Biochemical study of survivin -31G/C promoter polymorphism in breast Cancer

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Abstract

Breast cancer is the most common cancer and also the leading cause of cancer mortality in women worldwide.

Survivin is a member of the inhibitor of apoptosis (IAP) family, which has been identified recently. Unlike other IAP proteins, survivin is generally not found in normal adult tissues but notably expressed in the most common human cancers including stomach, colorectal, lung and breast.

In cancer cells, overexpression of survivin at both protein and mRNA levels is linked to genetic variant -31G/C in the survivin promoter.

P53-Abs were discovered 20 years ago during the course of tumor-associated antigens screening. The discovery of p53 mutation and accumulation of p53 in human tumors shed new light on the p53 humoral response.

The aim of work: investigate -31 G/C single nucleotide polymorphism (SNP)of survivin promoter in breast cancer patients .Measurement of the level of p53 antibodies in them and detecting the association between it and the polymorphism also.

Patients and methods: 100 subjects were enrolled in in this study. All were women in the age between 40 and 65 (60 breast cancer cases and 40 control divided into two groups according to age: Group 1 ≤ 50 years (27.3 ± 4.87) and Group 2 > 50(24.83 ± 6.12).

Results: Our study shows a significant difference in the prevalence of the survivin promoter polymorphism (–31G > C) between the case and control groups, P value (P = 0.005*). Notably, the combined prevalence of the GC and CC genotypes (GC + CC), reflecting the prevalence of the C allele, was significantly greater in the breast cancer group than in the control group (P= 0.002*) with rates of 29% and 6%, respectively. These results imply that the C allele at position –31 in the promoter region of the survivin gene increases an individual's susceptibility to breast cancer.

Also, the risk of developing cancer was 4.05 times higher in patients with the GC or CC genotype (GC + CC) than in patients with the GG genotype, and this difference was statistically significant (95% CI: (1.48 – 11.09).

Besides, in the breast cancer group, the GG genotype was present in 35 patients, whereas the GC + CC genotype was present in 25 patients.

As regard the level of p53 antibodies, there was a significant difference (p=0.025) between cases (11.67±11.96) and controls (4.65 ± 0.48). But, no significant difference in the different genotypes of breast cancer patients(p=0.83).

Conclusion Survivin promoter -31 G/C polymorphism is associated with the risk of developing breast cancer . C allele at position –31G/C in the promoter region of the survivin gene increases an individual's susceptibility to breast cancer. As regard the level of p53 antibodies, there was a significant difference between cases and controls. But, no significant difference in the different genotypes of breast cancer patients .The presence of p53 Abs it could be a useful marker to complement routine prognostic factors in breast cancer patients.

Introduction

Breast cancer, one of the most common malignancies affecting women, has attracted increasing attention by the international community and has evoked tremendous interest paritcularly in the medical and academic fields **(Siegel et al.,2015).**

An increasing number of studies have been performed to explore potential biomarkers which may be involved in the initiation and progression of breast cancer. Estrogen receptor (ER), progesterone receptor (PR), c-erbB-2, p53 and Ki-67 have been investigated in the conventional histopathological setting(**Ding.,2017 and Parsa et al.,2016).**

Survivin is an antiapoptotic protein belonging to the inhibitor of apoptosis protein family. It is a bifunctional protein that regulates cell division and suppresses apoptosis. Survivin is highly expressed in various human malignancies, but its expression is very low or below the level of detection in normal adult tissues **(Yazdani et al., 2012**).

The gene encoding human survivin was cloned by Ambrosini et al in 1997. Survivin spans 14.7 kb at the telomeric end of chromosome 17 and encodes the 16.5 kD wild-type survivin protein of 142 amino acids in length **(Ambrosini et al.,1997)**.

DNA polymorphisms with more than one variant (allele) having a frequency greater than 1 per cent in a human population have been estimated to occur on the average at one in every 1000 base pairs throughout the human genome **(Sherry et al.,1999**). The incidences of polymorphism in genomic DNA, their susceptibility to genetic alterations, and the risk of tumour progression in patients with cancer can vary substantially between different racial groups (**Perez et al .,2006 and Katkoori et al.,2009)**. Although most polymorphisms are functionally neutral, some affect regulation of gene expression or the function of the coded protein (**Costa et al. ,2008**).

The survivin gene codifies a multifunctional protein involved in the regulation of the cell cycle and inhibition of the apoptotic pathway, and a polymorphism located in its promoter region is associated with gene regulation. Most of the polymorphic studies are confined in promoter region among which the single nucleotide polymorphism (SNP) at -31G/C is most studied in all cancers in comparison to other polymorphic sites **(Jaiswal et al.,2012 and Theodoropoulos et al.,2010**). The aim of our work was to investigate -31 G/C single nucleotide polymorphism of survivin promoter in breast cancer patients .

P53-Abs were discovered 20 years ago during the course of tumor-associated antigens screening. The discovery of p53 mutation and accumulation of p53 in human tumors shed new light on the p53 humoral response. It is demonstrated that p53-Abs are found predominantly in human cancer patients with a specificity of 96%. Such antibodies are predominantly associated with p53 gene missense mutations and p53 accumulation in the tumor, but the sensitivity of such detection is only 30%. It has been demonstrated that this immune response is due to a self-immunization process linked to the strong immunogenicity of the p53 protein. The clinical value of these antibodies remains subject to debate, but consistent results have been observed in breast, colon, oral, and gastric cancers, in which they have been associated with high-grade

tumors and poor survival. The finding of p53-Abs in the sera of individuals who are at high risk of cancer, such as exposed workers or heavy smokers, indicates that they have promising potential in the early detection of cancer**(Angelopoulou et al.,1994).**

Patients and methods

Patients

The present study was carried out at Sohag university hospital and Sohag Cancer Institute in the period between 2016 and 2018 .100 subjects were enrolled in in this study. All were women in the age between 40 and 65 (60 breast cancer cases and 40 control divided into two groups according to age: Group 1 ≤ 50 years (27.3 ± 4.87) and Group 2 > 50(24.83 ± 6.12) .Clinical data were obtained from patients files including patient age, tumor grade ,tumor stage ,presence of lymph nodes and metastasis,estrogen and progesterone receptors positivity and CA 15-3 level. Exclusion criteriea were male breast cancer and prior chemotherapy or radiotherapy.

Blood samples were collected after taking the concent. Samples were divided between EDTA (Ethylenediaminetetraacetic acid) tubes and plane tubes. EDTA tubes were preserved at -20 c until the extraction of DNA and serum was obtained from the plane tubes after centrifugation and frozen at -20 c.

Methods

DNA extraction

By the use of : QIAamp DNA Mini Kits (QIAGEN, Lot No. 148046221)**.**

Polymorphism genotyping:

To identify genetic variants in the survivin gene promoter for screening of -31G/C polymorphism, a 329 bp region of promoter was sequenced. The survivin -31G/C polymorphism was then analyzed by polymerase chain reaction- restriction fragment containing the -31 polymorphic site was amplified using two primers;

the Forward primer:5′- TCC GTA GT GAA CCT GCG G -3′

The Reverse primer: 5′- TCC TCC GCT TAT TGA TAT GC -3′

Each PCR reaction was performed in total reaction volume of 50 µL containing25 µL .My Red Taq, 2.5 µL of each primer, 15 µL genomic DNA and 5 µL grade water .The conditions of PCR: initial denaturation step at 95ºC for 7 min, followed by 35 cycles of 95ºC for 1 minute seconds as the second melting step, 54ºC for 40 seconds for primer annealing and extension at 72ºC for 35 seconds and a final extension step of 72ºC for 7 min. Digestion was performed by incubating 10 µL of PCR products that were obtained by survivin primers with 1 µl of BsiSI enzyme in a final reaction volume of 13.5µl at 37ºC for 1 hour.

The amplified PCR products and the restriction fragments were separated by electrophoresis in a 2 % agarose gel.

ELISA kit for Detection of Anti-p53 Antibodies

P53 antibodies were measured by test kit supplied by SinoGeneClon Biotech cat Co.,Ltd.The test kit is an enzyme linked immunoabsorbant assay ELIZA for detection of p53 antibodies .We used Stat fax apparatus in measurement.Data was analyzed using IBM SPSS Statistics for Windows version 24 and Medcalc version 15.8.0.

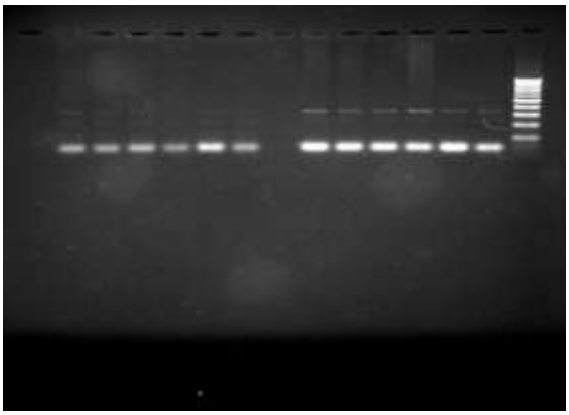
Quantitative data was expressed as means ± standard deviation. Qualitative data was expressed as number and percentage. Quantitative data was tested for normality by Shapiro–Wilk test. Mann–Whitney U test, Kruskal–Wallis H test and Spearman's correlation were used for data which wasn't normally distributed.

Results

Our present study included 100 subjects. All were women in the age between 40 and 57 (60 breast cancer cases and 40 control divided into two groups according to age: Group 1 ≤ 50 years and Group 2 > 50 . The age of patients ranged from(40 – 57) years with a mean±SEof 48.72 ± 4.94 while the age of the controls ranged from(40 – 56)years with a mean±SE of 47.55 ± 4.76.

Table (1): the clinicopathological features of the studied breast cancer patients

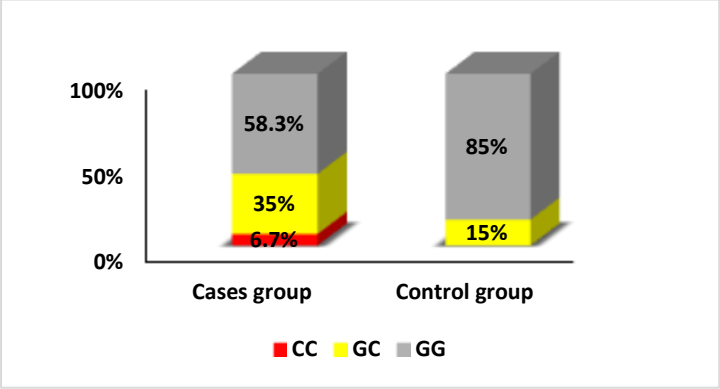
Parameter	No (%)
Tumor Stage	
1	**4 (6.7)**
2	**4 (6.7)**
3	**27 (45%)**
4	**25 (41.6)**
Tumor Grade	
1	**8 (13.3)**
2	**14 (23.3)**
3	**38 (63.4)**
Pathological Type	
Invasive Ductal Carcinoma	**52 (86.7)**
lobular carcinoma	**8 (13.3)**
Estrogen Receptor positivity	
Negative	**39 (65)**
Positive	**21 (35)**
Progesterone Receptor positivity	
Negative	**28 (46.7)**
Positive	**32 (53.3)**
Metastasis	
No	**36 (60)**
Yes	**24 (40)**
Lymph node	
No	**8 (13.3)**
Yes	**52 (86.7)**



Figure(1): PCR-RFLP to detect polymorphism of -31 G/C promoter of survivin gene of cases and controls after digestion by the restriction enzyme BiSI analyzed by 3% agarose gel. Lane 0 is the Puc8x ladder(.Lanes of cases after digestion

:1,2,3.4,5,6. Lane 1,3 are heterozygote G/C allele .Lanes 2,6 homozygote of G allele. Lane4,5 homozygote of C allele. Lanes of controls after digestion are 7,8,9,10,11,12,13. Lanes 8,9 are heterozygote G/C allele. Lanes 10,11,12are homozygote of G allele.

Figure (2): comparison between cases and control groups regarding Survivin genes.



Table(2): Univariate binary logistic regression analysis of polymorphism in predicting breast cancer

Characteristics	Cases	Control	OR (CI 95%)	P - value
GC+CC GG	25 (41.7%) 35 (58.3%)	6 (15%) 34 (85%)	4.05 (1.48 – 11.09) 1	0.007*

Table (3): comparison between cases and control groups regarding Survivin alleles.

Parameter	Cases	Control	P-value
Survivin alleles C G	29 (24.2%) 91 (75.8%)	6 (7.5%) 74 (92.5%)	0.002*

Table (4):Comparison between cases and control groups regarding the polymorphism

Parameter	Cases	Control	P-value
GC+CC GG	25 (41.7%) 35 (58.3%)	6 (15%) 34 (85%)	0.005*

Table (5): comparison between cases and control groups regarding age, family history and hormonal therapy

Parameter	Cases (N= 60)	Control (N= 40)	P-value
Age ≤ 50 years > 50 years	 37 (61.7%) 23 (38.3%)	 28 (70%) 12 (30%)	0.392
Age (years) Mean± S.D. Median (Range)	 48.72 ± 4.94 48.5 (40 – 57)	 47.55 ± 4.76 45.5 (40 – 56)	0.252**
Hormonal therapy No Yes	 28 (46.7%) 32 (53.3%)	 22 (55%) 18 (45%)	0.414
Family history No Yes	 55 (91.7%) 5 (8.3%)	 36 (90%) 4 (10%)	1*

Table (6): The relation between age and Survivin genotypes

Parameter	CC gene (N= 4)	GC gene (N= 21)	GG gene (N= 35)	P-value
Age ≤ 50 years > 50 years	 4 (100%) 0 (0.0%)	 19 (90.5%) 2 (9.5%)	 14 (40%) 21 (60%)	<0.001
Age (years) Mean± S.D. Median (Range)	 41 ± 1.16 41 (40– 42)	 45.48 ± 3.67 44 (43– 56)	 51.54 ± 3.55 52 (45 – 57)	<0.001*

Table (7): comparison between cases and control groups regarding p53 antibodies level and Survivin genotypes.

Parameter	Cases (N= 60)	Control (N= 40)	P-value
p53 (ng/ml) Mean± S.D. Median (Range)	 11.67 ± 11.96 5 (4 – 38)	 4.65 ± 0.48 5 (4 – 5)	0.025
Survivin genes CC GC GG	 4 (6.7%) 21 (35%) 35 (58.3%)	 0 (0.0%) 6 (15%) 34 (85%)	0.013*

Table (8): Receiver operating characteristic (ROC) curve of p53 (ng/ml) for optimum cut off point in predicting breast cancer

Marker	Cutoff	AUC	CI	Sensitivity	Specificity	PPV	NPV	P-value
p53 (ng/ml)	> 5	0.62	0.517 - 0.715	26.67	100	100	47.6	0.015*

Discussion

The differential expression of Survivin in tumors compared with normal cells and its requirement for cancer cell survival identify it as a potential marker in cancer diagnosis as well as an attractive therapeutic target.

Survivin is an antiapoptotic protein belonging to the inhibitor of apoptosis protein family. It is a bifunctional protein that regulates cell division and suppresses apoptosis. Survivin is highly expressed in various human malignancies, but its expression is very low or below the level of detection in normal adult tissues (**Yazdani et al., 2012).**

As a member of the family of the inhibitors of apoptosis proteins (IAPs), survivin forcefully inhibits cell apoptosis and facilitates the activation and proliferation of breast cancer cells (**Khan et al.,2017**). Admittedly, survivin is a downstream target of NF-κB, which in turn is activated by mTOR **(Kawakami et al.,2005**). Furthermore, survivin is a potent inactivator of caspase-9 and caspase-3 and is regulated via the Akt/mTOR signaling pathway (**Wilson.,2015 and Jin.,2007)**.

Several lines of evidence suggest that deregulation of Survivin expression occurs in cancer as a result of genetic (amplification of the Survivin locus on 17q25 in neuroblastoma), epigenetic (selective demethylation of Survivin exon 1 in ovarian cancer but not in normal ovaries), transcriptional (transcriptional repression by wild-type p53), and posttranslational (increased protein stability by phosphorylation on Thr34 of Survivin protein) molecular mechanisms **(Mirza et al.,2002)(Hoffman et al.,2002)(Zaffaroni.,2002)(Song et al.,2003)(Islam et al.,2000).**

DNA polymorphisms with more than one variant (allele) having a frequency greater than 1 per cent in a human population have been estimated to occur on the average at one in every 1000 base pairs throughout the human genome **(Sherry et al.,1999**). The incidences of polymorphism in genomic DNA, their susceptibility to genetic alterations, and the risk of tumor progression in patients with cancer can vary substantially between different racial groups **(Perez et al .,2006 and Katkoori et al.,2009**). Although most polymorphisms are functionally neutral, some affect regulation of gene expression or the function of the coded protein **(Costa et al.,2008).**

The survivin gene codifies a multifunctional protein involved in the regulation of the cell cycle and inhibition of the apoptotic pathway, and a polymorphism located in its promoter region is associated with gene regulation. Most of the polymorphic studies are confined in promoter region among which the single nucleotide polymorphism (SNP) at *-31G/C* is most studied in all cancers in comparison to other polymorphic sites **(Jaiswal et al.,2012 and Theodoropoulos et al.,2010).**

Our study shows a significant difference in the prevalence of the survivin promoter polymorphism (–31G > C) between the case and control groups, P value (P = 0.005*). Notably, the combined prevalence of the GC and CC genotypes (GC + CC), reflecting the prevalence of the C allele, was significantly greater in the breast cancer group than in the control group (P= 0.002*) with rates of 29% and 6%, respectively. These results imply that the C allele at position –31 in the promoter region of the survivin gene increases an individual's susceptibility to breast cancer.

Also, the risk of developing cancer was 4.05 times higher in patients with the GC or CC genotype (GC + CC)

than in patients with the GG genotype, and this difference was statistically significant (95% CI: (1.48 – 11.09).

Besides, in the breast cancer group, the GG genotype was present in 35 patients, whereas the GC + CC genotype was present in 25 patients. There were significant differences between these two groups in terms of age, hormonal therapy, stage of the tumor, metastasis and CA15-3 level.

In addition, there were no significant differences between these two groups as regard the family history, the grade of tumor, type, estrogen and progesterone receptor positivity, side, lymph node and p53 antibodies level.

As regard the relation between age, family history and clinicopathological features of the studied breast cancer patients and survivin alleles, there were significant differences between G allele and C allele in age ,progesterone receptor positivity and metastasis

But, no significant differences was found as regard the hormonal therapy ,family history ,stage of the tumor, grade , type, estrogen receptor positivity, side ,lymph node and p53 antibodies level.

Univariate binary logistic regression analysis of genetic polymorphism among breast cancer patients revealed that the age, hormonal therapy and metastasis could be a predictor variable. But, multiple binary logistic regression analysis results revealed that age and hormonal therapy are risk factors of genetic polymorphism among breast cancer patients.

Our results are in line with a study of the role of the functional polymorphism of survivin Gene (-31G/C) and risk of beast cancer in a north Indian Population that revealed that the variant genotype/allele was found in 54.1% of the cases compared with 46.5% of controls. The combined prevalence of genotype GC+CC was significantly higher in patients compared with the control group (P = .02). Analyses of odds ratios (ORs) in the patient and control groups indicated that the presence of homozygous CC genotype was associated with increased risk for development of breast cancer (OR, 2.04; 95% confidence interval [CI], 1.07-2.98). The gene frequencies for G and C alleles were statistically different between patient and control groups (OR, 1.37; 95% CI, 1.03-1.84**)(Rasool et al.,2017**). Also, results are consistent with those reported by **Yazdani et al. (2012) and Zahedi et al**. (**2012**).

In contrast to our results, a study of the association between survivin -31G/C promoter polymorphism and breast cancer in Eastern Azerbaijan, Iran, that revealed that the genotype frequencies and allele distribution of the survivin promoter -31G/C for both controls and cases were similar (**Rojhannejad et al.,2015**) .

In the present study, as regard the level of p53 antibodies, there was a significant difference (p=0.025) between cases (11.67±11.96) and controls (4.65 ± 0.48). But, no significant difference in the different genotypes of breast cancer patients(p=0.83).

There was no significant difference between the age, history and clinicopathological features of the studied breast cancer patients and the level of p53 antibodies. Besides, there was no significant difference between the level of p53 antibodies among the studied breast cancer patients (p= 0.887).

Previous studies in accordance with our results, **Yamamoto et al, (2012)** analyzed serum anti-p53 antibody levels in 124 patients with breast cancers and 7 patients with benign disease between April 2012 and March 2013, as well as levels of serum

carcinoembryonic antigen (CEA) and cancer antigen CA15-3. They found that twenty-two of 124 patients with breast cancer had an increased concentration of anti-p53 antibodies. By distribution of clinical stage, in stage 0-II the positive ratio of anti-p53 antibodies was significantly higher than that of CEA (p=0.03) and CA15-3 (p=0.01). There was a significant correlation between anti-p53 antibodies and family history (p=0.03). Triple-negative cancer also showed a significant correlation with anti-p53 antibodies (p=0.007). In patients with multiple and/or bilateral breast cancer, the level of anti-p53 was significantly higher than in unilateral breast cancer (62.5% vs 14.7%, p=0.004).

Also, **Ahmed et al., (2011**) detect antibody against p53 in the sera of eight patients (20%), the mean serum levels of p53-Abs showed significant increase in patients (32.8±5.4pg/ml) when compared to control group (4.1±0.3pg/ml) P<0.001. Moreover the positive rate of serum p53-Abs was not related to each of age, stage and histologic grade of tumor. Receiver operating characteristic (ROC) curve of p53 antibodies for optimum cut off point in predicting breast cancer shows high specificity (100) but no sensitivity (26.67). This is in line with the study done **by Müller et al., (2006**) for testing for anti-p53 antibodies increases the diagnostic sensitivity of conventional tumor markers. The aim of this study was to determine whether anti-p53 antibodies are of clinical significance as a serological marker in the diagnosis and monitoring of malignancies. A total of 1874 serum samples from 591 patients with various types of cancer, esophageal, gastric, colorectal, pancreatic, hepatocellular, breast, and urogenital cancer, and 436 control individuals were analyzed by immunoblot for antibodies against p53.The anti-p53 antibody test was correlated with expression of conventional tumor markers, survival and the clinicopathological features of malignant disease. Anti-p53 antibodies were found in 23.4% (138/591) of the sera of patients with malignant disease (range 11.5-34%). The detection of anti-p53 serum antibodies had a specificity of 100% for malignancy (p<0.0001). The overall sensitivity of measuring established tumor markers was 62.9% (372/591). The elevation of conventional tumor markers and the presence of anti-p53 antibodies in the sera of patients with malignant disease turned out to be an independent variable (p<0.05).

Combination of established tumor markers with the anti-p53 antibody test led to an increase in diagnostic sensitivity of 8% (49/591) (p<0.01). Thus, the independence of anti-p53 antibodies from established tumor markers allows the serological detection of additional tumor patients. Kaplan-Meier analysis revealed a trend toward a poorer prognosis in hepatocellular carcinoma and breast cancer patients who were anti-p53 serum positive.

Testing for anti-p53 antibodies can increase the diagnostic sensitivity when used in combination with measurement of conventional tumor markers. This increase is achieved without a parallel decrease in specificity.

However it can be used for monitoring the response of breast cancer patients to therapy and in detecting recurrent disease **(Keyhani et al., 2005).**

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