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Novel BRCA2-Interacting Protein, LIMD1, Is Essential for the Centrosome Localization of BRCA2 in Esophageal Cancer Cell

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Mutation of breast cancer 2, early onset (BRCA2) has been identified as a vital risk factor for esophageal cancer (EC). To date, several proteins have been reported as BRCA2-interacting proteins and are associated with multiple biological processes. This study's aim was to identify a novel interactive protein of BRCA2 and to explore its functional roles in EC. A yeast two-hybrid screening was performed to identify a novel BRCA2-interacting protein. Glutathione-*S*-transferase (GST) pull-down analysis was performed to find out how the binding domain of BRCA2 interacts with LIM domains containing 1 (LIMD1). The interaction between LIMD1 and BRCA2 at the endogenous level was confirmed by using coimmunoprecipitation and immunobloting. Furthermore, two different sequences of short hairpin RNAs (shRNAs) against LIMD1 were transfected into the human EC cell line ECA109. Afterward, the effects of LIMD1 suppression on the centrosome localization of BRCA2 and cell division were analyzed using an immunofluorescence microscope. Results showed that LIMD1 was a novel BRCA2-interacting protein, and LIMD1 interacted with the conserved region of BRCA2 (amino acids 2,750–3,094) in vitro. Importantly, after interfering with the protein expression of LIMD1 in ECA109 cells, the centrosome localization of BRCA2 was significantly abolished and abnormal cell division was significantly increased. These results suggested that LIMD1 is a novel BRCA2-interacting protein and is involved in the centrosome localization of BRCA2 and suppression of LIMD1, causing abnormal cell division in EC cells.

Key words: BRCA2; LIMD1; Centrosome localization; Esophageal cancer (EC)

INTRODUCTION

Esophageal cancer (EC) is a cancer arising from the esophagus. It is the eighth most common cancer worldwide, with 400,000 deaths every year (1). The major histological types include esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) (2). The risk factors for both types of EC have been proven to be different (3). The most common causes of ESCC are smoking, alcohol, hot drinks, and poor diet, while the most common causes of EAC are smoking, obesity, and acid reflux (4). Of note, the genetic aspect is a risk factor in both types of EC. The treatment of EC is based on the cancer's type, stage, and location. Surgery is generally accepted as the main treatment, and chemotherapy or radiation therapy is used along with surgery (5). Despite the amount of effort that has gone into developing treatments for this disease, outcomes remain unfavorable, and the overall 5-year survival rates are around 16.9% to 20.9% (6).

Breast cancer 2, early onset (BRCA2), together with breast cancer 1, early onset (BRCA1), are major hereditary breast cancer susceptibility genes (7). Multiple studies have demonstrated that alteration of the BRCA2 gene gives an increased risk factor for tumorigenesis and progression in a variety of cancers, including breast cancer, ovarian cancer, and EC (8,9). The germline mutation of the BRCA2 gene has been monitored in several high-risk EC populations from northwest China, northeast India, and Turkmen of Iran. The BRCA2 mutation has been identified as a vital risk factor in EC (10). Clinical investigations in patients with EC have shown that BRCA2 might impact patient survival and could be a potential genomic predictor of clinical response to DNA-damaging treatment (11). However, the detailed function of BRCA2 on EC cells and its underlying molecular mechanisms have not been well clarified.

The centrosome is an important organelle in cells and is closely associated to mitosis. A number of proteins that regulate mitosis have been reported to localize to the centrosomes, such as mitosis cyclin E, p53, poly-(ADP-ribose) polymerase (PARP), and BRCA2 (12). Usually,

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endogenous BRCA2 is localized in the nucleus (13,14), while recent studies have shown that BRCA2 also localizes to the centrosomes during the S and early M phases of the cell cycle (12). The localization of BRCA2 to centrosomes is associated with the functions of centrosome and directly participates in cell cycle regulation. In terms of cancer, centrosome defects are characteristic of many solid tumors and may be responsible for the origin of the mitotic spindle abnormalities (15) and DNA aneuploidy (16). Thus, investigating the effects of BRCA2 centrosome localization on EC cells might be helpful for us to understand the tumorigenesis of this cancer.

This study was aimed at understanding the functions of BRCA2. A yeast two-hybrid screening was performed to identify novel interactive proteins of BRCA2. We found that LIM domains containing 1 (LIMD1) was a novel BRCA2-interacting protein. The binding domain of BRCA2 interacting with LIMD1 was detected, and whether LIMD1 interacted with BRCA2 at an endogenous level was confirmed. Furthermore, two different sequences of short hairpin RNAs (shRNAs) against LIMD1 were transfected into the human EC cell line ECA109. The cells with BRCA2 located to the centrosome, and the cells with three or more centrosomes were analyzed to reveal the role of LIMD1 in BRCA2 centrosome localization in vitro. These findings might be helpful for us to understand the role of BRCA2 in the regulation of the centrosome cycle in EC cells.

MATERIALS AND METHODS

Yeast Two-Hybrid Screening

Novel BRCA2-interactng proteins were identified by using the yeast two-hybrid screening assay, and a ProQuest Two-Hybrid System (Invitrogen, Carlsbad, CA, USA) was used, according to the manual. Briefly, the conserved region (amino acids 2,750–3,094) of BRCA2 cDNA was cloned into the DNA-ding domain in the pDBLeu vector to generate a bait plasmid pDBLeu-BRCA2. Afterward, proteins that interacted with the bait were screened from the cDNA libraries of a 17-day mouse embryo and the human fetal brain (17).

Cell Culture and Transfection

The human EC cell line ECA109 was obtained from the Chinese Academy of Sciences' Type Culture Collection. Cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U/ml streptomycin, and 100 U/ml penicillin (Life Technologies, Cergy Pontoise, France) (18). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For transfection, cells were transfected with expression plasmids using FuGENE 6 (Roche Diagnostics, Tokyo, Japan) and Lipofectamine 2000 (Invitrogen) as previously described in detail (19).

Plasmids

LIMD1 cDNA was isolated by real-time polymerase chain reaction (RT-PCR) from human testis total RNA (BD Clontech, Palo Alto, CA, USA) and cloned into pcDNA3.1HA (BD Clontech). Flag-tagged BRCA2 protein was generated by fusing the Flag tag to the C terminus of the BRCA2 protein (12). Glutathione-*S*-transferase (GST)-tagged LIMD1 cloned into pGEX4T1 (Amersham Biosciences, Piscataway, NJ, USA).

Coimmunoprecipitation and Immunobloting

ECA109 cells were suspended in 1% Nonidet P-40 lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_3, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin; Sigma-Aldrich) and incubated for 15 min at 4°C with gentle shaking (20). Cell lysates were centrifuged at 12,000 rpm for 15 min, and the supernatant was incubated with anti-BRCA2 (Calbiochem, San Diego, CA, USA) or anti-LIMD1 (Abcam, Cambridge, MA, USA), Protein A-Sepharose (Zymed Laboratories, South San Francisco, CA, USA) or anti-HA (Roche Diagnostics) overnight at 4°C. Washes were carried out three times to reduce the nonspecific binding proteins bound to the heads. They were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Finally, the membranes were incubated with goat anti-rabbit coupled to horseradish peroxidase (HRP) (Bio-Rad), and the bands were visualized using chemiluminescence (PerkinElmer, Wellesley, MA, USA) (21).

GST Pull-Down Assay

GST pull-down assays were performed as described previously (22). Briefly, *Escherichia coli* strain BL21 was used for the production of GST fusion proteins, and glutathione agarose (Sigma-Aldrich) affinity chromatography was used for the purification of GST fusion proteins. The purified GST fusion proteins were centrifuged at 12,000 rpm for 3 min, and then resuspended in cell lysates for 8 h at 4°C with gentle shaking. The final adsorbates were analyzed by resolution on SDS-PAGE gels, followed by immunoblotting with anti-Flag antibody (Sigma-Aldrich).

shRNA Stable Line Production and shRNA Transfection

Cells were transfected with shRNA using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The shRNA sequences against LIMD1 were as follows: shRNA 1: hLIMD1 shRNA 3′-UTR, 5′-GCA

GAATGGCTGCAAATTTAA-3′; shRNA 2: hLIMD1 shRNA 5′-UTR, 5′-GTCTGCAGCATGGATAAGTA-3′. The scrambled shRNA acted as a blank control. All shRNA sequences were synthetized by Genomeditech (Shanghai, China). The knockdown of LIMD1 was verified by immunoblotting.

Immunofluorescence Microscopy

Cells were seeded onto 22×22-mm coverslips and incubated at 37°C until the cells grew to about 60% confluence. Cells were fixed with 100% formaldehyde for 3 min and then washed with phosphate-buffered saline (PBS) containing 1% normal goat serum three times. Afterward, cells were incubated with primary antibodies: anti-Flag (1:500; Sigma-Aldrich), anti-BRCA2 (1:200; Calbiochem), and anti-γ-tubulin (1:1,000; Sigma-Aldrich) for 1 h at room temperature. The cells were incubated with Alexa Fluor 488- or 594-conjugated secondary antibody (1:200; Molecular Probes) for 1 h at room temperature. DNA was stained with 1 μg/ml bis-benzimide (Hoechst 33258), and the cells were analyzed under an Olympus Power BX51 fluorescence microscope (Olympus Co., Tokyo, Japan) (12).

Statistical Analysis

Data were presented as mean ± standard derivation (SD) from three independent experiments and analyses. The data were analyzed by Student's *t*-test using the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). A value of $p < 0.05$ was defined as statistically significant.

RESULTS

LIMD1 Is a Novel BRCA2-Interacting Protein

In order to explore the novel interactive proteins of BRCA2, a yeast two-hybrid screen was performed using BRCA2 as a bait protein. After screening the cDNA libraries of a 17-day mouse embryo and the human fetal brain, LIMD1 was identified as an interactive protein of BRCA2 (Fig. 1A). To further confirm the results determined by the yeast two-hybrid screening, GST pull-down assay was performed to explore the interaction between BRCA2 and LIMD1. Results in Figure 1B show that LIMD1 interacted directly with the regions of BRCA2 (amino acids 2,750–3,094) and BRCA2 (amino acids 2,750–2,864). These results revealed that LIMD1 was a BRCA2-interacting protein, and LIMD1 interacted directly with the region of amino acids 2,750–3,094 of BRCA2.

LIMD1 Interacted With BRCA2 at an Endogenous Level

To determine the interaction between BRCA2 and LIMD1 at an endogenous level, the lysates of ECA109 cells were collected, and coimmunoprecipitation and immunoblot analysis were performed using antibodies against LIMD1 and BRCA2. Results in Figure 2 show that LIMD1 specifically interacted with BRCA2, but not with the immunoglobulin G (IgG) control. Simultaneously, BRCA2 interacted with LIMD1, but not with IgG. Thus, LIMD1 could interact with BRCA2 at an endogenous level.

LIMD1 Suppression Abolished the Centrosome Localization of BRCA2 and Caused Abnormal Cell Division

To explore the influences of LIMD1 on centrosome localization of endogenous BRCA2 in ECA109 cells, cells were transfected with two different sequences of shRNAs against LIMD1 or scrambled shRNA for 24 h. The efficiency of transfection was detected by immunoblot analysis, and the detailed functions of the suppression of LIMD1 on BRCA2 centrosome localization and cell division were detected by immunofluorescence microscopy. As predicted, the expression of LIMD1 was significantly downregulated by shRNA 1 and shRNA 2 compared with scrambled shRNA (all $p < 0.001$) (Fig. 3A). Importantly, shRNA 1 and shRNA 2 could significantly downregulate the percentage of the cells of BRCA2 located to the centrosome compared with scrambled shRNA (all $p < 0.001$) (Fig. 3B). Conversely, shRNA 1 and shRNA 2 could significantly upregulate the percentage of the cells with three or more centrosomes compared with scrambled shRNA (all $p < 0.001$) (Fig. 3C). These results revealed that LIMD1 could abolish the localization of BRCA2 to the centrosome and cause abnormal cell division.

DISCUSSION

In cells there are many intricate interactions between proteins, and these protein–protein interactions play diverse roles in many biological functions. Moreover, the protein–protein interactions are helpful for us to understand the roles of these proteins in biological processes. For example, the effect of BRCA2 on DNA damage repair was verified by investigating the interaction between BRCA2 and RAD51 recombinase (RAD51). In the current study, we identified the protein LIMD1 as a novel interactive protein of BRCA2 by using a yeast two-hybrid screening, and LIMD1 interacted with the conserved region of BRCA2 (amino acids 2,750–3,094) in vitro. Moreover, after downregulating the protein expression of LIMD1 by transfecting shRNAs into ECA109 cells, the location of BRCA2 to the centrosome was significantly abolished and abnormal cell division was significantly promoted.

BRCA2 is a tumor suppressor gene that is associated with a variety of cancers (23), including EC. BRCA2 is a large protein consisting of 3,418 amino acids, which is widely expressed in a variety of tissues (24). BRCA2 has several regions, including a nuclear localization signal (NLS), centrosomal localization signal (CLS), and

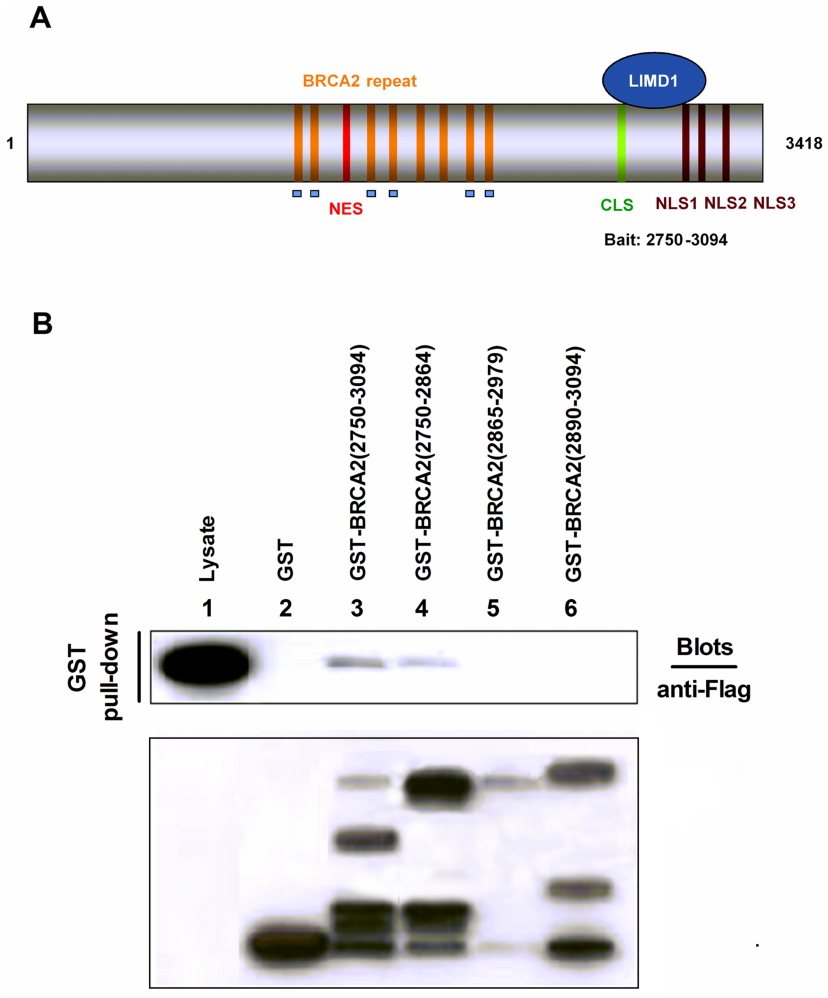

Figure 1. LIMD1 was a novel BRCA2-interacting protein. (A) Schematic representation of the structure of BRCA2; LIMD1 was identified as an interacting protein of BRCA2. (B) The interaction between BRCA2 and LIMD1 was analyzed by GST pull-down assay; LIMD1, LIM domains containing 1; BRCA2, breast cancer 2, early onset; NES, nuclear export sequence; CLS, centrosomal localization signal; NLS, nuclear localization signal; GST, glutathione-*S*-transferase.

nuclear export sequence (NES) (25). Several proteins have been reported to interact with these regions and play important roles in multiple biological processes (26). Interactions between BRCA2 and RAD51 could promote homologous recombination at the genetic and biochemical level in the causative agent of visceral leishmaniasis (27). BRCA2 was also found to interact with DNA meiotic recombinase 1 (Dmc1) and decrease sperm survival 1 (Dss1) in vitro, and that the N-terminal region of BRCA2 is responsible for these interactions (28). In

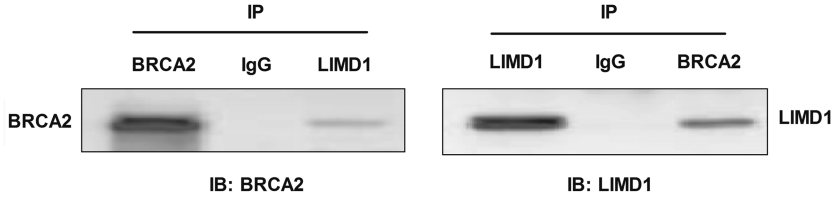

Figure 2. LIMD1 interacted with BRCA2 at the endogenous level. The interaction between BRCA2 and LIMD1 at the endogenous level was detected by coimmunoprecipitation and immunoblot analysis. LIMD1, LIM domains containing 1; BRCA2, breast cancer 2, early onset; IP, immunoprecipitation; IB, immunoblot; IgG, immunoglobulin G.

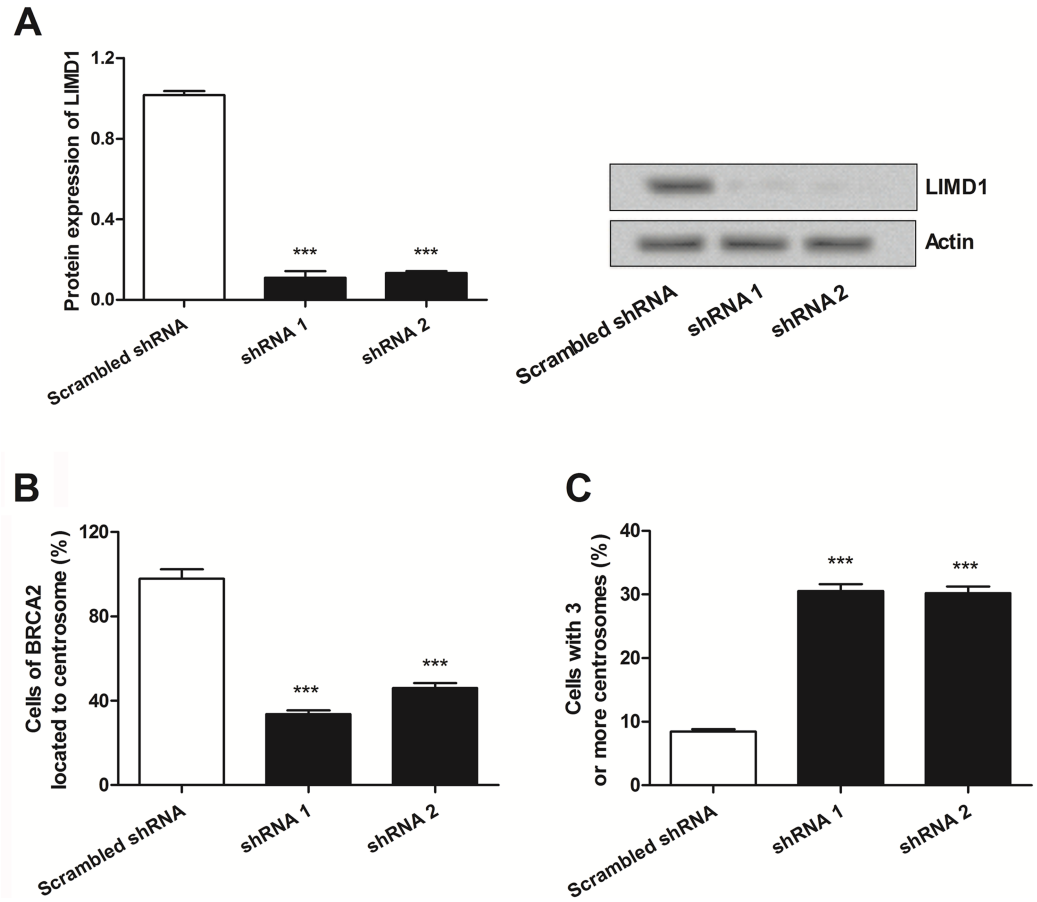

Figure 3. LIMD1 suppression abolished the centrosome localization of BRCA2 and caused abnormal cell division. Two difference sequences of shRNAs against LIMD1 or scrambled shRNA were transfected into ECA109 cells for 24 h, respectively. (A) The efficiency of transfection was detected by immunoblot analysis. (B) The cells of BRCA2 located to the centrosome were stained with bis-benzimide (Hoechst 33258) and analyzed under an immunofluorescence microscope. (C) The cells with three or more centrosomes were stained with bis-benzimide (Hoechst 33258) and analyzed under an immunofluorescence microscope. LIMD1, LIM domains containing 1; BRCA2, breast cancer 2, early onset; shRNA, short hairpin RNA. ***$p<0.001$.

the current study, we discovered a novel interactive protein of BRCA2, LIMD1, and this finding might be helpful for us to further understand the functional role of BRCA2 in EC cells.

LIMD1 is also widely reported as a tumor suppressor gene, which is encoded at chromosome 3p21.3 (29). Studies in vitro and in vivo have demonstrated that LIMD1 interacted with retinoblastoma protein (pRB), and the loss of LIMD1 promoted lung carcinogenesis (30). More recently, LIMD1 was found to interact with eIF4E and core proteins of the microRNA simultaneously, and LIMD1 was required for microRNA-mediated gene silencing and had been validated as a tumor suppressor in lung cancer (31). However, the role of LIMD1 in EC cells has not been determined yet. In this study, we found that LIMD1 could interact with the conserved region of BRCA2 (amino acids 2,750–3,094) in vitro. Moreover, interference with the protein expression of LIMD1 abolished the centrosome localization of BRCA2 and caused abnormal cell division. Thus, LIMD1 might be involved in these biological processes by interacting with BRCA2.

In mitosis, the centrosome plays a key role in the fixation of spindle and centromere, and the structure or function abnormalities of centrosome always results in abnormal nuclear division and tumorigenesis. BRCA2 is mainly localized in the nucleus where it plays an important role in DNA damage repair (13,14). During the S and early M phases of the cell cycle, BRCA2 is localized in the centrosomes and BRCA2 interacts with γ-tubulin, a component of the centrosome (12,25). The localization of BRCA2 to the centrosome is associated with the functions of centrosome and cell cycle regulation. A study by Tutt et al. found that BRCA2 acted as a cell cycle regulation gene, and the absence of BRCA2 causes centrosome amplification (32). Moreover, Nakanishi et al. found that

BRCA2 regulated the centrosome cycle, and suppression of BRCA2 led to abnormal nuclear division (12). Findings in the current study were partly consistent with the foregoing investigations that BRCA2 was a centrosome cycle regulating gene and BRCA2 was associated with abnormal cell division. In addition, we found that the role of BRCA2 in the centrosome might be involved in the interaction between BRCA2 and LIMD1. Therefore, we speculate that suppression of LIMD1 might be involved in the formation of EC. However, further experiments should be performed to support these inferences.

To sum up, we found that LIMD1 was a novel interactive protein of BRCA2, and it was involved in the centrosome localization of BRCA2 and thus caused abnormal cell division in ECA109 cells. Targeting LIMD1 might be a new treatment strategy for EC.

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