirmed that PTEN positive patients showed greater efficacy for trastuzumab (Fujita *et al*, 2006).

Activation of PI3K pathway as a consequence of PTEN loss correlated with poor prognosis and progression in breast cancer (Zhang *et al*, 2013; Saal *et al*, 2007). Also, several studies documented that PTEN inactivation/loss of expression served as a poor prognostic factor in several cancer types including renal cell carcinoma, prostate cancer, and breast cancer (Chen *et al*, 2014; Ocana *et al*, 2014; Yang *et al*, 2016). These results suggest that PTEN has the potential to serve as a predictor of treatment response and clinical outcomes in breast cancer patients.

Further, PTEN hypermethylation is found in both familial and sporadic breast cancer patients. A study by Lu et al revealed that hypermethylation of PTEN, resulting in loss of expression was detected in higher frequency in ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) of the breast as compared to normal controls, which suggests that PTEN has the potential to serve as a marker for early tumorigenesis of the breast (Lu *et al*, 2016). Lack of PTEN expression also significantly correlated with invasiveness of ductal carcinoma as well as estrogen receptor-negative status (Golmohammadi *et al*, 2016; Jones *et al*, 2013).

To date, 22,807 PTEN intronic variants with different physiological effects have been reported in NCBI. It was shown that variants in intronic region of PTEN lead to alternative splicing and pathogenic exon skipping which ultimately result in loss of PTEN expression accompanied by increased pAKT expression. Therefore, the present study has been planned to evaluate the

association of less characterized 32bp deletion variant which is located in intron2 of PTEN with the development of breast cancer.

MATERIALS AND METHODS:

469 primary breast cancer cases reported at Nizam Institute of Medical Sciences (NIMS), Hyderabad were recruited in the present study after obtaining informed consent and 391 controls were obtained from the local population without a family history of any cancers. Only histopathologically confirmed female patients with a confirmed diagnosis of primary breast cancer cases were included and secondary or bilateral breast cancer cases were excluded from the study. Epidemiological information was collected through personal interviews and clinical information was noted down from the tumor registry with the help of a medical oncologist. Further patients were followed for a brief period, to calculate overall survival time taking the death of the patient as an event. This study was approved by the institutional ethics committee of Osmania University and NIMS, Hyderabad.

Isolation of genomic DNA was performed by non-enzymatic salting-out method (Lahiri and Nurnberger 1991] from 5ml of blood samples collected from both controls and patients. Genotyping was carried out by using a combination of primers (Forward - 5′ CCAGCCCTCACTAAAA ACAAA-3′ and Reverse- 5′- CAAGTGTCCAAGCAGCAAA-3′). PCR reaction was performed using 50ng of DNA as a template in a 10µl reaction mix comprising of 10mM each of dNTP mix, 30pmol each of forward and reverse primers, 0.5U Taq Polymerase and Milli-Q water. The PCR conditions included initial denaturation (95^0C for 5 minutes), followed by 35 cycles of denaturation (95^0C for 30 seconds), annealing (61^0C for 45 seconds), extension (72^0C for 30 seconds) with an ultimate extension at 72^0C for 7 minutes. The PCR products were then analyzed on 3% agarose gel. Insertion allele and Deletion alleles were found to have a fragment length of 241bp and 209bp respectively. Genotyping has repeated on few randomly selected samples, and the reproducibility was 100%. The genotype data obtained were analyzed using various statistical analyses like SNPSTATs and SPSS version 20.

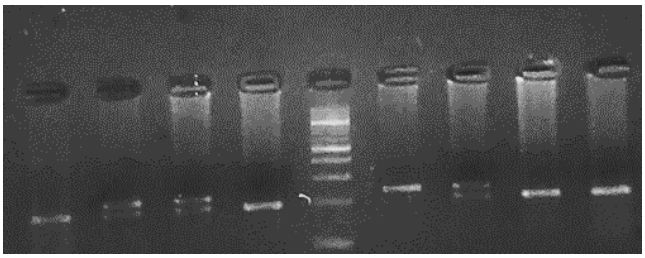

Figure No. 1: Representative 3% PCR-RFLP gel image of PTEN 32-bp Ins/Del polymorphism

Homozygous Wild Genotype, Ins/Ins: Lane 6 (241bp)
Heterozygous Genotype, Ins/Del: Lane2, 3and7 (241bpand209bp)
Homozygous Variant Genotype, Del/Del: Lane 1, 4, 8 and 9 (209bp)
100bpLadder: Lane5

RESULTS:

The demographic data of breast cancer patients are given in Table no 1. The median age of diagnosis was found to be 50 years (Range: 24-86 years), 64.6% of the patients were obese, 95.45% of breast cancer cases were found to be post-menopausal and 67% of them were lymph node-positive.

Table No. 1: Demographic data of breast cancer patients.

Epidemiological variables		**N (%)**
Diet (N=469)	Vegetarian	97 (20.7%)
	Non-vegetarian	372(79.3%)
BMI (N=342)	Non obese	121(35.4%)
	Obese	221(64.6%)
Menopause (N=286)	Premature menopause (≤40 years)	13 (4.55%)
	Post-menopausal (>40 years)	273(95.45%)
Clinical variables		
ER status (N=367)	Positive	184(50.1%)
	Negative	183(49.9%)
PR status (N=366)	Positive	178(48.6%)
	Negative	188(51.4%)
HER2 status (N=219)	Positive	86(39.3%)
	Negative	133(60.7%)
Triple negative receptor status	Triple –ve	69(31.5%)
(N=219)	Other combinations	150(68.5%)
Lymph node status (N=394)	Positive	264(67%)
	Negative	130(33%)
Tumor size (N=393)	<50mm	226(57.5%)
	>50mm	167(42.5%)
Stage of the cancer (N=422)	Stage 1&2	200(47.4%)
	Stage 3&4	222(52.6%)

Genotype distribution of PTEN 32-bp Ins/Del polymorphism among breast cancer cases and controls is given in Table (2). It was observed that frequencies of Del/Del genotype and Del allele were found to be elevated in breast cancer cases (49.7% and 69.09%) as compared to controls (40.1%, 63.55%) under the co-dominant model. However, the increase in Del/Delgenotype among cases was not significant. Along the same lines, in the recessive model Del/Del genotype conferred 1.47foldincreased risk significantly (OR=1.47; 95%CI: 1.12-1.93, $\chi 2$; p=0.0051), whereas in over dominant model, Ins/Del genotype showed 0.72fold reduced risk

for breast cancer significantly (OR=0.72; 95%CI: 0.55-0.95, *χ2; p*=0.018). However, the genotype distribution of PTEN 32-bp Ins/Del polymorphism showed deviation from Hardy Weinberg equilibrium in cases.

Table No. 2: Genotype distribution of PTEN 32-bp Ins/Del polymorphism between breast cancer cases and controls.

PTEN 32-bp Ins/Del polymorphism	**Controls (n=391)**	**Breast cancer (n=469)**	**OR(95% CI)**	***χ2; p***
Ins/Ins	51 (13%)	54 (11.5%)	1	
Ins/Del	183 (46.8%)	182 (38.8%)	0.94 (0.61-1.45)	0.77
Del/Del	157 (40.1%)	233 (49.7%)	1.40 (0.91-2.16)	
Dominant model				
Ins/Ins	51 (13%)	54 (11.5%)	1	
Ins/Del-Del/Del	340 (87%)	415 (88.5%)	1.15 (0.77-1.73)	0.5
Recessive model				
Ins/Ins- Ins/Del	234 (59.9%)	236 (50.3%)	1	
Del/Del	157 (40.1%)	233 (49.7%)	1.47 (1.12-1.93)	0.0051*
Over dominant model				
Ins/Ins- Del/Del	208 (53.2%)	287 (61.2%)	1	
Ins/Del	183 (46.8%)	182 (38.8%)	0.72 (0.55-0.95)	0.018*
Allele				
Ins	285(36.45%)	290(30.91%)	1	
Del	497(63.55%)	648(69.09%)	1.28(1.04-1.56)	0.015*
HWE (p)	0.91	0.051		
OR-Odds Ratio, χ2; p value indicates chi-squared test probabilities,*p <0 .05, #p < 0.10, *indicates that χ2 ; p value is less than 0.05; # indicates that χ2 ; p value is less than 0.10				

Further genotype data was stratified concerning various clinical and epidemiological characteristics to understand the confounding effects of the above-said genotypes. Neither

Del/Del genotype nor Del allele showed any significant association with epidemiological or clinical variables (Table 3 and Table 4).

Table No. 3: Genotype distribution of PTEN 32-bp Ins/Del polymorphism with respect to epidemiological variables.

PTEN 32-bp Ins/Del Polymorphism	**Ins/Ins (%)**	**Ins/Del (%)**	**Del/Del (%)**	**χ2; p**	**Ins (%)**	**Del (%)**	**χ2; p**
Diet							
Vegetarian	8(8.2%)	33 (34%)	56 (57.7%)		49(25.3%)	145(74.7%)	
Non-Vegetarian	46(12.4%)	149 (40%)	177 (47.6%)	0.17	241(32.4%)	503(67.6%)	0.05
OR(95%CI)	1.00(Ref)	0.79 (0.34-1.82)	0.55 (0.24-1.23)		1.00(Ref)	0.70(0.49-1.00)	
Menopausal Status							
Premature menopause	1(7.7%)	4(30.8%)	8(61.5%)		6(23.1%)	20(76.9%)	
Post-menopausal	30(11%)	109(39.9%)	134(49.1%)	0.07	169(61.2%)	377(38.8%)	0.01*
OR(95%CI)	1.00(Ref)	0.90(0.09-8.43)	0.14(0.01-1.19)		1.00(Ref)	0.31(0.12-0.80)	
BMI							
Normal	19(15.7%)	44 (36.4%)	58 (47.9%)		82(33.9%)	160(66.1%)	
Obese	21 (9.5%)	97 (43.9%)	103 (46.6%)	0.17	139(31.5%)	303(68.5%)	0.51
OR(95%CI)	1.00(Ref)	1.99 (0.98-4.08)	1.61 (0.80-3.23)		1.00(Ref)	1.11(0.80-1.56)	
Consanguinity							
Non consanguineous	40 (11%)	139 (38.3%)	184 (50.7%)		219(30.2%)	507(69.8%)	
Consanguineous	9 (12.9%)	32 (45.7%)	29 (41.4%)	0.36	50(35.7%)	90(64.3%)	0.19
OR-Odds Ratio, χ2; p value indicates chi-squared test probabilities,*p <0 .05, #p < 0.10, *indicates that χ2 ; p value is less than 0.05; # indicates that χ2 ; p value is less than 0.10							

Table No. 4: Genotype distribution of PTEN 32-bp Ins/Del polymorphism with respect to clinical variables

PTEN 32bp Ins/Del Polymorphism	**Ins/Ins (%)**	**Ins/Del (%)**	**Del/Del (%)**	**χ2; p**	**Ins (%)**	**Del (%)**	**χ2; p**
Progesterone Receptor Status							
Progesterone Receptor Positive	21 (11.8%)	68(38.2%)	89 (50%)		110(30.9%)	246(69.1%)	
Progesterone Receptor Negative	21 (11.2%)	79 (42%)	88 (46.8%)	0.76	121(32.2%)	255(67.8%)	0.71
OR(95%CI)	1.00(Ref)	1.16 (0.58-2.31)	0.99 (0.50-1.94)		1.00(Ref)	0.94(0.68-1.28)	
Estrogen Receptor Status							
Estrogen Receptor Positive	21 (11.4%)	75 (40.8%)	88 (47.8%)		117(31.8%)	251(68.2%)	
Estrogen Receptor Negative	21 (11.5%)	73 (39.9%)	89 (48.6%)	0.99	115(31.4%)	251(68.6%)	0.91
OR(95%CI)	1.00(Ref)	0.97 (0.49-1.93)	1.01 (0.52-1.98)		1.00(Ref)	1.01(0.74-1.38)	
HER2 Receptor Status							
HER2 Receptor Positive	10 (11.6%)	35 (40.7%)	41 (47.7%)		55(31.9%)	117(68.1%)	
HER2ReceptorNegative	17 (12.8%)	58 (43.6%)	58 (43.6%)	0.84	92(34.6%)	174(65.4%)	0.57
OR(95%CI)	1.00(Ref)	0.97 (0.40-2.37)	0.83 (0.35-2.00)		1.00(Ref)	0.88(0.59-1.33)	
Axillary Lymph Node Status							

Axillary Lymph Node Negative	17 (13.1%)	50 (38.5%)	63 (48.5%)		84(32.3%)	176(67.7%)	
Axillary Lymph Node Positive	25 (9.5%)	107 (40.5%)	132 (50%)	0.56	157(29.7%)	371(70.3%)	0.46
OR(95%CI)	1.00(Ref)	1.46 (0.72-2.94)	1.42 (0.72-2.83)		1.00(Ref)	1.12(0.82-1.55)	
Stage of the cancer							
Early stage (Stage I &II)	27 (13.5%)	73 (36.5%)	100 (50%)		127(31.7%)	273(68.3%)	
Advance stage (Stage III&IV)	23 (10.4%)	94 (42.3%)	105 (47.3%)	0.38	140(31.5%)	304(68.5%)	0.94
OR(95%CI)	1.00(Ref)	1.51 (0.80-2.85)	1.23 (0.66-2.29)		1.00(Ref)	1.01(0.75-1.35)	
Tumor Size							
<50mm	25 (11.1%)	87 (38.5%)	114 (50.4%)		137(30.3%)	315(69.7%)	
>50mm	16 (9.6%)	69 (41.3%)	82 (49.1%)	0.81	101(30.3%)	233(69.7%)	0.98
OR(95%CI)	1.00(Ref)	1.24 (0.61-2.50)	1.12 (0.56-2.24)		1.00(Ref)	1.00(0.73-1.36)	
OR-Odds Ratio, $\chi 2$; p-value indicates chi-squared test probabilities, ,*p <0 .05, #p < 0.10, *indicates that $\chi 2$; p value is less than 0.05; # indicates that $\chi 2$; p value is less than 0.10							

To understand the effects of PTEN 32-bp Intron2 Ins/Del polymorphism genotypes on the five-year overall survival rate of breast cancer patients, Kaplan-Meier survival analysis was performed taking death as an event. It was revealed that patients carrying Ins/Ins genotype showed a significant increase in median survival rate (61 months, Log Rank p value=0.003 and

Breslow p value=0.004) and patients with Del/Del genotype showed decreased median survival rate (54 months). Of note, Ins/Del genotype carriers showed 35 months of median survival rate. (Table 5) (Figure 2).

Table No. 5: Kaplan–Meier survival analysis for Overall survival of PTEN 32-bp Ins/Del polymorphism in breast cancer

Sl No	Genotype	N (%)	Death (%)	(OS in months) Mean ± SEM	Median	p-value
1	Ins/Ins	42(11.11%)	21(50%)	43.47±3.40	61	
2	Ins/Del	145(38.36%)	101(69.65%)	36.16±1.71	35	0.003[a]
3	Del/Del	191(50.53%)	107(56.02%)	43.51±1.47	54	0.004[b]
Total		378	229	40.68±1.07	48	

a) Log Rank (Mantle Cox) p value

b) Breslow (Generalized Wilcoxon) p value

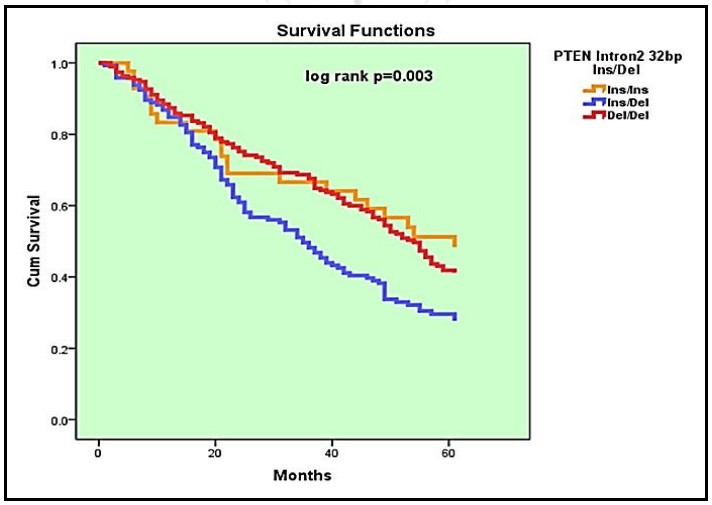

Figure No.2: Kaplan-Meier analysis survival curve for Overall survival of PTEN 32-bp Ins/Del polymorphism in breast cancer

DISCUSSION:

PTEN is a tumor suppressor that has a negative regulatory role in PI3K/AKT pathway. Polymorphisms in PTEN as well as deficiency of PTEN due to germline or somatic mutations have been linked to a variety of malignancies (Keniry *et al*., 2008). Hence, in our study, we have aimed to study the association of PTEN 32bp Ins/Del polymorphism (rs34421660) located in Intron2 with breast cancer development. The functional significance of this polymorphism is not clear. Since the location of SNP is present close to the splicing regulatory site, this might affect the splicing process leading to Intron retention or exon skipping which ultimately affects the function of PTEN (Ding *et al*., 2011).

In the present study, Del/Del genotype showed 1.47 fold increased risk for breast cancer in the recessive model. Along the same lines, Del allele carriers were found to have 1.28 folds of risk of breast cancer as compared to 'Ins' allele carriers. Of note, Ins/Del genotype showed 0.72 fold reduced risk for breast cancer in the co-dominant model. Our study did not show a significant association with any of the epidemiological or clinical variables studied. In contrast to our results, a study on hepatocellular carcinoma by Ding et al, in the Han Chinese population did not reveal any association with Del genotype or Del allele. Moreover, their study showed that the Del allele in combination with other alleles in a haplotype conferred reduced risk for HCC (Ding *et al*., 2011). Also, a study by Hashemi et al showed no association of Del/Del genotype or Del allele with susceptibility to metabolic syndrome in the Iranian population (Hashemi *et al*., 2013). Along the same lines, a study by Eskandari et al also revealed no association between PTEN Intron2 32bp Ins/Del polymorphism and chronic hepatitis B virus infection. Although not significant 'Del' allele was observed to be elevated in cases as compared to controls in their study, which is in concordance with the observations of our study (Eskandri *et al*, 2017). Further, Kaplan-Meier survival analysis revealed a significant decrease and increase in the median survival rate of breast cancer patients carrying Ins/Del and Ins/Ins genotypes respectively. These results suggest that 'Del' allele presence in the genotypes might be playing a role in decreasing the survival rate. Overall, it can be concluded that Del/Del genotype and Del allele pose a significant risk for breast cancer development in our population. However, the small sample size and lack of association with the epidemiological and clinical variables point towards the need for

study; on a large population and other critical variants in the PTEN gene that can affect its expression and function.

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