RESEARCH ARTICLE

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VNTR Polymorphism in the Intron 5 of SIRT3 and Susceptibility to Breast Cancer

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Abstract

Background: Breast cancer recurrence and metastasis are associated with alterations in the cellular stress responses that influence tumour signalling. Sirtuin3 (SIRT3), a mitochondrial deacetylase is the regulator of mitochondrial metabolism and oxidative stress affecting tumour cell responses. Genetic variants or dysregulation of SIRT3 was known to associate with poor prognosis of recurrence and relapse in few cancers. **Methods:** The current case-control study was conducted in Hyderabad, India. A total of 200 primary female breast cancer cases were recruited, irrespective of age and clinical subtype. However, secondary or recurrent breast cancer cases were excluded from the study. A total of 202 age and gender-matched healthy controls without any familial inheritance of either breast or other cancer and having similar ethnicity as cases were recruited. The blood samples of both cases and controls were collected from Nizam's Institute of Medical Sciences (NIMS), Hyderabad. Our study is an attempt to evaluate the association of SIRT3 VNTR polymorphism in intron 5 with the development and progression of breast cancer by PCR-based genotyping. **Result:** The statistical analysis of the results with respect to epidemiological and clinical phenotypes revealed significant association of 0R allele and 0R/0R genotype with breast cancer risk ($p<0.01$). The odds ratios also were found to be significant i.e., 0R/0R [OR(CI): 2.67(1.54-4.65); p=0.000005] genotype. Also, the epidemiological and clinical variables have shown significant association with the risk of onset of the disease. Therefore, the influence of lack of repeats at intron 5 harbouring enhancer site on altered expression of SIRT3 might confer increased susceptibility to breast cancer. **Conclusion:** The VNTR polymorphism in the intron 5 region of SIRT3 gene could serve as a molecular marker for detection of breast cancer onset. Further studies are warranted to study the prognostic and therapeutic significance of this SIRT3 polymorphism.

Keywords: Breast cancer- VNTR polymorphism- Sirtuin 3- Relapse- Prognosis

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Introduction

Breast cancer is highly prevalent among women worldwide and has become a primary concern lately. Although the survival rate of patients has improved over time, about 178361 new cases, have been reported in India in the year 2020, with a mortality rate of 72.3% (Malvia et al., 2017; Globacon, 2018; Bhattacharyya et al., 2020). This high prevalence of breast cancer may be due to relapse or recurrence followed by metastases despite definitive treatment (Valastyan and Weinberg et al., 2011; Riggio et al., 2021). This calls for immediate attention towards targeting new and potential therapeutic markers to effectively treat recurrent and metastatic cases and the aggressive forms of TNBC. Nevertheless, the cross-talk among the molecular mechanisms of chemoresistance has to be delineated to overcome acquired and intrinsic drug resistance (Zheng, 2017; Cosentino et al., 2021).

The peculiar behaviour of a cancer cell is influenced by various intrinsic and extrinsic cellular factors. Notably, the metabolome dynamics in malignant transformation is known to be associated with molecular reprogramming affecting cellular stress response mechanisms and genome integrity (Negrini et al., 2010; Pastor and Mostoslavsky, 2012; Faubert et al., 2020; Ponomarev et al., 2022;). The levels of key metabolic variables such as Pi, ATP, NADPH, and ROS (Reactive Oxygen Species) influence regulatory dependent activation of cancer signalling pathways (Presegue and Vaquero et al., 2011; German and Haigis, 2015). Among the diverse groups of regulatory proteins, Sirtuins, a family of NAD+ dependent deacetylases, mediate target-specific modifications to effectuate cell responses in particular oxidative stress pathways (Michishita et al., 2005; Presegue and Vaquero, 2011;

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Singh et al., 2018).

Current research indicated dysregulation of Sirtuins, mainly emphasizing the role of SIRT1 in various cancers (Lin and Fang, 2013; Choupani et al., 2018). Recent in vitro and in vivo studies on SIRT3, a critical mitochondrial regulator (Mas et al., 2017), demonstrating its dual role as an oncogene and tumour suppressor whose critical expression was known to interfere with suppression of glycolysis, increased production of ROS and proliferation of malignant cells in breast tissue (Finley and Haigis, 2012; Haigis et al., 2012; Zhang et al., 2020). However, limited studies were focused on the association of genetic variants in the gene encoding SIRT3 at chr11p15.5, known to modulate its function and activity, posing substantial cancer susceptibility. Identification and subsequent validation of these variants may aid clinicians in diagnosing the disease in both early stages and relapse conditions.

In view of our recent findings regarding the development of chronic myeloid leukaemia and its association study with variable number tandem repeat (VNTR) polymorphism in the intron 5 region of the SIRT3 gene (Velpula et al., 2021), we attempted to analyse this variant in breast cancer patients to assess its role in the onset and progression of the disease.

Materials and Methods

For the current case-control study, 200 clinically confirmed breast cancer cases and 202 healthy control samples were studied. The patients were recruited from Nizam's Institute of Medical Sciences (NIMS), Hyderabad and ethnically matched normal healthy individuals without family history of any cancers were recruited as control group. The control group included volunteers from neighbourhood, local health centres, various institutions and localities in Hyderabad during the period of 2010-2014. The study was approved by the ethical committee of the Department of Genetics, Osmania University and NIMS, Hyderabad.

After obtaining informed consent from each case, 5ml of peripheral blood samples in EDTA vacutainers were collected. As per structured proforma, detailed epidemiological as well as clinical information of each patient was recorded with the help of oncologists for genotype-phenotype comparisons to evaluate the association of the variables.

Genomic DNA was isolated from blood and tissue by rapid non-enzymatic/salting out method and concentration is estimated by nanodrop (Thermo-Fisher Nanodrop Lite). Polymerase chain reaction using target specific primers to amplify the VNTR region at intron 5 of SIRT3 gene. The forward primer sequence 5'-TTCCTGAAGCTGGGTACA-3' and reverse primer sequence 5'-CATTCACCTT CCCAAAGTGG-3' were designed using primer3 primer designing tool. The reaction mixture (10µl) is composed of 10X PCR buffer, 25mM MgCl2, 25mM dNTP mix, 25pM of each forward and reverse primer, 0.25-0.5 U of Taq DNA polymerase and 1µL genomic DNA (50ng). PCR cycle program was 5 min at 95°C, 32 cycles of 40 sec at 95°C, 40 sec at 52.9°C, and 1 min at 72°C, and the terminal extension was 5min at 72°C. Amplified PCR products were electrophoresed on 2% agarose gel containing 0.5 µg/ mL ethidium bromide and visualized on a UV transilluminator and the gel picture was captured on gel doc (Bio-Rad).

The samples were genotyped for VNTR polymorphism based on the sizes of the PCR product. The repeat size of the VNTR was 72bp. Samples with zero repeats (0R) corresponded to PCR product size of 421bp. With an increase in each repeat number, the respective bands were observed; 493bp (1R), 565bp (2R), 637bp (3R) and so on.

The allelic and genotypic frequencies among cases and controls of breast cancer patients were calculated. The data was distinguished with respect to age at onset, familial history, tumour size, clinical subtype, receptor status (estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2) and nodal status for appropriate statistical comparison. The chi-square, odds ratios and p values were calculated using SPSSv26 and MEDCALC online software tools. p value of 0.05 is considered significant for all comparisons.

Results

The genotypic analysis of 200 breast cancer cases and 202 healthy controls revealed in total 4 VNTR alleles (0R, 1R, 2R and 3R) and 7 genotypes (Figure 1). The genotype distribution did not deviate from that of controls (Chi-square value=34.6; p=0.000005) and 2R/2R genotype was the most common type among controls (24.75%), hence, considered as reference genotype. Comparison of controls with respect to this genotype has clearly shown elevation of 0R/0R heterozygote among cases (45.5%) when compared to controls (22.77%) with significant odds ratio of 2.67 (95%CI: 1.54-4.65) indicating 0R/0R as risk conferring genotype for breast cancer. Similarly, the allelic frequency distribution revealed significant heterogeneity (Chi-square=53.7; p=0). The 0R allele has shown elevation among cases (46.75%) when compared to controls (24.25%) with a significant odds ratio of 2.79 (95%CI: 1.94-4.01) indicating 0R as risk conferring allele (Table 1).

To understand the probable risk nature of 0R repeat allele relative to 2R (the wild type and the most common allele), the data was graded into four genotype groups i.e., 2R/2R as reference group (group1), 0R/0R as risk group (group 2), the low repeat genotypes as group 3 (0R/1R, 1R/1R) and the high repeat genotypes as group 4 (1R/2R, 2R/3R, 3R/3R). The genotype group distribution revealed significant variation in the frequency for group 2 (0R/0R) emphasizing that 0R allele may pose relatively greater risk [OR: 2.67 (95%CI: 1.54-4.65)]. With regard to epidemiological variables, the genotype frequency distribution was found to be slightly elevated in patients of ≤40 years of age (39.13%) when compared to patients of >40 years of age (35.54%) thus indicating its risk at early age. This trend is also observed in the other higher repeat groups (group 3 and 4), indicating the risk of both low repeat and high repeat genotype risk. With respect to familial history, the genotype distribution was found to be insignificant. Likewise, with respect to tumour

Table 1. Frequency Distribution of Genotypes and Alleles of SIRT3 Intron 5 VNTR Polymorphism among Breast Cancer Cases and Controls. * Indicates significance at p<0.05.

	Cases (n=200)		Controls (n=202)		Odds ratio (95% CI)	Odds ratio (p value)	Chi square test value (p)
Genotype	N	%	N	%	Reference		
2R/2R	37	18.5	50	24.75			34.6 (0.000005)*
0R/0R	91	45.5	46	22.77	2.67 (1.54-4.65)	0.0005*	
0R/1R	5	2.5	6	2.97	1.13 (0.32-3.98)	0.8535	
1R/1R	46	23	47	23.26	1.32 (0.73-2.38)	0.3515	
1R/2R	5	2.5	17	8.41	0.39 (0.13-1.17)	0.0952	
2R/3R	12	6	16	7.92	1.01 (0.43-2.39)	0.9756	
3R/3R	4	2	20	9.9	0.27 (0.08-0.86)	0.0264*	
Alleles	N	%	N	%	Odds ratio (95% CI)	Odds ratio (p value)	53.7 (0)*
2R	91	22.75	133	32.92	Reference		
0R	187	46.75	98	24.25	2.79 (1.94-4.01)	0.0001	
1R	102	25.5	117	28.96	1.27 (0.87-1.85)	0.2069	
3R	20	5	56	13.86	0.52 (0.29-0.93)	0.027	

In table 1, the frequency distribution of both genotypes and alleles have been calculated among breast cancer cases and controls

size, the genotype frequency distribution was found to be insignificant. However, the genotype frequencies were elevated in patients with tumour size >3mm when compared to patients with tumour size <3mm. And the genotype specific risks were observed in both the low repeat (group 2 and 3) and high repeat (group 4) groups when odds ratios were calculated. With respect to clinical subtype too, the genotype frequency distribution was found to be insignificant. The 0R/0R risk genotype and group 2 genotype frequencies were elevated in lobular

Table 2. Frequency Distribution of Genotypes of SIRT3 Intron 5 VNTR Polymorphism with Regard to Genotype Groups and Epidemiological Variables (age at onset, familial history, tumour size) among breast cancer cases and controls. * Indicates significance at p<0.05.

Genotype groups	Cases N=200 (%)	Controls N=202 (%)	Odds ratio (95% CI)	Odds ratio (p value)	Chi square test value (p)
Group 1 2R/2R	37 (18.50)	50 (24.75)	Reference		0.000001*
Group 2 0R/0R	91 (45.50)	46 (22.77)	2.67 (1.54-4.65)	0.0005	
Group 3 0R/1R, 1R/1R	51 (25.50)	53 (26.23)	1.30 (0.73-2.31)	0.3691	
Group 4 1R/2R, 2R/3R, 3R/3R	21 (10.50)	53 (26.23)	0.53 (0.27-1.03)	0.0637	
Genotype groups (Age at onset)	≤40 years N=42 (%)	>40 years N=134 (%)	Odds ratio (95% CI)	p-value	
Group 1	8 (19.04)	29 (21.64)	Reference		0.3323 [Yates' chi square p value- 0.5129]
Group 2	23 (54.76)	53 (39.55)	0.63 (0.25-1.60)	0.3361	
Group 3	8 (19.04)	34 (25.37)	1.17 (0.39-3.51)	0.7765	
Group 4	3 (7.14)	18 (13.43)	1.65 (0.39-7.06)	0.4962	
Genotype groups (Familial history)	Familial N=48 (%)	Non-familial N=133 (%)	Odds ratio (95% CI)	p-value	
Group 1	10 (20.83)	26 (19.4)	Reference		0.2606
Group 2	16 (33.33)	64 (48.12)	0.65 (0.26-1.62)	0.3546	
Group 3	16 (33.33)	28 (21.05)	1.48 (0.57-3.85)	0.4158	
Group 4	6 (12.50)	15 (11.27)	1.04 (0.31-3.43)	0.9487	
Genotype groups (Tumour size)	<3mm N=81 (%)	>3mm N=59 (%)	Odds ratio (95% CI)	p-value	
Group 1	20 (24.69)	10 (19.60)	Reference		0.6875
Group 2	33 (40.74)	25 (42.37)	1.51 (0.60-3.80)	0.376	
Group 3	16 (19.75)	15 (25.42)	1.87 (0.66-5.28)	0.2342	
Group 4	12 (14.81)	9 (15.25)	1.50 (0.47-4.74)	0.4896	

In table 2, the genotypes were stratified into following groups and the frequency distribution is calculated; Group 1- 2R/2R (Reference group); Group 2- 0R/0R (Risk group); Group 3- 0R/1R, 1R/1R (at least one low repeat group); Group 4- 1R/2R, 2R/3R, 3R/3R (High repeat group); In addition, the frequency distribution of epidemiological variables (age at onset, familial history and tumour size) has been evaluated.

Table 3. Distribution of Genotype Groups of SIRT3 Intron 5 VNTR Polymorphism with Respect to Clinical Variables (clinical subtype, metastasis, estrogen receptor status, progesterone receptor status, HER2NEU receptor status, triple negative receptor status, nodal status) among breast cancer patients. * Indicates significance at p<0.05.

Genotype groups (Clinical subtype)	Ductal N=155 (%)	Lobular N=19 (%)	Odds ratio (95% CI)	p-value	Chi square test value (p)
Group 1	31 (20.00)	4 (21.05)	Reference		0.4453
Group 2	68 (43.87)	10 (52.63)	0.88 (0.25-3.01)	0.8356	[Yates' chi square p value- 0.6820]
Group 3	37 (23.87)	5 (26.31)	0.95 (0.23-3.87)	0.9484	
Group 4	19 (12.25)	0 (0)	5.57 (0.28-109.23)	0.2579	
Genotype groups (Metastasis)	Metastatic N=77 (%)	Non-Metastatic N=70 (%)	Odds ratio (95% CI)	p-value	
Group 1	14 (18.18)	16 (22.85)	Reference		0.6271
Group 2	33 (42.85)	32 (45.71)	1.18 (0.49-2.80)	0.7102	
Group 3	17 (22.07)	15 (21.42)	1.29 (0.48-3.51)	0.6115	
Group 4	13 (16.88)	7 (10.00)	2.12 (0.66-6.81)	0.2057	
Genotype groups (Estrogen receptor)	Positive N=85 (%)	Negative N=100 (%)	Odds ratio (95% CI)	p-value	
Group 1	18 (21.17)	18 (18.00)	Reference		0.8218
Group 2	36 (42.35)	48 (48.00)	1.33 (0.61-2.92)	0.4716	
Group 3	20 (23.52)	24 (24.00)	1.20 (0.49-2.90)	0.6856	
Group 4	11 (12.94)	10 (10.00)	0.91 (0.31-2.67)	0.8623	
Genotype groups (Progesterone receptor)	Positive N=80 (%)	Negative N=105 (%)	Odds ratio (95% CI)	p-value	
Group 1	20 (25.00)	16 (15.23)	Reference		0.4047
Group 2	35 (43.75)	49 (46.66)	1.75 (0.79-3.85)	0.1637	
Group 3	17 (21.25)	27 (25.71)	1.98 (0.81-4.86)	0.133	
Group 4	8 (10.00)	13 (12.38)	2.03 (0.67-6.09)	0.2063	
Genotype groups (HER2NEU)	Positive N=81 (%)	Negative N=101 (%)	Odds ratio (95% CI)	p-value	
Group 1	21 (25.92)	15 (14.85)	Reference		0.0499*
Group 2	31 (38.27)	50 (49.50)	2.26 (1.01-5.02)	0.0459	
Group 3	16 (19.75)	28 (27.72)	2.45 (0.99-6.04)	0.0519	
Group 4	13 (16.04)	8 (7.92)	0.86 (0.28-2.59)	0.791	
Genotype groups (Triple negative receptor status)	TNBC N=45 (%)	Non-TNBC N=155 (%)	Odds ratio (95% CI)	p-value	
Group 1	6 (13.33)	31 (20.00)	Reference		0.4724
Group 2	25 (55.55)	66 (42.58)	1.95 (0.73-5.25)	0.1828	[Yates' chi square p value- 0.6463]
Group 3	10 (22.22)	41 (26.45)	1.26 (0.41-3.84)	0.6842	
Group 4	4 (8.88)	17 (10.96)	1.21 (0.30-4.91)	0.784	
Genotype groups (Nodal status)	Positive N=93 (%)	Negative N=95 (%)	Odds ratio (95% CI)	p-value	
Group 1	18 (19.35)	18 (18.94)	Reference		0.6342
Group 2	45 (48.38)	40 (42.10)	0.89 (0.41-1.94)	0.7672	
Group 3	19 (20.43)	27 (28.42)	1.42 (0.59-3.42)	0.4329	
Group 4	11 (11.82)	10 (10.52)	0.91 (0.31-2.67)	0.8623	

In table 3, the frequency distribution of clinical variables (clinical subtype, metastasis, receptor status and nodal status) has been also evaluated.

type (52.63%) when compared to ductal type (43.87%) suggesting dominant genotype specific risk for breast cancer in lobular clinical subtype (Table 2).

Comparison with clinical variables, with regard to metastasis, though the genotype frequency distribution was found to be insignificant, there is a trend of genotype frequency distribution elevation with increase in the repeats. With respect to estrogen receptor status, the genotype distribution was found to be insignificant. However, the 0R/0R risk genotype and group 2 genotype frequencies were elevated in patients without estrogen receptor (48.0%) when compared to patients with estrogen receptor (42.35%) suggesting dominant genotype specific risk for breast cancer in ER-ve patients. With respect to progesterone receptor status, the genotype distribution was found to be insignificant. However, the 0R/0R

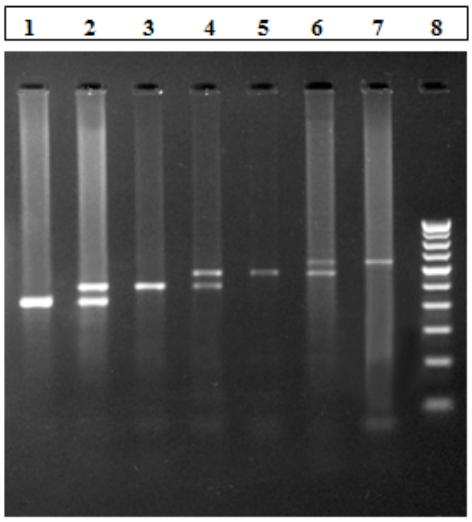

Figure 1. Gel Picture Representing 7 Different Genotypes of SIRT3 VNTR Polymorphism

risk genotype and group 2 genotype frequencies were slightly elevated in patients without progesterone receptor (49.66%) when compared to patients with progesterone receptor (43.75%) suggesting dominant genotype specific risk for breast cancer in PR-ve patients. With respect to HER2 status, the genotype frequency distribution has revealed significant heterogeneity (Chi- square p value=0.0499). The 0R/0R risk genotype and group 2 genotype frequencies have shown significant association with a statistical p value of 0.0459 indicating genotype specific risk. The overall triple negative status indicated the genotype frequency distribution was insignificant. However, the genotype frequencies were elevated in TNBC patients when compared to non-TNBC patients. And the genotype specific risks are observed in both the low repeat (group 2 and 3) and high repeat (group 4) groups when odds ratios are calculated. And with respect to nodal status, the genotype distribution was found to be insignificant. Therefore, these results indicate strong association of 0R allele with the development of breast cancer (Table 3).

Discussion

Undoubtedly, an essential prognostic aspect for minimizing breast cancer prevalence and recurrence is its diagnosis, presented at any stage. Our study attempts to evaluate association of VNTR polymorphism in intron 5 region of SIRT3 gene with the progression of breast cancer in order to assess its role as a prognostic and therapeutic marker. We observed a strong association of 0R repeat allele with breast cancer development and prognosis.

The metabolic shift in malignant cell transformation and metastasis is critically determined by intracellular ROS (Reactive Oxygen Species), ATP and NADH/FADH2 (cellular energy currencies), regulated by various regulatory proteins such as PARPs, Sirtuins, HATs etc. Metabolic reprogramming during therapy can influence genetic and epigenetic changes enhancing genomic instability, thereby inducing drug resistance. A comprehensive understanding of metabolic vulnerability and linked molecular mechanisms in resistance/relapse is being emphasized to gain insights into the design and development of novel therapeutic approaches over the past few decades. In this aspect, polymorphism in SIRT3 regulatory elements could serve as a therapeutic approach and our study is first of its kind in observing repeat variations in the intronic regions of sirtuins (SIRT3).

The repeat variations in the intronic region could result in allelic imbalance, transcriptional dysregulation and mRNA instability. Many instances from the analysis of germline non-coding variants in breast cancer major gene BRCA 1/2 have summarized the retention of introns due to aberrations in the processing of mRNA (splice mutations) mediated by integral factors (Bougie and Weberpals, 2011). The non-coding variants of high penetrant BRCA1 (in the introns 2, 5, 12, 16 and 19), BRCA2 (in the intron 17) and other genes were reported in breast and ovarian cancer, indicating their functional relevance to transcriptional and post-transcriptional vulnerability (Theri et al., 2011; Dos Santos et al., 2018). In particular, germline repeat variants might interfere to a greater extent with allelic imbalance due to differential cis/trans regulations. The polymorphic variants responsible for this dysregulated processing are widely under study. Also, intronic retention (IR) may affect the genome's structural topology, hindering mRNA's post-transcriptional signatures. Hence this IR proportion in the pre-mRNA could serve as a biomarker in evaluating the tumorigenic progress and recurrence in the breast tissue. Recent studies also evaluated a reduction in gene expression in the tumour tissues with an increase in IR (Grabski et al., 2021). Likewise, the enhancer binding sites at the intron 5 region of SIRT3 may get altered due to the repeat variations (eVNTRs), affecting the binding of enhancer regulatory proteins, thus leading to dysregulation of transcriptional efficiency.

Our study circumscribed genotype and allele-specific risk; however, it could not reveal the association of polymorphic variation specifically in the intronic region with the anomaly in the expression of the SIRT3 gene. Nonetheless, various studies on low penetrant intronic variant genes such as p53-intron3, IL4-intron3, IFNG-intron1, CYP19-intron4 etc., have reported their association with breast cancer susceptibility (Baxter et al., 2001; Gohrke et al., 2002; Saha et al., 2005; Duan et al., 2014).

Further systemic and large-scale analysis is warranted to understand the regulatory function of this variant and evaluate its prognostic and therapeutic significance.

Author Contribution Statement

SA conceived the idea and designed the study. HY and DJ did the experimental work through genotyping, analyzed the data, interpreted and wrote the manuscript. SV provided technical guidance for the genotyping and statistical analysis. RD extended research support by clinical collaboration and providing breast cancer samples. VS team helped in sample collection and isolation of DNA. SA and VS helped in interpretation of results and have critically refined the manuscript. All authors have critically analyzed the manuscript, read and approved it.

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Ethical Approval

The research work and design has been approved by the ethical committees of NIMS Hospital and Department of Genetics, Osmania University.

Data availability statement

The data that supports the findings of this study are available with SA.

Study registration

Any part of this study has not been registered under clinical trials or any meta-analysis or from any data base.

Conflict of interest

The authors declare no conflict of interest.

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