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Original Article

Hsa-mir-1269 genetic variant contributes to hepatocellular carcinoma susceptibility through affecting SOX6

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Abstract: MiR-1269 is an essential oncogene that plays crucial roles in regulating the development of hepatocellular carcinoma (HCC). In this study, we mainly focused on the polymorphisms (rs73239138) in miR-1069 to explore its potential role in regulation of target genes in liver cancer. We detected increased level of miR-1269 in 80 HCC patients. SOX6 was predicted as a potential target gene of miR-as. Notably, Pearson correlation analysis indicated that patients harbored with miR-1269 wild type (rs73239138, GG genotype), positively correlated with SOX6 expression. Over-expression of miR-1269 with GG genotype promoted cell proliferation comparing with AA genotype, which is acompanied by a decreased level of SOX6. Further dual luciferase reporter assay showed that miR-1269 with GG genotype have a stronger binding ability with SOX6. SNP rs73239138 in miR-1269 was very likey to be involved in the development of HCC by acting as a protective factor, as the carriers of GA and GG genotype resulted in a smaller tumor size. In conclusion, our results support that SNP rs73239138 in miR-1269 is a protective factor which prevents binding to 3'UTR of SOX6 and there by suppresses tumor growth in HCC.

Keywords: Genotype, miR-1269, SOX6, tumor growth, polymorphism

Introduction

Hepatocellular cell carcinoma is one of the commonest cancers all over the world, especially in Asia. It accords for more than 60,000 deaths, and about 750,000 cases are diagnosed newly every year [1]. Meanwhile, HCC contains numbers of characteristics including aggressiveness, invasiveness, especially intrahepatically, and frequent recurrence after resection [2, 3]. Nowadays, liver transplantation has been recognized as the most effective therapy of hepatocellular carcinoma, but only small number of patients is available to liver transplantation [4]. Although hepatocellular carcinoma is now increasingly diagnosed at the earlier stage due to the routine screening, effective chemotherapeutic agents for hepatocellular carcinoma are still lacking [5]. Therefore, the potential mechanism, prognostic biomarkers and therapeutic targets of HCC remain to be determined.

MiRNAs are small, non-coding RNA molecules of 19-25 nucleotides which have been reported to play important roles by regulating cell differentiation, proliferation, migration and apoptosis [6, 7]. MiRNAs negatively regulate their target genes expression at the post-transcription level through binding to 3'untranslated regions (UTRs) of their targets message RNAs [8, 9]. Various miRNAs have been identified involving in multiple biological behavior of HCC. For example, the up-regulated miR-1269 was proved to be a potential biomarker for the prognosis prediction of HCC [10] while the overexpression of miR-1269 promotes cell proliferation in HCC through directly suppressing FOXO1, and functions as an oncomiR in HCC [11]. Besides, recently studies were focusing on the additional factor such as single-nucleotide polymorphisms (SNPs) in miRNA or in the 3'UTR region of target genes which might be responsible for the abnormal regulation. MiRNAs could regulate the 3'UTR region who harbored SNPs, SNPs located

Table 1. The clinicopathological relevance analysis of miR-1269 in HCC patients

	miR-1269		
Feather	Low	High	*P* value
All cases	40	40	
Age			
<60	15	13	0.639
≥60	25	27	
Gender			0.723
Male	35	36	
Female	5	4	
HBV			0.692
Positive	37	36	
Negative	3	4	
Tumor size (cm)			0.001
≤5 cm	35	21	
>5 cm	5	19	
Tumor capsular			0.692
Incomplete	4	3	
Complete	36	37	
TNM stage (I:II:III)	19:16:5	15:17:8	0.551

in the miRNA (miRSNPs) binding sites through affecting the binding of miRNAs with the target genes resulted in reduction or increase in the target mRNA translation, and thus being associated with the susceptibility to cancers [12, 13]. Researchers have found that the T allele of FOXO3a (rs4946936) significantly increased the expression levels in luciferase assays and affected the binding affinity of miR-223 to the FOXO3a 3'UTR [13]. On the other hand, SNPs located in the mature region especially in the binding site of miRNA might cause a major effect on their target genes. Previous studies have shown that a common polymorphism (rs895819) in hsa-mir-27a, by modulating miR-27a and ZBTB10 levels, acted as an important factor of the gastric cancer susceptibility [14]. In addition, the rs2910164 CC and GC genotype in miR-146a was found to be associated with an improved lung function and milder disease stages, at least partially, mediated by its ability to increase COX2 expression and PGE2 production [15].

In this study, we mainly focused on SNP rs73239138, which is located in the mature region of miR-1269 and has not been reported before. We aimed to explore the potential function of this SNP and the effect on its target genes as predicted by bioinformatics analysis.

Materials and methods

Study subjects

The hospital-based case-control study consists of 590 patients newly diagnosed with HCC and 549 cancer-free controls. All the subjects were genetically unrelated Han Chinese recruited from the Second Affiliated Hospital of Nanjing Medical University, China between January 2007 and December 2012. Patients with other hematological disorders, previous history of cancers, radiotherapy and chemotherapy were excluded. The cancer-free control subjects from the same geographic area showed no evidence of genetic relationship with the cases. The patients were classified according to World Health Organization classification. This study was approved by the Second Affiliated Hospital of Nanjing Medical University, and every patient had written informed consent. The clinical feather of all the cases and controls were presented in **Table 1**.

Cell lines and cell culture

HCC cell lines and HepG2 and Huh7 were purchased from the Chinese Academy of Sciences Cell Bank. All cells were cultured in Dulbecco modified Eagle medium (DMEM) purchased from Gibco (CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, USA) and grown in humidified 5% CO_2 at 37°C. MiR-1269 mimics and normal control (both GG type and AA type) were obtained from Genepharma (Shanghai, China). The transfection was conducted by using Lipofectamine 2000 (Invitrogen Corp, CA, USA).

Prediction of miRNAs binding to the SNP

Bioinformatics analysis (Targetscan, PicTar, miRbase and miRTarget) was used to predict the potential target gene of miR-1269 and was listed in **Table 2**.

Construction of luciferase-based reporter plasmids

The total fragment of the SOX6 3'UTR was amplified. The PCR production was cloned into the pGL3-promoterless luciferase-based plasmid (Promega, CA, USA) at the cloning site between KpnI and XhoI. The amplified fragment was verified by DNA sequencing.

Table 2. Bioinformatics prediction of potential target genes

microRNA	Related Gene	miRbase	Target Scan	PicTar	miRNA Target
hsa-miR-1269	FOXO1	√	√	√	√
	SOX6	√	√	√	√
	DAZ1		√	√	√
	MEF2D		√	√	√
	CCND2	√	√	√	
	DAZL	√	√	√	

Dual-luciferase reporter assay

Cells were plated onto 24-well plates and transfected with 100 ng of pGL3-SOX6, miR-1269 mimics (GG genotype and/or AA genotype) and the corresponding control plasmid, respectively by using Lipofectamine 2000 (Invitrogen Corp, CA, USA). A Renilla luciferase vector pRL-SV40 (5 ng) was also co-transfected to normalize the differences in transfection efficiency. Transfection was repeated three times in triplicate.

Cell proliferation assays

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Roche, Molecular Biochemicals, Mannheim, Germany) assay was used to detect the cell proliferation. Cells were plated at 1×10^4 cells per well into 96-well plate after treated for 48 hours, and was incubated at 37°C for 4 hours. Then, 150 μL of dimethylsulfoxide was added to each well. Absorbance at 490 nm was determined by the TECAN infinite M200 Multimode microplate reader. Each assay was performed in triplicate and repeated 3 times independently.

Genotype

We extracted genomic DNA from peripheral whole blood of every validation subject by using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genotyping was performed with the TaqMan SNP Genotyping Assay. The PCR reactions were carried out in a total volume of 5 μL containing TaqMan Universal Master Mix, SNP Genotyping AssayMix, Dnase-free water and genomic DNA. The PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The 384-well ABI 7900HT Real Time PCR System was applied (ABI, CA, USA).

Statistical analysis

Patients with HCC were divided into two groups by using the median of the expression of miR-1269 as cutoff. The association between rs73239138 genotypes and the risk of HCC was evaluated by calculating the odds ratios (ORs) and their 95% confidence intervals (CIs) using univariate and multivariate logistic regression analysis. Stratification analysis was performed according to the clinical characteristic and risk classification to determine the genotype distribution in cases and controls as well as their association with the risk of HCC. All statistical tests were two-sided and $P<0.05$ was considered statistically significant. Statistical analysis was performed with SPSS 13.0 and SAS software (version 9.1.3; SAS Institute, Cary, NC, USA). The graphs were generated by Graphpad Prism 5.0 (Graphpad Software, Inc.).

Results

Up-regulated miR-1269 was associated with tumor size in HCC patients

We firstly identified the expression level of miR-1269 in 80 pairs tissues (tumor tissues and corresponding adjacent tissues). Aberrant up-regulated miR-1269 was obtained which was consistence with previous research (**Figure 1A**). We next divided the HCC patients into two groups by using to the median expression level of miR-1269 as cutoff to explorer the potential function of miR-1269 in HCC patients. We found that the increased level of miR-1269 was associated with tumor size (**Table 1**).

SNP in the mature region of miR-1269 associated with the expression of SOX6

In order to investigate the miRNA associated SNPs in the mature region of miR-1269; we first screened the potential target gene of miR-1269 by bioinformatical prediction. As listed and ranked in **Table 2**, FOXO1, SOX6, DAZ1, MEF2D, CCND2 and DAZL was picked as candidates. Since FOXO1 has been reported to be regulated by miR-1269 in HCC, we chose SOX6 as another candidate. Aberrant decreased level of SOX6 was identified in tumor tissues (**Figure 1B**).

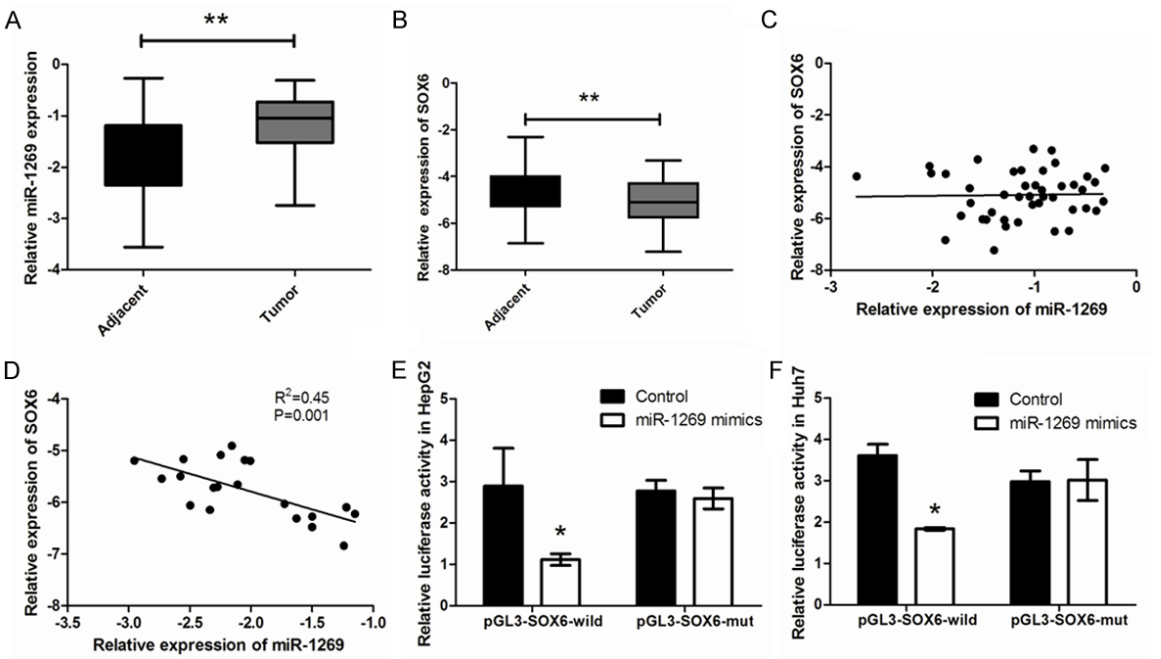

Figure 1. SNP in the mature region of miR-1269 associated with the expression of SOX6. (A) The expression level of miR-1269 was determined by RT-PCR in patients. (B) Relative expression level of SOX6. Data was log-transformed as presented with Data was presented as the mean ± SEM. (C, D) Pearson correlation analysis between the expression level of miR-1269 and SOX6 in different patients (C in patients carrying GG genotype; D in patients carrying all genotype). (E, F) Cells were co-transfected with miR-1269 mimics or control, Renilla luciferase vector pRL-SV40 for 48 h. Both firefly and Renilla luciferase activities were measured in the same sample. Firefly luciferase signals were normalized with Renilla luciferase signals. Left panel indicated the HepG2 cell line (panel C) while the right indicated Huh7 (panel D) cell lines. Data was presented as the mean ± SEM. * indicates P<0.05, ** indicated P<0.01.

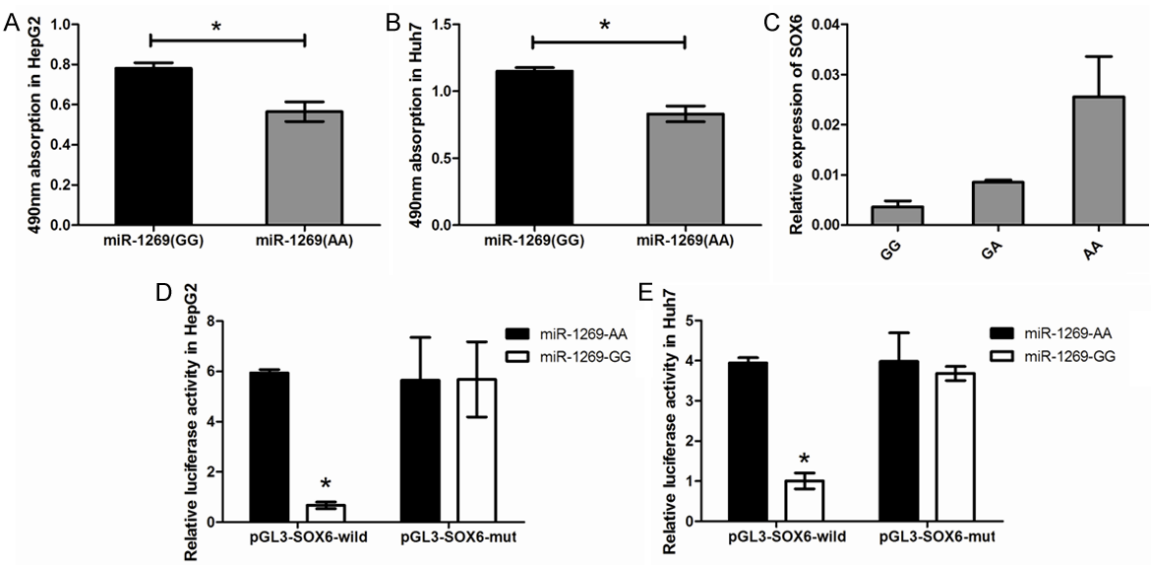

Figure 2. SNP rs73239138 abrogated the specific function of miR-1269 in regulation of SOX6. (A, B) Cell proliferation was measured by MTT assay. Cells were treated with miR-1269 harboring GG or AA genotype. (C) Relative expression of SOX6 in patients carrying with different genotype of SNP rs73239138 in miR-1269. Data was presented with mean ± SEM. (D, E) Cells were co-transfected with miR-1269 with different genotypes and Renilla luciferase vector pRL-SV40 for 48 h. Both firefly and Renilla luciferase activities were measured in the same sample. Firefly luciferase signals were normalized with Renilla luciferase signals. Left panel indicated the HepG2 cell line (panel D) while the right indicated Huh7 cell lines (panel E). Data was presented as the mean ± SEM. * indicates a significant difference (P<0.05).

Table 3. Frequency distributions of selected variables in patients and cancer-free controls

Variables	Cases (*n*=590)		Controls (*n*=549)		*P**
	N	%	*N*	%	
Age (years)					
≤ 60	331	56.1	311	56.6	0.853
> 60	259	43.9	238	43.3	
Gender					
Male	331	56.1	312	56.8	0.804
Female	259	43.9	237	43.2	
Smoking exposure					
Negative	260	44.1	302	55.0	<0.001
Positive	330	55.9	247	45.0	
HBV/HCV					
Negative	46	7.8	516	94.0	<0.001
Positive	544	92.2	33	6.0	
Drinking exposure					
Negative	248	42.0	321	58.5	<0.001
Positive	342	58.0	228	41.5	
Family history of cancer					
Negative	277	46.9	296	53.9	0.019
Positive	313	53.1	253	46.1	

*Two-sided chi-square test for either genotype distributions or allele frequencies between cases and controls.

Further Pearson correlation indicated a reverse correlation between the HCC patients who harbored GG genotype instead of the whole patients (GG, GA and AA genotypes) in miR-1269 and SOX6 (**Figure 1C**, **1D**). Unfortunately, this correlation was not observed in analyzing correlation between miR-1269 and FOXO3 (data not shown).

We further designed the pGL3-plasmid containing the full length of 3'UTR of SOX6 as well as the mutant type and co-tranfected with miR-1269 mimics and control to further investigate the potential regulation mechanism between miR-1269 and SOX6. We found that miR-1269 could directly bind with the 3'UTR region of SOX6 which resulting in the decreased fluorescence activity of plasmid containing the wild type of the 3'UTR region of SOX6 (**Figure 1E**, **1F**).

SNP rs73239138 abrogated the specific function of miR-1269 in regulation of SOX6

In order to understand the detailed function of SNP rs73239138 in miR-1269, we order the over-expression vector with either the GG genotype or AA genotype and transfection into both HepG2 and Huh7. The cell proliferation was measured by MTT assay. Interestingly, we found that cells treated with wild type (GG genotype) presented an increased proliferation (**Figure 2A**, **2B**). The expression of SOX6 in HCC patients harboring different genotypes was also investigated. We found that patients with the A allele presented an increased level SOX6 expression indicated that the A allele might prevent the binding of miR-1269 to SOX6 (**Figure 2C**). The dual-luciferase report assay was also employed, both miR-1269 GG genotype and AA genotype was treated in cells by co-transfected with pGL3-SOX6 wild type and mutant type. The results indicated that the existence of an allele could cause a disability of miR-1269 on binding with SOX6 which interfered the specific function of miR-1269 (**Figure 2D**, **2E**).

Clinical significant of rs73239138 with hepatocellular carcinoma

We next investigate the clinical significant of rs73239138 as well as the genotype frequencies in 590 HCC cases and 549 controls. The characteristics of the patients and healthy controls are summarized in **Table 3**. No statistically significant differences were observed between cases and controls in terms of sex and age (both P>0.05). Patients suffering from smoking exposure, drinking exposure, the history of cancer and the HBV and/or HCV infection indicated to be the susceptible population by comparing with controls.

As listed in **Table 4**, Chi-square statistical analysis results showed that the genotypes of rs73239138 were in Hardy-Weinberg equilibrium distribution pattern in the healthy control group (P=0.33). Further, logistic regression analysis results revealed that The GA genotype and AA genotype presented a significant decreased risk of HCC as compared with the GG genotype. Logistic regression analyses indicated that individuals with the rs73239138 GA genotype was significantly associated with HCC risk (OR=0.69, 95% CI=1.03-1.12; P=0.0001). Individuals carried A allele had an OR of 0.87 (95% CI=1.01-1.34; P=0.0001) for HCC compared with individuals having the rs73239138 GG genotype. All ORs were adjusted for sex, age, and smoking status, drinking history HBV/HCV infection or family cancer history.

Table 4. Genotype distribution of the miR-1269 rs73239138 polymorphism in HCC patients and cancer-free controls

Genotype	Cases (*n*=590)		Controls (*n*=549)		OR (95% CI)[a]	*P* value[a]
	N	%	*N*	%		
rs2866943						
GG	272	46.1	301	54.8	1.00	0.0001
GA	228	38.6	224	40.8	0.69 (1.03-1.12)	
AA	90	15.3	24	4.4	0.87 (1.01-1.34)	
A carrier	318	53.8	248	45.2	0.81 (1.02-1.31)	0.003

[a]The ORs, 95% CIs and *P* value were calculated after adjusting for age, gender, parental smoking, drinking and family caner history.

Table 5. Stratified analysis of rs73239138 genotype with clinicopathological parameters of HCC

Feather	Genotype				GA vs GG	AA vs GG	A carrier vs GG
	GG	GA	AA	A carrier	*P* value*	*P* value*	*P* value*
Age (years)							
≤ 60	153	136	42	178	0.44	0.11	0.95
> 60	119	92	48	140			
Gender							
Male	151	124	56	180	0.81	0.27	0.79
Female	121	104	34	138			
Differentiation grade							
Well	157	128	52	180	0.65	0.88	0.66
Moderate	95	78	30	108			
Poorly	20	22	8	30			
Tumor size (cm)							
≤3 cm	123	173	57	230	<0.001	0.003	<0.001
>3 cm	149	55	33	88			
HBV							
Positive	250	210	84	294	0.94	0.66	0.81
Negative	22	18	6	24			
Tumor capsular							
Incomplete	155	142	49	191	0.16	0.67	0.45
Complete	117	86	41	127			

*Two-sided chi-square test for either genotype distributions or allele frequencies between cases and controls.

Stratified analysis of correlation between miR-1269 polymorphism and HCC risk

Last, we conducted the stratified analysis to understand the correlation between of the SNP rs73239138 genotypes with the clinicopathological parameters of HCC (**Table 5**). We found a significant association of the rs73239138 genotypes with the tumor size. Compared with the GG homozygote, patients carried with GA and AA genotype presented a small tumor size.

Discussion

The regulation pattern of miRNAs in human malignant tumor was mainly restricted in the binding in the 3'UTR of target region which caused an abnormal decreased/increased level and resulted in the inactivation/activation of signaling pathway. It is very important for researchers to explore the functional miRNAs in human disease; however, the regulation patter cannot always be same as previous.

In the present study, we began our experiment based on the abnormal expression of miR-1269 in HCC patients which was consistence with previous report [16]. During the exploration of the potential target gene of miR-1269, we found that the expression level of miR-1269 in HCC patients harboring the GG genotype was correlated with the expression level of SOX6; however, in patients with GA or AA genotype presented no significant correlation which indicated that the SNP in miR-1269 might associated with SOX6. Further dual-luciferase report assay confirmed the binding ability of miR-1269 on SOX6 3'UTR region. Researchers have identified that over-expressed miR-1269 could promote cell proliferation, here; we further investigated the function of miR-1269 carrying different genotypes. We found that miR-1269 with GG genotype presented a stronger proliferation ability by binding with SOX6.

As a member of the D subfamily of SOXs, SOX6 plays critical roles in cell fate determination, differentiation, and proliferation [17]. Recent studies have demonstrated that SOX6 functions as tumor suppressor in human HCC. For example, Xie et al. indicated that SOX6 may be a novel target of miR-155 which could enhance liver cell tumorigenesis at least in part through the novel miR-155/SOX6/p21waf1/cip1 axis [18]. Besides, Regarding to the prognostic value of SOX6, we found that the prognosis of HCC patients with a low expression level of SOX6 was poor, and Cox regression analysis indicated that a low expression level of SOX6 was a significant prognostic factor for both poor disease-free and overall survival of HCC patients, suggesting that SOX6 may become a novel prognostic marker for HCC [19]. In this study, the decreased level of SOX6 induced by miR-1269 resulted in a tumor suppressor decreasing affection which was consistence with the research above.

We further observed that miR-1269 rs73239138 GA or AA genotype is associated with decreased HCC risk compared with GG genotype. We further found that the SNP rs73239138, locating in the mature region of miR-1269 may cause a interference on the binding ability on regulation SOX6. Considering about the oncogene function of miR-1269, our founding indicated that the G>A SNP might be acted as protective factor in the pathogenesis of HCC.

In summary, we reported the first evidence that the SNP rs73239138 in miR-1269 might be a protective factor to prevent its binding to 3'UTR of SOX6 which might suppress tumor growth in HCC.

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Disclosure of conflict of interest

None.

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