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Original Article

miR-15b facilitates the progression of colorectal cancer via targeting β-catenin and Axin2

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Abstract: Colorectal cancer (CRC) is a prevalent and malignant tumor type worldwide with an increasing incidence in China. MicroRNA-15b (miR-15b) may be a potential biomarker that has been reported to be associated with CRC development. Meanwhile, growing evidence suggests that Wnt/β-catenin signaling pathway is a critical player in CRC progression. The role of miR-15b in CRC via Wnt/β-catenin signaling pathway remains to be fully elucidated. Preliminary studies showed that the expression of miR-15b increased significantly in SW620 with high metastasis than SW480 with low metastasis (P<0.01). Subsequently, transfected SW620 cells were prepared, and it could be observed that miR-15b mimics were supportive of the proliferation and metastasis in CRC cell, and miR-15b inhibitor might be opposed to the growth of CRC cell. Further, miR-15b mimics was affirmed to significantly stimulate β-catenin expression (P<0.01), and obviously suppress Axin2 expression (P<0.01). Also, we found that miR-15b mimics promoted dramatically β-catenin protein expressions by 4.2-fold (P<0.01) and significantly inhibited Axin2 protein expression by 1.6-fold (P<0.05) compared with the control in an animal experiment. These results made a deep insight into the association of miR-15b with Wnt/β-catenin signaling pathway in the development, invasion and metastasis of CRC.

Keywords: Colorectal cancer, miR-15b, Wnt/β-catenin, metastasis, Axin2

Introduction

Colorectal cancer (CRC), one of the most common malignancies in gastrointestinal tract, is currently the third leading diagnosed cancer in males and the second in females worldwide with high mortalities [1]. Several risk factors contribute to the CRC occurrence, such as age, sex, dietary habit, preexisting health problems, smoking, alcohol, etc. [2]. Although the notorious metastasis and prognosis render CRC recalcitrant to cure, reliable biomarkers can be efficiently employed to predict the potential cancer challenge early, making CRC treatable by surgery [2].

MicroRNAs (miRNAs or miRs) have been widely recognized to be the most promising candidates of biomarker for CRC detection. miRNAs are a group of endogenously small, non-coding RNAs (~22 nt), playing pivotal regulations in posttranscriptional gene silencing or degradation via incomplete binding to 3' untranslated region (3'UTR) of target mRNA [3]. The regulation network of miRNA are rather complex, as each miRNA can bind to no less than one target mRNA, the activity of which can also be adjusted by more than one miRNA [4]. A variety of miRNAs have been reported to regulate cell proliferation, apoptosis, metastasis and resistance in multiple myeloma [5], breast cancer [6], gastric cancer [7], liver cancer [8], pancreatic cancer [9], as well as CRC [10-12]. The miR-15 family consisting of miR-15a/15b/16/195/322/424/457/497 has been confirmed to serve key functions in human cancer [13]. In this family, miR-15b was observed to be one of the reliable miRNA biomarkers to distinguish patients with adenomatous polyps from healthy controls, and advanced colorectal adenoma from CRC patients [14]. More importantly, it was demonstrated that the expression of miR-15b was obviously upregulated in CRC patients [15, 16] and could lead to tumor metastasis, recurrence, and poor patient prognosis [17]. However,

the potential anti-CRC mechanism of miR-15b remains poorly understood.

Wnt signaling pathway is central to the maintenance of intestinal homeostasis as well as carcinogenesis and is frequent in aberrant hyperactivation. As known, the Wnt/β-catenin signaling pathway (the canonical Wnt pathway) is a major contributor to the occurrence, invasion and metastasis of CRC and mutated in almost 90% CRC patients. Axin2 and β-catenin are deemed to be usually mutated in CRC, although the mutations of other pathway components can also promote CRC progression. We presume that miR-15b may aggravate CRC by targeting critical genes in Wnt/β-catenin signaling pathway.

In this study, we investigated the effects of miR-15b on the expressions of Axin2 and β-catenin in *in vitro* cell lines with different metastasis as well as in *in vivo* rat subcutaneous injection test, and further analyzed its functions in regulating the invasion and metastasis of CRC via Wnt/β-catenin signaling pathway.

Materials and methods

Cell culture

The SW620 and SW480 cell lines were purchased from typical culture preservation commission cell bank, Chinese academy of sciences. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 1% of antibiotic-antimycotic solution (containing 10000 units/mL of penicillin base, 10000 mg/mL of streptomycin base, and 25000 ng/mL of amphotericin B) (Gibco, Grand Island, NY, USA) under standard conditions of 37°C, 95% humidity and 5% CO_2.

Cell transfection by miR-15b inhibitor and mimics

To alter the expression levels of miR-15b, miR-15b mimics, miR-15b inhibitor (30 nM) and control molecules (30 nM) (Invitrogen Life Technologies) were transfected into the SW620 using Lipofectamine® 3000 Transfection Reagent following the manufacturer's instructions. The cells were incubated for 48 h prior to further experiments.

MTT assay

The SW620 and SW620 transfected cells as well as corresponding controls were seed at a density of $5×10^3$ cells/mL into a 96-well plate. After 0, 24, 48 and 72 h of incubation, the cells were combined with 10 μL sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL; Beyotime, Shanghai, China) for 4 h at 37°C. Subsequently, the cells were mixed with 150 μL dimethyl sulfoxide (DMSO) for 10 min. The plate was read spectrophotometrically at a wavelength of 570 nm by a Bio-Rad model 550 microplate reader (Bio-Rad Laboratories, Inc.) to detect proliferation rate. Cell viability (%) was equal to absorbance value of transfected cells/absorbance value of control cells.

Transwell chamber assay

Matrigel was thawed at 4°C overnight, and then diluted to the final concentration of 1 mg/mL with pre-cold serum-free DMEM. The diluted Matrigel (=100 μL) and DMEM (=200 μL) were co-incubated to coat upper transwell chamber at 37°C. The SW620 and SW620 transfected cells as well as corresponding controls were trypsinized, washed by PBS, and resuspended in serum-free medium. Subsequently, these cells were plated on the upper chamber which was put into 24-well plate. The lower chamber contained DEME medium and 10% FBS. After 48 h of incubation at 37°C, cells that did not pass the pores were removed by a cotton swab. Then the chamber was fixed with 4% cold methanal and stained with 0.1% crystal violet for 30 min. The cells were counted by an inverted microscope (OLYMPUS, Tokyo, Japan) for 3 fields of view.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

After PBS washing, total RNA was isolated from SW620 and SW480 by RNA extraction kit-DP430 (TIANGEN, Beijing, China) according to the manufacturer's instructions, and quantified by a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). The isolated RNA was transcribed into cDNA using One Step PrimeScript® miRNA cDNA Synthesis Kit (Takara, Japan) by following the manufacturer's instructions. Reverse transcription was subsequently performed with a reaction mixture (20 μL) containing 10 μL 2× SYBR Fast qPCR Mix, 0.8 μL PCR

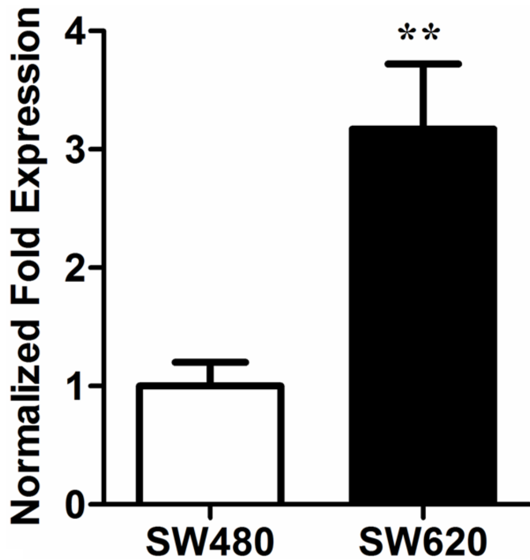

Figure 1. miR-15b expression in SW620 and SW480 cells. **P<0.01, compared with SW480.

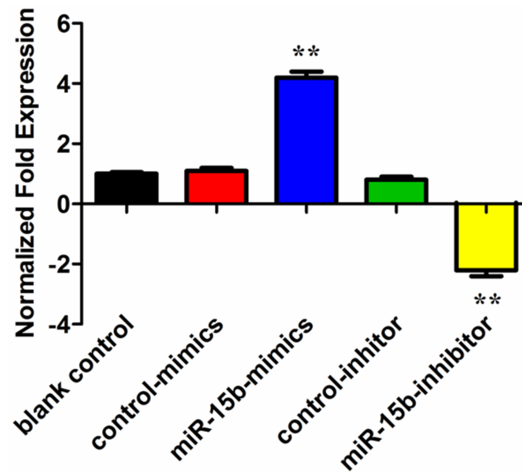

Figure 2. miR-15b expression in transfected SW620 cell. **P<0.01, the difference of miR-15b mimics transfected cell was calculated compared with control mimics; the difference of miR-15b inhibitor transfected cell was calculated compared with control inhibitor.

Forward/Reverse Primer, 0.4 μL 50× ROX Reference Dye II, and 2 μL cDNA template. The PCR reaction was performed at 95°C for 30 s, followed by 40 cycles of 95°C for 3 s and 60°C for 13 s. The reaction was run on Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument (Thermo Scientific, Wilmington, DE, USA). The expressions of miR-15b, β-catenin and Axin2 were normalized to U6 (endogenous control). The primer sequences were as follows: miR-15b, forward 5'-ATGAACTTTCTCTGTCTTGG-3', reverse 5'-TCACCGCCTCGGCTTGTCACA-3'; β-catenin, forward 5'-CCTATGCAGGGGTGGTCAAC-3', reverse 5'-CGACCTGGAAAACGCCATCA-3'; Axin2, forward 5'-CAGCCATTCAGGAACTACCC-3', reverse 5'-ATATAGGGCGACACACGGAG-3'; U6, forward 5'-CTCGCTTCGGCAGCACA-3', reverse 5'-AACGCTTCACGAATTTGCGT-3', which were synthesized by Sangon Biotech (Shanghai, China) The relative target gene expression was calculated as a fold change of $2^{-\Delta\Delta Ct}$ value, in which $\Delta\Delta Ct = \Delta Ct^{tarfet\ gene} - \Delta Ct^{endogenous\ control}$.

Immunoblot assay

The levels of β-catenin, Axin2 and GAPDH proteins were determined by western blotting. Briefly, the proteins were extracted from the cells by EpiQuik Total Histone Extraction Kit (Epigentek, Farmingdale, NY, USA) and quantified by a BCA Protein Quantification Kit (Vazyma, Nanjing, Jiangsu, China) according to the manufacturer's instructions. Approximately 50 mg of total protein extracts were separated by 12% SDS-PAGE electrophoresis prior to being transferred to PVDF membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked by 5% milk solution for 2 h at room temperature and washed three times by TBST (Sigma-Aldrich, USA). Then the membrane was respectively incubated with β-catenin, Axin2 and GAPDH primary rabbit monoclonal antibody at 4°C overnight at a dilution of 1:1000 (Abcam, Cambridge, British). Followed by TBST wash for three times, the membrane was incubated with secondary anti-rabbit antibody conjugated with horseradish peroxidase (ZSGB-BIO, Beijing, China) at 1:2000 for 30 min and washed by TBST. The strip gray levels were quantified using a Quantity one v4.62 software. Each sample was performed in triplicate.

Animal experiments

The animal experiments performed in the present study were preapproved by Central South University, and all experiments were performed in accordance to the animal care guidelines of the Chinese Council. Forty BALB/c nude mice (=20-22 g) purchased from the animal center of Shanghai Biological Science Institution (Shanghai, China) were housed in the animal facility of Xiangya Hospital with food and water provided ad libitum. These mice were divided into two groups: one group received SW620 cell transfected with miR-15b mimics (n=20), the

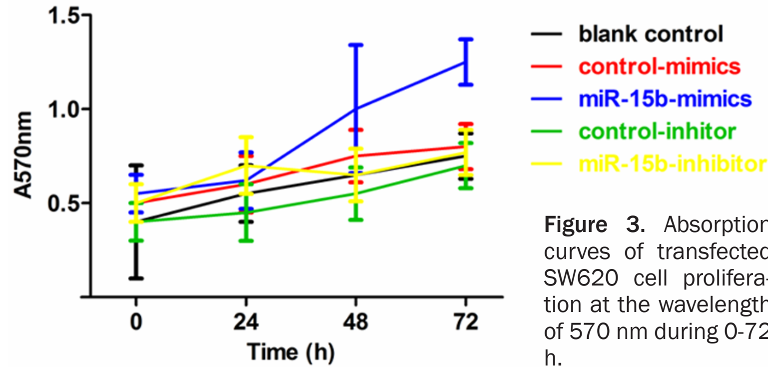

Figure 3. Absorption curves of transfected SW620 cell proliferation at the wavelength of 570 nm during 0-72 h.

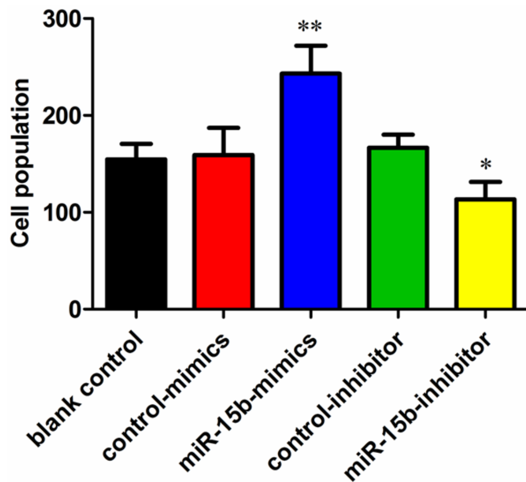

Figure 4. Transfected SW620 Cell population of transfected cell metastasis by MTT assay. **P<0.01, the difference of miR-15b mimics transfected cell was calculated compared with control mimics; the difference of miR-15b inhibitor transfected cell was calculated compared with control inhibitor.

other group with no tumor cell injection was set as control (n=20). The CRC model was established by injecting 1×10^6 cells into the flanks of mice, which were maintained in a sterile animal facility for 2 weeks. After the mice were sacrificed, the tumor and corresponding normal tissue were removed from the mice for subsequent experiments.

Statistical analysis

The experiments were performed triplicate in three independent occasions. The data were presented as the mean ± standard deviation and analyzed using a two-tailed Student's t-test. Statistical analyses were conducted by SPSS 11.5 (SPSS, Chicago, IL, USA). A value of $P<0.05$ was considered statistically significant.

Results

miR-15b expression in SW620 and SW480

We observed 3.17-fold upregulation of miR-15b in SW620 with high metastasis potential compared with SW480 with low metastasis potential (P<0.01, **Figure 1**). After respective transfections of miR-15b mimics and inhibitor into SW620, it could be shown that the expression of miR-15b was upregulated significantly in SW620 transfected with miR-15b mimics compared with the control transfected with random miR mimics sequence (P<0.01, **Figure 2**), while was inhibited evidently in SW620 transfected with miR-15b inhibitor compared with the control transfected with random miR inhibitor sequence (P<0.01, **Figure 2**).

miR-15b impact on SW620 proliferation and metastasis

MTT assay was employed to detect the impact of miR-15b on SW620 proliferation. Results showed that miR-15 mimics transfection notably enhanced absorption during 24-72 h, while miR-15b inhibitor transfection did not cause marked absorption change during the incubation time (**Figure 3**). These results indicated that miR-15b mimics in SW620 were in favor of the proliferation of CRC cell, and miR-15b inhibitor in SW620 appeared to be of no impact on the proliferation of CRC cell. Transwell chamber assay was then used to survey the effect of miR-15b on SW620 metastasis. Results manifested that the cell population through transwell chamber significantly increased in miR-15b mimics cells (P<0.01), and apparently decreased in miR-15b inhibitor cells (P<0.01, **Figure 4**). These findings suggested that miR-15b could make a deep impact on CRC cell metastasis.

miR-15b impact on β-catenin and Axin2 mRNA in SW620 cell

qRT-PCR was employed to detect target gene expressions of β-catenin and Axin2 in SW620 transfection cells. As shown, miR-15b mimics could significantly promote β-catenin expression (P<0.01), and largely suppress Axin2

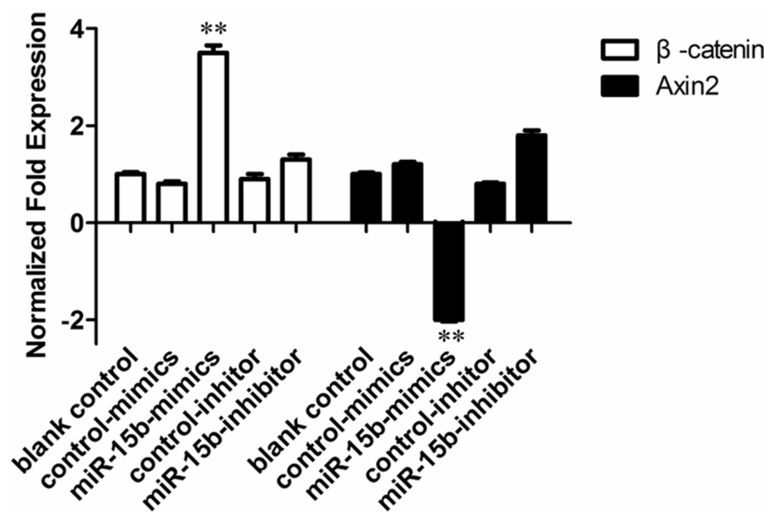

Figure 5. Expressions of β-catenin and Axin2 genes detected by qRT-PCR in transfected SW620 cell. **P<0.01, the difference of miR-15b mimics transfected cell was calculated compared with control mimics; the difference of miR-15b inhibitor transfected cell was calculated compared with control inhibitor.

expression (P<0.01, **Figure 5**). Surprisingly, miR-15b inhibitor seemed to have no evident impact on both β-catenin and Axin2 expressions (**Figure 5**). These observations demonstrated that miR-15b overexpression had a decisive consequence on β-catenin and Axin2 mRNA *in vitro*.

miR-15b impact on β-catenin and Axin2 protein expressions in animal experiment

To further investigate the influence of miR-15, the β-catenin and Axin2 protein expressions were inspected in rats. It could be found that miR-15b mimics promoted dramatically β-catenin protein expressions by 4.2-fold (P<0.01) (**Figure 6A** and **6B**), and significantly inhibited Axin2 protein expression by 1.6-fold (P<0.05) compared with the control (**Figure 6A** and **6B**). Meantime, we observed that miR-15b level increased by nearly 2-fold in rats injected by SW620 transfected with miR-15b mimics compared with the control (P<0.01, **Figure 6C**). These results implied that overexpressed miR-15b could also significantly inhibit the β-catenin and Axin2 protein expressions.

Discussion

CRC, a global tumor type, becomes a challenging health problem in developing countries. The incidence of CRC is still increasing in China. Accumulated evidence showed that abnormal expressions of microRNA accounted for the progression of CRC including late invasion and metastasis [10, 11, 18-20]. miR-15b has been demonstrated to be overexpressed in CRC [15, 16]. In this work, we also observed the notable upregulation of miR-15b in SW620 with high metastasis compared with SW480 with low metastasis (**Figure 1**) and in an *in vivo* test (**Figure 6C**). Furthermore, we affirmed that miR-15b remarkably stimulated the proliferation and metastasis of transfected SW-620 (**Figures 3** and **4**). More importantly, inhibition of miR-15b could significantly decrease the metastasis of transfected SW620 (**Figure 4**), indicating miR-15b might be a potential target in CRC development. Recently, miR-15b was considered one of the underlying manipulator of CRC metastasis through suppressing the expressions of Klotho protein [17], which was a tumor suppressor by inhibiting insulin/IGF1, p53/p21, as well as Wnt signaling [21]. Additionally, it was pointed that miR-15b could regulate, at least in part, Wnt signaling pathway [22]. Therefore, we tried to make an insight into the relationship of miR-15b with several critical gene expressions in Wnt/β-catenin signaling pathway.

Wnt/β-catenin signaling pathway is the canonical Wnt pathway. In the absence of Wnt ligand, APC, Axin and GSK3-b will form a “destruction complex” to phosphorylate cytosolic β-catenin, which is ultimately degraded by the ubiquitin-proteasome system. In the presence of Wnt ligand, the concentration of β-catenin will increase in the cytoplasm, and a series of target gene transcriptions will be induced after subsequent β-catenin translocation to the nucleus [23]. Increased proofs indicated the pivotal status of Wnt/β-catenin signaling cascade in the progression of CRC. The β-catenin encoded by CTNNB1 is a cytoskeletal protein and a decisive regulator in Wnt/β-catenin cascade [24]. The aberrant upregulation of β-catenin has been proved to accelerate the invasion and metastasis in CRC [25, 26], and

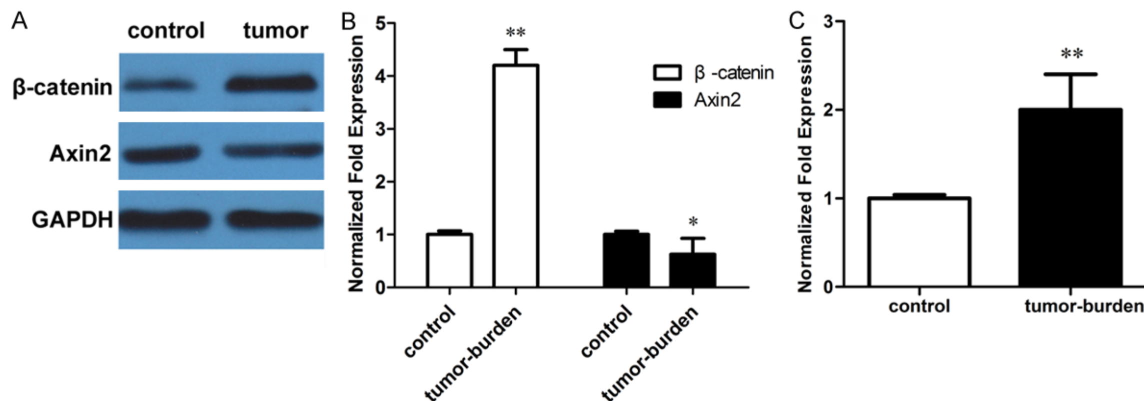

Figure 6. (A) β-catenin and Axin2 protein expressions by western blot, (B) the analyses of β-catenin and Axin2 protein expression, and (C) miR-15b expression in tumor-transfected rat. **P<0.01, compared with the control; *P<0.05, compared with the control.

probably an independent factor for poor prognosis [26]. Axin2 is a scaffolding protein, providing several binding sites to the components in Wnt/β-catenin cascade [24]. Axin2 was firstly recognized as a negative regulator in Wnt signaling pathway [27]. To our knowledge, the roles of miR-15b in regulating the expressions of β-catenin and Axin2 have not been intensively investigated. In this paper, it was found that the expression of β-catenin was significantly unregulated, while the expression of Axin2 was dramatically downregulated in transfected SW620 cells (**Figure 5**), as per in *in vivo* test (**Figure 6A** and **6B**). To our regret, we did not observe significant decrease of β-catenin when miRNAs inhibitor used and obvious increase of Axin2 when miRNAs mimics used.

Previous study manifested that Axin2 had opposite functions in the regulation of Wnt signaling pathway: on one hand, Axin2 participated to form the "destruction complex" promoting β-catenin degradation and therefore impeded the Wnt signal transduction; on the other hand, Axin2 accelerated to recruit GSK3 to the plasma membrane and phosphorylated LRP5/6, initiating Wnt signaling ultimately [28]. In present study, Axin2 seemed to function via degrading β-catenin. How Axin2 acts in other cell lines needs to expand the sample bank.

In conclusion, the present study suggested that miR-15b could be a useful biomarker for CRC prediction, and might be a potential target for CRC prevention and treatment. miR-15b could enhance proliferation and metastasis of CRC via upregulating the expression of β-catenin and downregulating the expression of Axin2 in Wnt/β-catenin signaling pathway.

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Disclosure of conflict of interest

None.

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