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Significance of Serum Survivin and -31G/C Gene Polymorphism in the Early Diagnosis of Breast Cancer in Egypt

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Abstract

The majority of breast cancer cases are discovered in later disease stages, thus affecting survival rate. We studied the impact of survivin -31 G/C single nucleotide polymorphism and its serum level alteration. Minor allele C and the GC + CC genotype occurred more frequently in breast cancer patients; further, increased breast cancer risk with associated with elevated serum survivin level. These data could help in early diagnosis and understanding of the pathogenesis of breast cancer.

Background: Breast cancer is one of the most relevant malignancies among women. Molecular abnormalities in promotor region of survivin gene may account for overexpression of survivin and increased breast cancer risk. This study aimed to explore the potential association between survivin promotor gene -31G/C single nucleotide polymorphism (rs9904341) and its serum level alteration on one hand, and the risk of breast cancer in Egyptian patients on the other hand. It also aimed to assess the usefulness of survivin as an early noninvasive diagnostic biomarker and in breast cancer staging. **Patients and Methods:** A total of 135 patients with physically and pathologically confirmed breast cancer and 40 unrelated control subjects as well as 40 patients with benign breast mass were recruited from the early detection unit at National Cancer Institute, Cairo University. Genotyping was performed using allelic discrimination probes by real-time quantitative PCR and serum survivin by enzyme-linked immunosorbent assay. **Results:** The minor allele C of -31G/C survivin single nucleotide polymorphism was more frequent in breast cancer patients (19.3%) compared to the control group (7.5%). Furthermore, subjects with the GC + CC genotype were at increased risk of breast cancer compared to the GG genotype of the control group and also the benign group. Moreover, those patients exhibited higher serum levels of survivin compared to GG genotype. There was also significant elevation of serum survivin in different breast cancer stages. **Conclusion:** Genetic variation in -31G/C of the survivin gene may contribute to the disposition of breast cancer in the Egyptian population. Serum survivin alteration played a pivotal role in the pathogenesis of breast cancer.

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Introduction

Breast cancer is the most relevant endocrine-related cancer associated with death in women.[1] A complex combination of environmental and genetic factors contributes to its prevalence.[2] Incidence rates are rapidly rising in developing countries, though it is more common in developed countries.[3] In Egypt, it is the most common cancer in female subjects, and the second most common cancer in both sexes.[4]

Survivin—a bifunctional protein—is a unique member among the inhibitor of apoptosis proteins (IAPs) family that exhibits cell-

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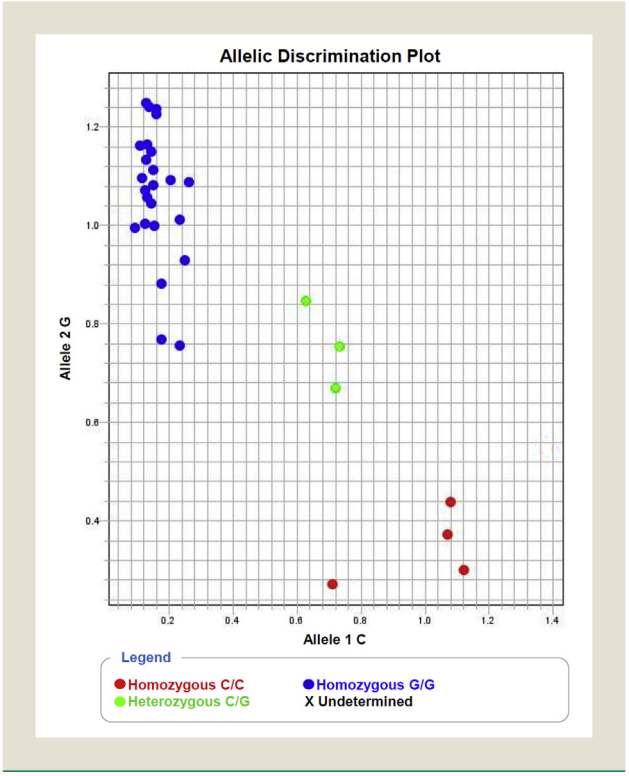
Figure 1 Allelic Discrimination Plot for rs9904341 SNP on Applied Biosystems Step One Plus 7500 qPCR System. (Blue Dots Represent G/G Genotype Near *Y*-Axis; C/C Genotype is Plotted Near *Y*-Axis as Red Dots; G/C Genotype is Plotted as Green Dots in Middle Between Both Axes)



Abbreviations: SNP = single nucleotide polymorphism; qPCR = real-time quantitative PCR.

cycle—regulated expression peaking at mitosis. Also, it plays a pivotal role in suppressing apoptosis.[5] Survivin plays a vital role in chromosomal attachment, spindle-assembly checkpoint, S-phase progression, inhibition of caspase-dependent/independent cell death, and inhibition of mitochondrial and death receptor (tumor necrosis factor-related apoptosis inducing ligand—mediated apoptosis).[6]

Similar to other members of IAPs, survivin inhibits the terminal effector of caspase-3 and caspase-7 and interferes with caspase-9 activity and processing, thus blocking a common step downstream of mitochondrial cytochrome C release. Overexpression of survivin in malignancies could bypass an apoptotic checkpoint and favor abnormal progression of transformed cells through mitosis.[7]

The human survivin/baculoviral IAP repeat-containing 5 (*BIRC5*) gene spans 14.7 kb on the telomeric position of chromosome 17, localized to band q25 and encoding a 16.5 kDa protein of 142 amino acids.[8] Structurally, it consists of a single baculovirus IAP repeat domain, together with an extended COOH-terminal α-helical coiled-coil domain. Meanwhile, it does not have a RING-finger domain, as do other members of IAPs.[9]

The survivin gene promoter region contains several cell-cycle—dependent elements (CDEs) and a cell-cycle homology region (CHRs), which are characteristic of G2M-expressed genes. The cell-cycle—dependent expression of the survivin gene is mediated by CDEs and CHRs, located in the proximal promotor region of survivin gene.[10] Deletion in the promotor region results in the lack of cell-cycle—dependent expression of survivin in HeLa cells. Likewise, functional polymorphisms might affect survivin gene expression or enzymatic activity, and consequently augment the susceptibility to cancer.[11]

Several single-nucleotide polymorphisms (SNPs) have been extensively studied in the promotor region of survivin. Compared to other SNPs, -31G/C polymorphism (rs9904341), located at the CDE/CHR repressor binding site (231) from the first nucleotide of the ATG start codon, has been proven to have functional significance. In addition, other survivin gene promoter polymorphisms (-644T > C, -625G > C, and -241T > C; MAF > 5%) have been found to be in linkage disequilibrium with -31 G > C.[12] In this regard, earlier studies have revealed that the -31C allele has a significantly higher promoter activity than the -31G allele.[13] Furthermore, the overexpression of survivin is thought to be linked with -31G/C polymorphism at both messenger RNA and protein levels, owing to its ability to modify cell-cycle—dependent transcription via functional disruption of binding at the CDE/CHR repressor motifs.[14]

Survivin is highly expressed in cancer cells but absent in normal cells. This observation makes survivin a suitable target for cancer therapy and suggests that its reexpression may occur during the early malignant transformation or following the imbalance between cell proliferation and death.[15] Therefore, the present work was designed to explore for the first time the potential association between survivin promotor gene -31G/C SNP (rs9904341) and its serum level alteration in Egyptian breast cancer patients. It also aimed to assess the usefulness of survivin as an early noninvasive diagnostic biomarker that could be effective in breast cancer staging.

Patients and Sample Collection

Study Participants

This study comprised 135 Egyptian women with ages ranging from 18 to 72 years who had malignant breast cancer and who were selected from the early detection unit at the National Cancer Institute (NCI),

Table 1 Age and Histopathologic Features of Study Groups

Characteristic	Controls	Benign Tumor	Malignant Breast Cancer		
			T1	T2	T3 and T4
No. of women	40	40	45	45	45
Age (years)	36.85 ± 13.09	40.35 ± 13.80	39.02 ± 14.04	37.4 ± 12.76	41.13 ± 14.04
Tumor size (mm)	0	15.85 ± 6.26	14.29 ± 2.87	36.37 ± 9.00	69.69 ± 9.94

Data are expressed as mean ± SD.

Table 2 Differences in Genotype Frequency and Allele Distribution of Survivin -31 G/C Polymorphism by Group

Characteristic	Controls (N = 40), N (%)	BC Patients (N = 135), N (%)	OR (95% CI)	χ^2	*P*
Genotype					
GG	35 (87.5)	85 (62.9)	4.11 (1.51-11.19)	8.62	.003*
GC + CC	5 (12.5)	50 (37.1)			
Allele					
G	74 (92.5)	218 (80.7)	2.94 (1.21-7.13)	6.17	.013*
C	6 (7.5)	52 (19.3)			

Abbreviations: BC = breast cancer; CI = confidence interval; OR = odds ratio.
*Statistically significant.

Cairo University, Egypt. They were recruited between April 2015 to January 2017. Patients did not receive radiotherapy or chemotherapy before surgery and during sampling. The study also included an age-matched control group comprising 40 apparently healthy women with no history of malignancy. Additionally, the benign group included 40 patients diagnosed with a benign breast mass. Physical examinations and diagnoses were processed by physicians in the medical oncology unit of the NCI, where routine clinical and pathologic examinations were performed. Once the diagnosis was confirmed, clinical staging of the disease was done after mastectomy according to the American Joint Committee on Cancer tumor, node, metastasis classification system. The malignant breast cancer group was further divided into 3 groups according to tumor size: T1 (< 20 mm), T2 (20-50 mm), and T3/T4 (> 50 mm), where each group included 45 breast cancer patients. Informed consent was obtained from the participating patients in adherence with the guidelines of the ethical committee of the NCI, Cairo, Egypt. The study was also approved by the ethical committee of the Faculty of Pharmacy, Cairo University, Egypt (approval Bio 7.4.1), and in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Sample Collection

Whole blood was collected from recruited subjects by trained laboratory technicians in Vacutainers and was divided into 2 aliquots. Serum was obtained from the first aliquot by centrifugation of Vacutainers at 5000 rpm for 15 minutes at 25°C. The separated serum samples were stored at −80°C until used for survivin analysis. The second aliquot was collected in EDTA Vacutainers and was stored at −80°C, to be used for DNA extraction.

Methods

DNA Extraction and Genotyping

The DNA extraction was performed using Genomic DNA Purification (Wizard Promega; Promega, Madison, WI) following the manufacturer's instructions. The concentration of the extracted DNA was measured using the Nano Drop (ND-1000) Spectrophotometer (Nano Drop Technologies, Wilmington, DE). The ratio of absorbance of extracted DNA at 260 and 280 nm was 1.7 to 1.9. Genotyping was performed by real-time quantitative PCR with TaqMan allelic discrimination assay software (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA) using the Applied Biosystems Step One Plus 7500 real-time quantitative PCR System. The PCR was used to target the 183 base pair region using the forward primer 5′-GGGTGGACCGCCTAAGA-3′ and reverse primer 5′-GGGCCAGTTCTTGAATGTAGAG-3′. Primers were designed using the NCBI/Primer-BLAST database (National Center for Biotechnology Information, Bethesda, MD, USA; https://blast.ncbi.nlm.nih.gov/Blast.cgi). In each well, the basic master mix volume was 3.75 µL containing 2.5 µL 2× TaqMan Universal PCR Master Mix (Thermo Fisher Scientific), 0.25 µL 20× SNP assay mix (Tamra quencher was prepared using 4 µM for each of the VIC and FAM probe and 18 µM for each of the nonlabeled primer), and 1 µL of nuclease-free water. A volume of 1.3 µL of individual DNA sample was then added to each well. The program was started with heating at 95°C for 10 minutes for denaturation, followed by 40 cycles (at 95°C for 15 seconds and at 60°C for 1 minute) for annealing and extension. Allelic discrimination was assessed according to Applied Biosystems software. Genotypes were quantified using the allelic discrimination plot shown in Figure 1.

The integrity of the amplified DNA was assessed by DNA agarose gel electrophoresis using 3% agarose gel (Invitrogen;

Table 3 Differences in Genotype Frequency and Allele Distribution of Survivin -31 G/C Polymorphism by Group

Characteristic	Benign Tumors (N = 40), N (%)	BC Patients (N = 135), N (%)	OR (95% CI)S	χ^2	*P*
Genotype					
GG	33 (82.5)	85 (62.9)	2.77 (1.14-6.74)	5.36	.02*
GC + CC	7 (17.5)	50 (37.1)			
Allele					
G	71 (88.8)	218 (80.7)	1.88 (0.88-4.01)	2.75	.1*
C	9 (11.2)	52 (19.3)			

Abbreviations: BC = breast cancer; CI = confidence interval; OR = odds ratio.
*Statistically significant.

Table 4 Serum Survivin Concentration by Group

Group	N	Survivin Concentration (ng/L), Mean ± SD
Control	40	50.9 ± 13.9
Benign tumor	40	63.2 ± 14.9
Malignant Breast Cancer		
Total	135	569.6 ± 193.1[a]
T1	45	425.2 ± 74.4[a]
T2	45	503.9 ± 120.6[a,b]
T3 and T4	45	779.7 ± 150.3[a,c,d]

[a]Significantly different from controls and benign tumor group at $P \leq .0001$.
[b]Significantly different from T1 at $P \leq .01$.
[c]Significantly different from T1 at $P \leq .0001$.
[d]Significantly different from T2 at $P \leq .0001$.

Thermo Fisher Scientific) in single-strength Tris–acetic acid–EDTA buffer. Regarding electrophoresis, 5 μL of the PCR products were mixed with 1 μL of 6× loading dye, and the samples were loaded into the wells. Electrophoresis was operated at 110 V for 15 minutes. The PCR products were visualized using an ultraviolet transilluminator.

Determination of Serum Survivin Level

Survivin in serum was measured by enzyme-linked immunosorbent assay (Bioassay Technology Laboratory; Korain Biotech, Shanghai, China) following the manufacturer's instructions.

Statistical Analysis

Comparisons of genotype and allele frequencies between control, benign, and breast cancer groups were performed by the chi-square test. Hardy-Weinberg equilibrium (HWE) was used to examine whether the population was representative or not. Statistical data were reported as mean ± SD, frequencies (number), and percentages when applicable. Comparisons of numerical variables between the studied groups were performed by the Student *t* test to compare independent samples from 2 groups when the samples were normally distributed and the Mann-Whitney *U* test to compare independent samples when the samples are not normally distributed. The receiver operating characteristic curve was plotted to determine the cutoff values and to analyze the diagnostic utility of serum survivin. $P < .05$ was considered statistically significant. Statistical analysis and comparison between groups were performed by GraphPad Prism 6.01 software (GraphPad Software, La Jolla, CA).

Results

Characteristics of Study Participants

Table 1 lists the count, age, and tumor size of studied participants. The HWE was used to analyze the polymorphic distribution of various genotypes in the study population. Because the observed and expected frequencies of the GG, GC, or CC genotype were not statistically significant, the study population was deemed consistent with HWE.

Genotypes and Allele Distribution of Survivin -31G/C Polymorphism

Table 2 shows significantly higher frequencies of survivin -31 GC + CC genotype and C allele in breast cancer patients compared to the control group (37.1% vs. 12.5% and 19.3% vs. 7.5%, respectively).

Table 3 shows significantly higher frequencies of survivin -31 GC + CC genotype in breast cancer patients compared to benign tumors group (37.1% vs. 17.5%).

Survivin Serum Levels

As shown in Table 4, a significant increase in serum level of survivin was observed in malignant breast cancer patients collectively and individually in each stage compared to the benign and control groups. Interestingly, the serum level of survivin was significantly increased with increasing the stage of the disease. It is thus clear that as the tumor size increases, the survivin level tends to increase as well.

Receiver-Operating Characteristic Curve Analysis

On the basis of the analysis of receiver operating characteristic curves, a serum survivin cutoff value of ≥ 136.4 ng/L may be used as a cutoff point at which 92.7% of breast cancer patients (T1 and T2 stage disease) can be correctly diagnosed early, but 13% of normal subjects have false-positive findings. According to the receiver operating characteristic curve, the sensitivity was 92.7% and the specificity 86.9% at $P \leq .001$, with an area under the curve value of 0.89.

Correlation Analyses Among Serum Survivin Level, Age, and Tumor Size

From Table 5, it is evident that there was strong positive correlation between serum survivin concentrations in different breast cancer stages and tumor size. However, there was no correlation between age and serum survivin concentration.

Association Between -31G/C Survivin Gene Polymorphism and Serum Levels of Survivin

Table 6 indicates that combined C variants of survivin polymorphism (GC + CC) were associated with significantly high values of serum survivin. The same trend of change was detected in both control and benign tumor groups.

Discussion

Survivin has become an attractive molecule for the early detection and prognosis of breast cancer because of its differentiated expression between normal and cancerous tissues. Additionally, the CDEs and CHRs located at the promotor region of the survivin gene control the survivin expression.[16] Although many SNPs were studied in the survivin gene promotor region, only the -31G > C polymorphism has shown functional significance, and it thus has been extensively studied for its impact on cancer risk.

This study aimed to explore the potential association between the survivin promotor gene -31G/C SNP (rs9904341) and its impact on the risk and serum levels of survivin in Egyptian breast cancer patients. We found that the percentage of the -31C variant allele was relatively higher in breast cancer patients (19.3%) than in both benign (11.2%) and control (7.5%) groups. Moreover, the percentage of the GC + CC genotype was higher in breast cancer patients (37.1%) than in benign (17.5%) and control (12.5%) subjects. Thus, subjects carrying -31G/C + CC genotype and allele

Table 5 Correlation Between Serum Survivin Level With Age and Tumor Size

Characteristic	Serum Survivin (ng/L)											
	Control		Benign Tumors		Malignant Breast Cancer		T1		T2		T3 and T4	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Age (years)	0.190	NS	0.127	NS	0.009	NS	−0.132	NS	−0.010	NS	−0.102	NS
Tumor size (mm)	0	NS	0.035	NS	0.862	<.01	0.650	<.01	0.606	<.01	0.776	<.01

Abbreviations: NS = not significant; *r* = Spearman rank correlation coefficient.

C were at higher risk of developing breast cancer compared to those carrying the -31GG genotype in an Egyptian population. In addition, our results showed that the serum level of survivin increased positively with tumor size, and consequently with breast cancer staging. There was also a significant association between GC + CC genotype and elevated serum survivin levels. The selected population complied with the HWE, which indicates that it is representative to the whole population and is not biased. Also, it is worth noting that statistical significance was more prominent and useful when comparing the breast cancer group as a whole to both the benign and control groups in terms of genotype.

The survivin gene promotor -31G/C polymorphism has been extensively studied in various types of cancer in different population, although these studies have showed conflicting results. A study performed by Guo et al[17] on Chinese lung cancer patients showed that survivin -31G/C polymorphism was associated with increased lung cancer risk. Also, the functional survivin -31G/C genetic variant was reported to have a substantial influence on prostate cancer susceptibility and evolution in a Chinese population.[18] Another study on a Chinese population postulated that survivin -31G/C polymorphism might contribute to the risk of developing colorectal cancer.[19] Moreover, a study on a Turkish population stated that individuals carrying the survivin -31G/C genotype exhibited a significantly lower risk of non−small-cell lung cancer.[20] Qin et al[21] added that the development and progression of renal-cell carcinoma is significantly enhanced by this polymorphism in Chinese individuals. Upadhyay et al[22] have reported that survivin promoter region polymorphism (-31G > C) is associated with increased susceptibility to esophageal cancer in a Northern Indian population.

Regarding studies carried out on breast cancer patients, a study conducted on a North Indian population suggested that there is an association of -31G/C survivin polymorphism with breast cancer at a genotypic and allelic level, where the homozygous CC genotype illustrated increased risk for development of breast cancer.[23] Another study on a Turkish population assumed that carrying the -31C allele was statistically significant in terms of susceptibility to breast cancer and recommended the use of survivin gene polymorphism as a risk factor in breast cancer.[24]

However, some studies have failed to find any associations. A study carried on a Serbian population concluded that -31G/C polymorphism in the promoter of the survivin gene could not be considered as a risk factor for oral squamous-cell carcinoma and skin basal-cell carcinoma.[25] Similarly, Borges Bdo et al[26] found no significant differences in the -31G > C genotype or allele frequencies between patients with gastric cancer and the control group. Also, Borbely et al[27] reported that this polymorphism did not increase the prevalence of developing cervical cancer.

A precise mechanism for the up-regulation of survivin expression is not completely understood. The major control for survivin expression is thought to be at a transcriptional level. Polymorphism at -31G/C in the survivin promoter could affect the gene transcriptional activity by affecting the binding of elements regulating cell-cycle−dependent transcription of this gene.[27] As a result, higher transcriptional activity is more likely to be associated with the -31C allele and the -31G/C + CC genotypes in Egyptian breast cancer patients.

Table 6 Genotypes of -31G/C Survivin Gene Polymorphism and Serum Levels of Survivin

Group	Genotype	N (%)	Survivin (ng/L), Mean ± SD
Control	GG	34 (85)	47.22 ± 2.7
	GC + CC	6 (15)	71.96 ± 5.33[a]
Benign tumor	GG	30 (75)	57.15 ± 2.68
	GC + CC	10 (25)	81.20 ± 5.22[b]
Breast cancer	GG	85 (63)	477.6 ± 15.25
	GC + CC	50 (37)	726.1 ± 23.84[c]

[a]Significantly different from GG genotype at $P \leq .01$.
[b]Significantly different from GG genotype at $P \leq .001$.
[c]Significantly different from GG genotype at $P \leq .0001$.

It is worth mentioning that the serum level of survivin in this study was significantly higher in different stages of breast cancer patients compared to benign and control groups. Our results are in agreement with Khan et al,[28] who found that survivin serum levels were significantly higher in breast cancer samples compared to controls, particularly exosomal survivin-2B. Thus, it could serve as a diagnostic marker in patients with early breast cancer. Another study suggested the possibility of using serum survivin autoantibody as a biomarker for diagnosis of breast cancer.[29] Similarly, Lv et al[30] found that survivin was one of the 4 marker genes detected in circulating tumor cells in the blood of Taiwanese women with breast cancer. Yie et al[31] postulated that circulating breast cancer cells express survivin in the peripheral blood of breast cancer patients, but not in healthy women. The presence of survivin-expressing circulating breast cancer cells was found to be significantly associated with various clinicopathologic features such as vessel infiltration, histologic grade, tumor size, nodal involvement, estrogen receptor/progesterone receptor status, HER2 expression, and clinical stages of the disease. However, the exact mechanism that the -31 G/C SNP possibly affects the secretory characteristics of survivin needs to be elucidated by further functional studies. Hence, genetic variation in the survivin promotor region could affect the survivin gene expression and influence tumor development.[32]

An earlier study investigated survivin serum levels in early-stage breast cancer and stated that there was no difference in its levels between patient and control subjects.[33] Another study examined survivin levels in serum and urine of breast cancer patients and found that there was no significant difference in serum and urine levels between patients with breast cancer and healthy controls.[34] This discrepancy may be due to the relatively small sample size, the different methods used for assay, and the various subcellular localizations of survivin in breast carcinoma.

Conclusion

The -31G/C polymorphism in the survivin gene promotor is associated with elevated levels of serum survivin and consequently increased susceptibility to breast cancer. Moreover, increased levels of serum survivin could be regarded as an early diagnostic biomarker of breast cancer.

Because genetic polymorphisms may differ between ethnic groups, further studies are required to examine the associations between survivin gene polymorphism, serum survivin, and breast cancer on other ethnicities and larger populations.

Clinical Practice Points

- Early diagnosis of breast cancer has been a challenge in many countries; it is probably diagnosed in later stages, which makes treatment harder. Moreover, the etiology of breast cancer is not well defined.
- We found that survivin could be used as an early noninvasive diagnostic biomarker for breast cancer. Moreover, the studied polymorphism in the promotor region of the survivin gene was found to contribute to the increased risk of breast cancer in an Egyptian population.
- This study provides substantial evidence for the use of survivin in the early diagnosis and staging of breast cancer patients. In addition, it could aid in finding new approaches and strategies in its treatment.

Disclosure

The authors have stated that they have no conflict of interest.

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