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Role of p73 polymorphism in Egyptian breast cancer patients as molecular diagnostic markers

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ABSTRACT:

Background:

The incidence of breast cancer in Egyptian women is rising; to date, a few susceptibility genes have been identified. p73 protein (also known as p53-like transcription factor or p53-related protein) is one of the ancestors of the tumor suppressor p53 protein, whose gene is located within the chromosomal loci 1p36; a region most frequently deleted in human cancers. As a consequence of sharing same domain architecture with p53; p73 might regulate p53- response genes and induced cell cycle arrest/ apoptosis in response to DNA damage. A commonly studied non-coding polymorphism consisting of a double nucleotide substitutions (G→A) and (C→T) at position 4 and 14 exon 2, situated upstream of the initial AUG regions of p73. This functional consequence of p73 polymorphism may serve as a susceptibility marker for human cancer, but the results are inconsistent.

Patients and Methods:

Eighty newly diagnosed females representing Egyptian population confirmed breast cancer patients and forty healthy controls, recruited from the departments of Experimental and Clinical Surgery and Cancer Management and Research, Medical Research Institute, Alexandria University. Single Nucleotides Polymorphism (SNP) in p73 gene (G4C14-to-A4T14) was determined in these samples by PCR-CTPP techniques.

Results:

Insignificant differences in the distributions of p73 genotypes between patients and controls were observed ($p = 0.126$). When p73 GC/GC genotype was used as the reference, the combined variant genotypes (AT/AT)/(GC/AT) was significantly associated with the risk for breast cancer [OR= 2.418, 95% CI (1.018-5.746); p= 0.042]. p73 [(GC/AT) /(AT/AT) genotypes] was found to be associated with increased risk for breast cancer among women with pathological grade III, clinical stage III, tumor size ≥ 5 cm, axillary lymph node involvement and the +ve (Her2/neu) expression, but not significantly associated with +ve ER/PR status, vascular invasion and metastasis. Furthermore, patients carrying AT variant has a favorable prognosis ($p <0.001$) and longer survival (41.33±1.45 months) than did patients carrying GC/GC genotype (24.0±1.13 months).

Conclusion:

In conclusion, this study provides the first indication that p73 variants (AT/AT)/ (GC/AT) are risk factors for breast cancer susceptibility in Egyptian women. Thus analysis of p73 G4C14- to- A4T14 polymorphism may be useful for identifying females with higher risk to develop cancer. Additional studies are needed to confirm these findings.

Keywords:
p73, Cyclin D1, polymorphism, diagnosis, Egypt.

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INTRODUCTION:

The global burden of breast cancer is growing larger in recent years .It is represent 31% of all cancers diagnosed and 15% of all cancer death in women (Coral and Amy, 2010). In Alexandria, Egypt, breast cancer accounts for 42.7% of malignancies among females (Alexandria Cancer Registry Annual Report, 2010). Molecular epidemiology is an emerging new field that for study not only the genetic and environmental causes of carcinogenesis, but also interaction between the two (Perera and Weinstein, 2000). Therefore medicine is facing a new challenge, which is the identification of determinations for genetic susceptibility to cancers including breast cancer and the informations needed to accomplish this role require an understanding of human genetic variation (Lyla and Dan, 2006).

Recent breast cancer epidemiologic studies provide some genetic and epigenetic factors that play a role in the development of this disease, moreover, they reported that individuals carrying breast carcinoma have a high probability to carry one of these factors(Coral and Amy, 2010).

p73 (Jost *et al.,* 1997), tumor suppressor gene encoded protein that shares structural and functional homology with p53 but not identical. p73 gene located on chromosomal region 1p63, locus is deleted in a variety of tumorigenesis. Because of these similarities to p53; p73 possiblely might activate p53 response genes and induced cell cycle arrest or apoptosis in response to DNA damage (Kaghad *et al.,*1997). The wild -type isoform p73 α , contain 14 exons and gives rise to protein containing 636 amino acids; it exhibits the same structure of p53 and both have a transactivation domain (TA), a DNA binding domain (DBD), and an oligomerization domain (OD) (Kaghad *et al.,*1997; Barry Trink *et al.,* 1998; Thanos and Bowie, 1999). The supreme similarity among all p53 family members present within the DNA binding domain indicated that p73 may bind the same DNA sequences like p53 and strengthen transcription activation (Kaghad *et al.,*1997). A part of p73 structure not present in p53 gene with an expanded c-terminal region of p73 contains SAM (sterile alpha motif) which acts as oligomerization domain and involved in protein- protein interactions and developmental regulation (Schultz *et al.,* 1997; Ishimoto *et al.,* 2002).

p73 gene is characterized by two promoters realizing different classes of proteins, the TAp73 protein is generated by alternative splicing in the p1 promoter region located upstream of exon 1, while the other alternative splicing located in intron 3 in the p2 promoter region is produceing the acidic NH_2 terminally truncated isoform (ΔNp73) which lack of all or most of the transactivation domain (Ishimoto *et al.,* 2002; Yang *et al.,* 2000; Stiewe *et al.,* 2002).

This ΔNp73 acts as a negative inhibitor towards TAp73 and p53 (Grob *et al.,* 2001). Observed that overexpression of p73 wild type is common alteration in carcinogenesis particularly in patients with poor prognosis (Stiewe and Putzer, 2002; Dominguez *et al.,* 2001), rather, ΔTA-p73 isoform is significantly detected excessively in many types of cancers including breast cancer (Alex *et al.,* 2002; Uramoto *et al.,* 2004; Douc-Rasy *et al.,* 2002; Casciano *et al.,* 2002).

Two silent single nucleotide polymorphisms affect the five untranslated region in exon 2 at position 4/14 (G4C14-to-A4T14) produced different variants of p73 mRNAs (Kaghad *et al.,*1997). This p73 two linked polymorphisms located upstream of the initiation AUG codon of exon 2, causing stem-loop like structure during transcription initiation thus, altering gene expression [(Kaghad *et al.,*1997; Melino *et al.,* 2002). Many of the studies have examined the correlation between p73 (GC/ AT) polymorphism and the risk of carcinogenesis (De Feo *et al.,* 2009; Niwa *et al.,* 2004; Li *et al.,* 2004; Pfeifer *et al.,* 2005).

Though, few studies have been conducted to investigate the impact of p73 dinucleotides

polymorphism on breast cancer susceptibility (Huang *et al.,* 2003; Li *et al.,* 2006). These studies producing a confused results. the aim of our study is to determined whether the p73 GC/AT dinucleotides polymorphism are the risk factors for breast cancer susceptibility in Egyptian females, and whether there were any relationships of the p73 polymorphic variants with clinicopathological status.

METHODS:

Patients:

All patients (n=80) who have experienced primary invasive breast carcinoma, with a median age 52.0 (range 32.0-77.0) years, at the Experimental and Clinical Surgery and Cancer Management and Research Departments, Medical Research Institute, Alexandria University From 2008 to 2012, were enrolled in this study. The samples were collected before starting any cancer treatments. Non tumor control group (n=40), with median age 49.50 (range 36.0-71.0) years, was composed of healthy women volunteers clinically free from any chronic disease. Other tools used to confirm our information were questionnaires and medical reports. This study protocol was approved by the Local Ethical Committee at Alexandria University.

p73 genotyping: 5-mL blood samples were obtained from cases and controls. The samples were collected in tubes containing EDTA and genomic DNA was purified from peripheral whole blood using a ready-for use DNA extraction kit (QIA amp DNA Blood mini kit, Qiagen, Hilden, Germany). Genotyping was performed by Polymerase Chain Reaction with Confronting Two-Pair Primers (PCR-CTPP) [(Hamajima *et al.,* 2000; Tamakoshi *et al.,* 2003), using semi quantitatively conventional Polymerase Chain Reaction (PCR) kits (Qiagen, Germany) according to producer's instructions.

According to the published sequence of the human p73 gene, we designed four primers (Forward primer (F1):5´-CCACGGATGGGTCTGATCC-3´; Reverse primer (R1): 5´-GGCCTCCAAGGGCGACTT-3´ and (F2) Forward primer (F2): 5´-CCTTCCTTCCTGCAGAGCG-3´; Reverse primer (R2): 5´-TTAGCCCAGCGAAGGTGG-3´; the p73 G4C14-to-A4T14 polymorphism specific primers were ordered from QIAGEN system (QIAGEN, Germany) to amplify a 260-bp fragment of p73 gene. The PCR reactions were performed on a thermal cycler (Biometra- TProfessional Thermocycler-Germany) and the cycling program was programmed according to the manufacturer's protocol. Specifically, these reactions were carried out in a total volume 50 µl of QIAGEN Multiplex PCR Master Mix 25 µl, primer mix (2 µl taken from each 20µM primer working solution) 8 µl , Template DNA 17 µl. Each PCR started within the initial heat- activation program to activate HotStar Tag DNA polymerase (95°C for 15 min), followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 90 sec, and extension at 72 C° for 90 sec, with a final extension step at 72 °C for 10 minutes. Agarose gel electrophoresis was used as the appropriate detection system. This gave a satisfactory signal with our PCR product. The DNA fragments were separated using 2% agarose gel containing ethidium bromide and the bands on the gel were visualized by using UV Transilluminator. The allele types were determined as follows: two fragments of (270-, 428-bp) for the AA genotype, three fragments of (193- , 270-, 428- bp) for the GA genotype and two fragments of (193 -, 428- bp) for the GG genotype.

Statistical Analysis:

Data were analyzed using the Predictive Analysis Software (PASW statistics) for windows (SPSS Inc. Chicago, USA). Association between categorical variables was tested using Chi – square test and Firsher's exact test if more than 20% of the cell has expected account less than five. Range, mean, standard deviation and median were used with quantitative data. Parametric tests were applied that reveals normal data distribution. If

data were abnormally distributed, the non parametric tests were used. Odd ratio (OR) and 95% confidence interval were used and the P value was assumed to be significant at the 5% level.

RESULTS:

The clinical profile of breast cancer patients included in the current study is presented in table (1). Clinical characteristics of normal healthy female volunteers and patients with breast cancer were depicted in table (1). Because the cases and control were frequency- matched for age, there were no significant differences in the distributions of age between cases and control (p=0.45). The genotype frequencies of P73 G4C14/A4T14 polymorphism were analyzed among the controls and breast cancer patients. The frequencies of GC/GC, GC/AT and AT/AT genotypes were 31(77.5%), 8(20.0%) and 1(2.5%) for healthy controls and 47 (58.8%), 29(36.3%) and 4(5.0%) for breast cancer patients, respectively, table (2).

The GC/AT genotypes of p73 G4C14/A4T14 were not correlated with age, table (3a) and Premenopausal status, table (3b). When p73 GC/GC genotype was used as the reference, the combined variant genotypes (AT/AT) / (GC/AT) was significantly associated with the risk for breast cancer [OR= 2.418, 95% CI (1.018-5.746); p= 0.042] table(3).

Table 1: Characteristics of normal healthy controls and breast cancer patients

Clinical characteristics	Normal subjects (n = 40)		Breast cancer patients (n = 80)		Test of significance (*P*- value)
	No	%	No	%	
Age (years)					
< 45	15	37.5	11	13.8	X^2 **test** (*P* = 0.454)
≥ 45	25	62.5	69	86.3	
Range	36.00 –71.00		32.00 – 77.00		
Mean ± SD	50.15 ± 9.43		52.62 ± 10.07		***Student T test*** (*P* = 0.198)
Median	49.50		52.0		
Menopausal status					
Premenopausal	20	50.0	37	46.3	X^2**test** $X^2P = 0.698$
Postmenopausal	20	50.0	43	53.8	

x^2p: p value for Chi square test *: Statistically significant at $p < 0.05$

Table 2: Frequencies of *P73* (G4C14/A4T14) genotype in breast cancer patients and healthy controls

	Normal healthy controls (n=40)		Breast cancer patients (n = 80)		*p*
	No.	%	No.	%	
Polymorphic variants					
GC/GC	31	77.5	47	58.8	0.042*
GC/AT	8	20.0	29	36.3	0.069
AT/AT	1	2.5	4	5.0	FEp =0.664
p	0.126				

p: p value for Chi-square test FEp: p value for Fisher Exact test *: Statistically significant at $p \leq 0.05$

Table (3): Association of P73 (G4C14/A4T14) polymorphism with breast cancer risk

	Normal healthy controls		Breast cancer patients		Test of sig.	OR (95% CI) (lower– upper)
	No	%	No	%		
All participants						
GC/GC®	31	77.5	47	58.8		1.000 (reference)
GC/AT	8	20.0	29	36.3	P = 0.055	2.391 (0.968-5.908)
AT/AT	1	2.5	4	5.0	FEp = 0.644	2.638 (0.968-5.908)
AT/AT+GC/AT	9	22.5	33	41.3	P = 0.042*	2.418 (1.018-5.746)

p: p value for Chi-square test FEp : p value for Fisher Exact test *: Statistically significant at $p \leq 0.05$

Table (3a): Association of P73 (G4C14/A4T14) polymorphism with breast cancer risk

	Normal healthy controls		Breast cancer patients		Test of sig.	OR (95% CI) (lower– upper)
	No	%	No	%		
Women age < 45years						
GC/GC®	12	80.0	6	54.5		1.00 (reference)
GC/AT	2	13.3	4	36.4	FEp = 0.192	4.00 (0.563-28.396)
AT/AT	1	6.7	1	9.1	FEp = 1.000	2.00 (0.106-37.830)
AT/AT+ GC/AT	3	20.0	5	45.5	FEp = 0.218	3.33 (0.588-18.891)
Women age ≥ 45 years						
GC/GC®	19	76.0	41	59.4		1.00 (reference)
GC/AT	6	24.0	25	36.2	p = 0.322	1.931 (0.680-5.484)
AT/AT	0	0.0	3	4.3	FEp = 0.547	1.463 (1.232-1.738)
AT/AT+ GC/AT	6	24.0	28	40.6	p = 0.139	2.163 (0.767-6.094)

p: p value for Chi-square test FEp : p value for Fisher Exact test *: Statistically significant at $p \leq 0.05$

Table (3b): Association of P73 (G4C14/A4T14) polymorphism with breast cancer risk

	Normal healthy controls		Breast cancer patients		Test of sig.	OR (95% CI) (lower– upper)
	No	%	No	%		
Premenopausal status						
GC/GC®	16	76.2	22	64.7		1.00 (reference)
GC/AT	4	19.0	10	29.4	FEp = 0.524	1.181 (0.483-6.850)
AT/AT	1	4.8	2	5.9	FEp = 1.000	1.455 (0.121-17.462)
AT/AT+ GC/AT	5	23.8	12	35.3	p = 0.371	1.745 (0.512-5.948)
Postmenopausal status						
GC/GC®	15	78.9	25	54.3		1.00 (reference)
GC/AT	4	21.1	19	41.3	FEp = 0.153	2.850 (0.813-9.986)
AT/AT	0	0.0	2	4.3	FEp = 0.530	1.600 (1.259-2.034)
AT/AT+ GC/AT	4	21.1	21	45.7	FEp = 0.093	3.150 (0.906-10.953)

p: p value for Chi-square test FEp : p value for Fisher Exact test *: Statistically significant at $p \leq 0.05$

Association of different p73 (G4C14/A4T14) polymorphic variants among breast cancer patients with clinicopathological features were shown in table (4). Compared with GC/GC genotype, the combined variant p73 GC/AT or AT/AT genotypes was significantly associated with tumor pathological grade, clinical stage, tumor size, lymph node involvements and Her2/neu expression. Patients with AT allele (GC/AT or AT/AT genotype) were potentially to be a positive lymph node status, advanced tumor stage or recurrence than patients

Table (4): Association of *p73* (G4C14/A4T14) polymorphism with clinicopathological features of breast cancer

	GC/AT+AT/AT®		GC/GC®		Test of sig	OR (95% CI) (lower– upper)
	No	%	No	%		
Tumor pathological grade						
II ®	24	72.7	44	93.6	FEp= 0.023*	
III	9	27.3	3	6.4		5.500 (1.359-22.261)
Clinical stage						
II ®	6	18.2	35	74.5	p <0.001*	
III	27	81.8	12	25.5		13.125 (4.364-39.473)
Tumor size (cm)						
< 5®	4	12.1	36	76.6	FEp <0.001*	
≥ 5	29	87.9	11	23.4		23.727 (6.836-82.361)
Lymph node involvements						
-ve®	3	9.1	15	31.9	FEp= 0.028*	
+ve	30	90.9	32	68.1		4.688 (1.232-17.829)
Estrogen receptor status						
-ve®	2	6.1	2	4.2	FEp=1.000	
+ve	31	93.9	45	95.7		0.689 (0.092-5.155)
Progesterone receptor status						
-ve®	4	12.1	4	8.5	FEp=1.000	
+ve	29	87.9	43	91.5		0.674 (0.156-2.915)
Her2/neu expression						
-ve®	25	75.8	44	93.6	FEp= 0.044*	
+ve	8	24.2	3	6.4		4.693 (1.140-19.316)
Vascular invasion						
-ve®	6	18.2	10	21.3	P= 0.733	
+ve	27	81.8	37	78.7		1.216 (0.394-3.754)
Metastasis						
-ve®	24	72.7	34	72.3	p = 0.970	0.981 (0.362-2.660)
+ve	9	27.3	13	27.7		

p: p value for Chi-square test FEp: p value for Fisher Exact test *: Statistically significant at $p \leq 0.05$

with the GC/GC genotype. Kaplen Meir Disease Free Survival (DFS) curve was constructed to study the prognostic value of p73 (G4C14/A4T14) genotypes. After a median fallow up period of 25 months (range 18-48 months), 22(27.5%) out of 80 patients had metastasis. The incidence of metastasis was observed in 27.7% of patients with GC/GC genotype and 27.3% of patients carrying AT variant (AT/AT) / (GC/AT) genotypes table (5). A significant association between the genotypes and survival was found in the patients (p <0.001), figure (1). Furthermore, patients carrying AT variant (AT/AT)/ (GC/AT) genotypes has a favorable prognosis and longer survival (41.33±1.45 months) than did patients carrying GC/GC genotype (24.0±1.13 months).

DISCUSSION

p73 protein was considered as one among the p53 family , the high level of similarity between p53 and p73 is appeared in the DBD domain which revealed that p73 can bind and activate p53 target genes , thus induced cell cycle arrest and apoptosis (Kaghad *et al.,*1997).

Table (5): Association of p73 (G4C14/A4T14) genotypes with breast cancer disease free survival (DFS)

	Metastasis N =22	Non Metastasis N = 58	Median (Mean ± SE) DFS (months)	Log rank	p
GC/GC (N=47)	13 (27.7)	34 (72.3)	24.0 (24±1.13)	20.557*	<0.001
[(GC/AT)/(AT/AT)](N=33)	9 (27.3%)	24 (72.7)	40.0 (41.33±1.45)		

*: Statistically significant at p<0.05

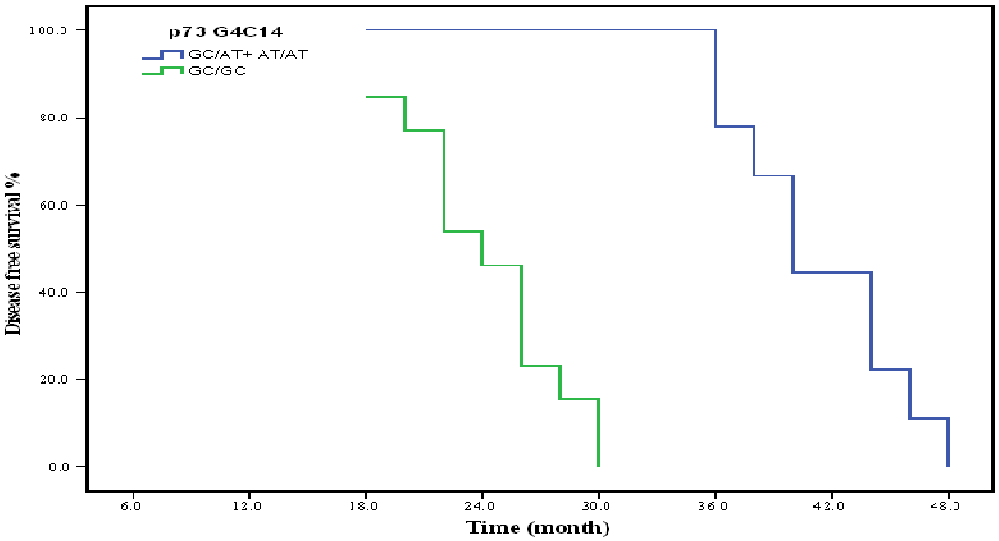

Figure (1): Kaplan-Meier disease free survival for p73 (G4C14/A4T14) genotypes

Because of alternative N- and C- terminal splicing of transcription, p73 gives a variety of isoforms. Formation of ΔN-isoform (shorter amino terminus lacking the TA domain) requires activation of the alternative P2 promoter in exon 3 / intron 3` (Zaika *et al.,* 2002). The p73 amino-terminally truncated (ΔN) isoform is commonly called ΔTA-p73 and strongly established as an oncogene. Therefore it is involved in the oncogenesis by inhibiting tumor suppressive modulations of p53 and TA p73 (Zaika *et al.,* 2002).

Numerous studies have proven that p73 protein is a classic tumor suppressor (Grob *et al.,* 2001; Zaika *et al.,* 2002; Benard *et al.,* 2003). Surprise investigations proved that the NH2-terminal truncated isoform of human p73 (Np73) owning an opposite activities of TAp73 indicated that Np73 likely has an oncogenic function (Zaika *et al.,* 2002). It is found that p73 is over-expressed in many cancer types including breast carcinoma (Zaika *et al.,* 1999; Cai *et al.,* 2000; Kang *et al.,* 2000). Dinucleotides polymorphisms have been found in the p73 gene (designated as G4C14-to-A4T14). This functional polymorphism lies upstream of the codon AUG of exon 2, region which might form a stem-loop like structure and affect translation efficiency (Kaghad *et al.,*1997).

The associations of p73 G4C14-to-A4T14 Polymorphism and cancer susceptibility have been investigated in different molecular epidemiological studies, and produce conflicting results (Douc-Rasy *et al.,* 2002; Casciano *et al.,* 2002; De Feo *et al.,* 2009; Niwa *et al.,* 2004; Li *et al.,* 2004; Pfeifer *et al.,* 2005; Huang *et al.,* 2003;Li *et al.,* 2006).

Therefore, this study was objective to examine the association of p73 G4C14→A4T14 polymorphism with breast cancer susceptibility and survival in 80 breast cancer Egyptian females with a median follow up of 25 months.

In this study, we noticed that the two genotypes p73 (GC/AT) and (AT/AT) were more frequently observed in breast cancer patients whereas p73 GC/GC

genotype was significantly higher in controls. However, insignificance difference in the genotypes distribution between patients and controls was observed. Also found that the combined variant genotypes (GC/AT) / (AT/AT) were more frequent in breast cancer patients [OR 2.418, p=0.042] than those with GC/GC genotype. These results indicated possible relationship between p73 G4C14–to–A4T14 polymorphism and breast cancer in Egyptian population.

Moreover, we found that the combined variant genotypes (GC/AT) / (AT/AT) were more frequent in breast cancer patients [OR 2.418, p=0.042] than those with GC/GC genotype. These results indicated possible relationship between p73 G4C14–to–A4 T14 polymorphism and risk of breast cancer.

Many experimental studies showed that individual carries AT allele is associated with increased risk of developing breast cancers in Japanese population (Li *et al.,* 2004), gastric cancer in Caucasians population (De Feo *et al.,* 2009), colorectal cancer in Korean population (Pfeifer *et al.,* 2005) and lung cancer in a non -Hispanic white population (Huang *et al.,* 2003). But few studies showed no correlations between p73 G4C14-to-A4T14 Polymorphism and cancer risk (Choi *et al.,* 2006; Hu *et al.,* 2005). Furthermore, very recently, Hu Y *et al.,* (2012) conducted a Meta Analysis study and found that Tp73 polymorphism (GC/AT) is probability associated with cancer risk in most cancer types and ethnicities (Hu *et al.,* 2012).

Also we evaluated the association of p73 genotypes with pathological parameters of breast cancer patients. Compared with GC/GC genotype, the combined variant genotypes (GC/AT) / (AT/AT) were found to be associated with increased risk for breast cancer among women with pathological grade III [OR= 5.500, p= 0.023], clinical stage III [OR= 13.125, $p < 0.001$], tumor size $\geq$ 5 cm [OR= 23.727, $p < 0.001$], axillary lymph node involvement [OR= 4.688, p= 0.028] and the +ve (Her2/neu) expression [OR= 4.693, p= 0.044]. These results suggest that AT variant allele has an important role in breast cancer progression, and may provide the clinician with additional information regarding patients carrying AT variant with the risk of recurrence.

Results from the present study showed that patients with (AT/AT) / (GC/AT) genotypes had a more favorable disease free survival than those with GC/GC genotype. Unexpectedly, our results taken together seem to show that there was a higher risk in developing breast cancer of females carrying the AT/AT genotype, but once affected, the patient has a better prognosis. Few studies have shown that Tp73 polymorphism is a poor prognostic factor in carcinogenesis (Grob *et al.,* 2001; Dominguez *et al.,* 2001). Study in relationship between ΔNp73 expression and prognosis in patient with lung cancer have concluded that positive expression of ΔNp73 might be a possible marker in predicting poor prognosis (Uramoto *et al.,* 2004; Casciano *et al.,* 2002). These funding might be due to the negative effect of p73 polymorphism in translation efficiency; further research with large number of samples are needed to confirm these preliminary results.

In summary, we found that p73 exon 2 G4C14-to -A4T14 polymorphism seem to have a major gene effect on risk of breast cancer in Egyptian females. p73 GC/ GC genotype were significantly associated with shorter disease free survival in breast cancer patients . Larger prospective studies are needed to further confirm our results.

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