RESEARCH ARTICLE

Correlation Between Auto-antibodies to Survivin and MUC1 Variable Number Tandem Repeats in Colorectal Cancer

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Abstract

Aim: The aim of this study was to investigate the frequency and correlation between auto-antibodies to survivin and MUC1 variable number tandem repeats (VNTR) in colorectal cancer (CRC), which can provide valuable information for the design of immunotherapeutic vaccines for this disease. Methods: Enzyme-linked immunosorbent assays (ELISA) were used to examine the level of auto-antibodies against survivin and MUC1 VNTR in the serum of 135 CRC patients and 95 healthy volunteers. Results: Using mean absorbance + 2 standard deviations (SD) of the healthy samples as a cut-off value, the positive rates of survivin and MUC1 VNTR auto-antibodies in CRC were 31.1% and 18.5%, respectively. Altogether, the survivin and MUC1 VNTR positive samples accounted for 36.3% of the CRC patients, and 7.4% were positive for both. Conclusion: A significant positive correlation was found between levels of specific antibodies against survivin and MUC1 VNTR in the serum of CRC patients (r = 0.3652, P < 0.0001), suggesting that vaccines against both targets would elicit immune responses more effectively.

Keywords: Survivin - MUC1 VNTR - ELISA - colorectal cancer - auto-antibody

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Introduction

Colorectal cancer (CRC) is one of the most common malignancies, representing 15% of all diagnosed cancers with an annual incidence of one million new cases worldwide. In developed countries, CRC is the third most common cancer in men, and the second most common cancer in women1. Although the incidence of CRC in China is lower than in Western countries, it has increased in recent years, becoming a substantial burden, particularly in the more developed areas of China (Wan, 2009).

Thus far, surgery, chemotherapy and radiotherapy are the main treatments of choice for CRC (Penland et al., 2004; Hobday, 2005; Meyerhardt et al., 2005). However, more than half of the patients that are initially believed to be cured by surgery and adjuvant therapy may develop recurrences and eventually die of the disease(Midgley et al., 2009). A more advanced treatment is needed, and increasing attention is being paid to the activation of the patient's immune system and enhancement of tumor-specific immunity (Speetjens et al., 2011).

Tumor immunotherapy is a novel strategy which employs vaccines targeting tumor-associated antigens (TAAs). These TAAs can be expressed in the host cells and presented to the immune system by antigen-presenting cells (APC), which can induce immune responses to tumor cells by producing TAA-specific antibody and cytotoxic T lymphocytes (CTL) through activation of B cells and T cells (Green et al., 2000; Dermime et al., 2002) . Therefore, selecting an ideal antigen is the key to the success of such targeted therapies for the prevention and treatment of cancer.

Survivin is a structurally unique inhibitor of apoptosis (IAP) gene family, characterized by developmentally regulated expression during human and mouse differentiation. While nearly undetectable in normal adult tissues, survivin is abundantly expressed in transformed cells and a variety of human tumors (Ambrosini et al., 1997), which renders it a favorable target for cancer vaccines. Some studies have shown that patients expressing survivin have significantly shorter overall survival times (Chakravarti et al., 2002). Recently, antibodies to survivin have been found in patients with lung, gastric, breast, bladder and colorectal cancer (Rohayem et al., 2000; Zhang et al., 2003; Yagihashi et al., 2005; Yip et al., 2006) .

Like survivin, the MUC1 mucin core protein is aberrantly expressed in malignancies, such as in adenocarcinomas (Hull et al., 1989; Apostolopoulos et al, 1994; Finn et al., 1995). MUC1 is a type I transmembrane protein with an extracellular domain consisting of a variable number of 20-amino acid tandem repeats (VNTR) rich in Ser, Thr and Pro. One tandem repeat has been shown to have at least two O-glycosylation sites, resulting

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in a unique extracellular domain with high carbohydrate content (Gendler et al., 1988). Nevertheless, the glycons present on MUC1-expressing cancer cells are shorter than those on normal cells, which makes MUC1 an ideal cancer vaccine target (Finn et al., 1995).

However, the prerequisite for a tumor vaccine is the ability to break immune tolerance, since most antigens are also expressed in normal host cells. Therefore, it is essential to find antigens which have already been recognized by the immune system in cancer patients. There are several reports showing that survivin auto-antibodies have been detected in some cancer patients, such as lung, breast and colorectal cancer patients (Al-Joudi et al., 2006; Karanikas, 2009; Chen et al., 2010), as well antibodies to MUC1 (Hirasawa et al., 2000; Tang et al., 2010). Moreover, several studies have revealed that vaccines targeting survivin or MUC1 VNTR can elicit both specific humoral and cellular immune responses both in pre-clinical and clinical trials (Tang et al., 2008; Yuan et al., 2010; Ishizaki et al., 2011; Kameshima et al., 2011), indicating that they are potent cancer vaccine targets. However, the correlations between auto-antibodies to survivin and MUC1 VNTR remain unknown. Therefore, this study was aimed towards clarifying this issue by detecting the frequency and levels of circulating anti-survivin and anti-MUC1 VNTR auto-antibodies using a large cohort of patients with CRC and normal controls, which may provide guidance for vaccine design.

Materials and Methods

Serum samples

Before treatment for cancer, we obtained sera from 135 patients with CRC, including 67 men and 58 women, 29–80 years of age (median age 62.5 years), consecutively admitted to The Second Hospital of Jilin University, Chang Chun, Jilin Province in 2009-2010. There were 74 colon cancer patients and 61 rectal cancer patients among them, all of whom were adencarcinoma cases. Written informed consent from patients was obtained, and the study was performed in accordance with medical ethics. Following surgery, the clinical stage of each patient was estimated from surgical pathology and clinical reports using the Dukes classification system. The controls were selected to approximately match both the median age of the CRC population and the proportion of male and female subjects. Serum samples from 95 healthy individuals, 47 men and 48 women between 24–84 years of age (median age 65 years) were obtained as controls.

Preparation of recombinant protein

The recombinant survivin fusion protein was prepared as reported previously (Zhang et al., 2008) . Briefly, the cDNA of survivin amplified from total RNA of HEK293 cells by RT-PCR was cloned into the pGEM-T vector. After confirming the size and sequence of survivin cDNA by restriction enzyme digestion and sequencing, the survivin cDNA was subsequently subcloned into an prokaryotic expression vector pRSET-B (Invitrogen) for protein expression. Expression of the recombinant protein was confirmed by immunoblotting using a mouse monoclonal antibody (MAb) (Gene Tex). For MUC1, a cDNA fragment containing nine identical peptide tandem repeats (sequence HGVTSAPDTRPAPGSTAPPA) was synthesized by GENERAY Corporation and ligated into pET-26B (Novagen). The plasmid was then transformed into an Escherichia coli strain, BL21DE3 (Invitrogen). The cells were grown in LB medium until OD600 reached 0.6, and then isopropyl b-D-thiogalactopyranoside (IPTG) was used to induce protein expression. Five hours after induction, the cells were harvested by centrifugation at 4,000 × g. The cells were then disrupted by sonication at 4°C in lysis buffer containing 0.2 mol/L Tris-HCl, pH 7.5, 200 mL/L glycerol and 1 mmol/L phenylmethylsulfony fluoride. Expression of the recombinant protein was confirmed by immunoblotting using a mouse MAb against MUC1 VNTR (BD Pharmingen). The two recombinant His-tagged protein were purified using a Hi Trap chelating HP column (Invitrogen).

The concentrations of the purified recombinant survivin and MUC1 VNTR were determined by measuring UV absorbance using a spectrophotometer (Thermo Scientific NanoDrop 2000C).

ELISA

Purified recombinant protein was diluted in 50 mM bicarbonate buffer (pH 9.5) to a final protein concentration of 20 μg/mL. The survivin and MUC1 solutions were dispensed at 20 μg/mL into ELISA plates (Jet Biofil) (100 μL/well) and incubated overnight at 4°C. After removal of the protein solution, plates were blocked with 5% skim dry milk solution in phosphate buffered saline (PBS) for 1.5 h at 37°C (100 μL/well). The plates were washed five times with PBST (PBS containing 0.1% Tween 20). Serum samples diluted 1:100 in PBS were added at 100 μL/pre-coated well. After 2 h, the serum was removed, and the plates were washed five times with PBST. Each well was then incubated for 1 h with 100 μL of a 1:20,000 dilution of goat anti-human IgG1 labeled with horseradish peroxidase (Jackson Immuno Research), washed five times with PBST, and developed by adding 100 μL of TMB substrate (Tiangen). After a 25-min incubation in the dark, the reaction was stopped with 50 μL of 2 M H2SO4, and the absorbance at 450 nm was measured. All serum samples were run in duplicate and randomly distributed on the plates. Sera from cancer patients and sera from healthy donors were tested simultaneously.

Western blot analysis

Purified protein (50 μg) was boiled in loading buffer for 5 min, subjected to 13.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked in PBS containing 30 g/L skim dry milk for 40 min at room temperature. After decanting the blocking buffer, the membrane was cut into strips. The strips were then incubated separately with patient serum (1:20 dilution each in PBS containing 10 g/L skim dry milk) overnight at 4°C with agitation. After washing, the strips were incubated with horse radish peroxidase-conjugated anti-human IgG1 (Jackson Immuno Research) for 1 h at 37°C, and then stained routinely. Anti-survivin

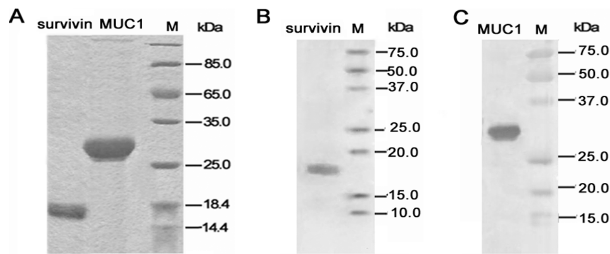

Figure 1. Purification and Identification of Recombinant Survivin and MUC1 VNTR. (a) Analysis of recombinant survivin and MUC1 VNTR protein expressed in E. coli on an SDS–13.5% PAGE gel. Lane M, marker. (b) Identification of purified survivin protein by Western blotting with anti-survivin MAb; lane M, marker. (c) Identification of purified MUC1 VNTR protein by Western blotting with anti-MUC1 VNTR MAb

or anti-MUC1 VNTR MAb were used as positive controls.

Competitive indirect ELISA (CI-ELISA)

Serum samples (100 μl of a 1:100 dilution) were incubated with 50 μg/ml of recombinant survivin or MUC1 antigen for 1 h at 37°C and then subjected to the anti-survivin or anti-MUC1 ELISA described above.

Statistical analysis

Statistical analysis (unpaired Student's t-test) of differences in the absorbance of anti-survivin and anti-MUC1 VNTR antibodies was performed using GraphPad Prism 5.01 (GraphPad Software) and data analysis software (Microsoft Excel 2003). The correlation between two experimental groups was evaluated by Spearman analysis. Significance in a two-tailed test was defined as $P < 0.05$.

Results

Purification and identification of survivin and MUC1 VNTR recombinant proteins

In the present study, two constructs expressing recombinant proteins were made, one of which was the full-length survivin and the other was MUC1 VNTR (containing nine MUC1 tandem repeats). Survivin was prepared for expression as a recombinant C-terminal 6His-tagged protein from E. coli and for purification using the Ni-NTA Purification System (Invitrogen) under denaturing conditions, while MUC1 VNTR were prepared under natural conditions. The expressed survivin and MUC1 VNTR were analyzed by SDS-PAGE (Figure 1A), and the purified proteins were identified by Western blotting (Figure 1B and C). The results showed that survivin and MUC1 VNTR were successfully purified in E. coli and could be specifically recognized by anti-survivin and anti-MUC1 VNTR MAb.

Auto-antibodies against survivin and MUC1 VNTR are detected in CRC patients

A total of 135 CRC patients were recruited after histopathological confirmation of the tumor. Means + 2 standard deviations (SD) taken from healthy donors' index were selected to determine the cut-off for positivity in the ELISA. The cut-off value for positivity in the anti-

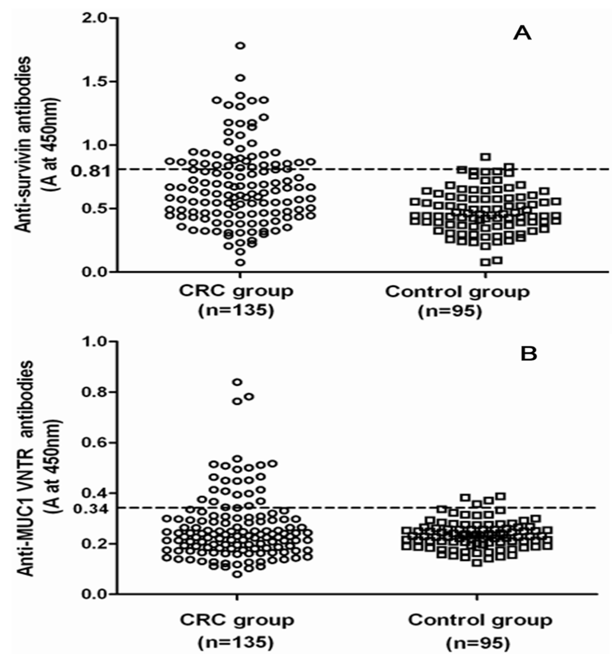

Figure 2. ELISA Analysis of Anti-survivin and Anti-MUC1 VNTR in Sera from CRC Patients (n = 135) and Healthy Controls (n = 95). (a) Data represent mean values (A450) from three determinations. The horizontal line indicates the cut-off value for seropositivity (A450 > 0.81). (b) Data represent mean values (A450) from three determinations. The horizontal line indicates the cut-off value for seropositity (A450 > 0.34)

survivin ELISA, determined from healthy donor samples, was 0.81. Based on this criteria, sera from 42 of 135 CRC patients were positive by ELISA using recombinant survivin protein, indicating that the sensitivity of this assay was 31.1%; whereas for the control group, only 1 out of 95 samples was positive, indicating the specificity was 98.9% (Figure 2A). The cut-off value for positivity in the anti-MUC1 VNTR ELISA was 0.34. Sera from 25 of 135 CRC patients (18.5%) were positive by ELISA using recombinant MUC1 VNTR protein, with a specificity of 96.8% (Figure 2B). There were significant differences between CRC patients and healthy controls both in anti-survivin and MUC1 VNTR auto-antibodies ($P < 0.01$), which suggested that a significant proportion of the cancer patients had generated antibodies against survivin and MUC1 VNTR in the context of tumorigenesis.

Specificity of auto-antibodies against survivin and MUC1 VNTR in CRC patients

To determine the specificity of the survivin and MUC1 VNTR ELISAs, several randomly selected positive sera were pre-absorbed with recombinant survivin (Figure 3A) or MUC1 VNTR (Figure 3B) for competitive ELISA. Data were obtained in triplicate for each sample. Reactivity of sera decreased significantly ($P < 0.05$) after absorption with recombinant survivin or MUC1 VNTR protein (t-test). The negative control (NC) was processed with no protein incorporated into the assay to show non-specific binding in the assay. The results of sera from nine CRC patients and three healthy controls are shown in Figure 3.

To confirm the results above, sera from randomly selected ELISA-positive CRC patients were examined

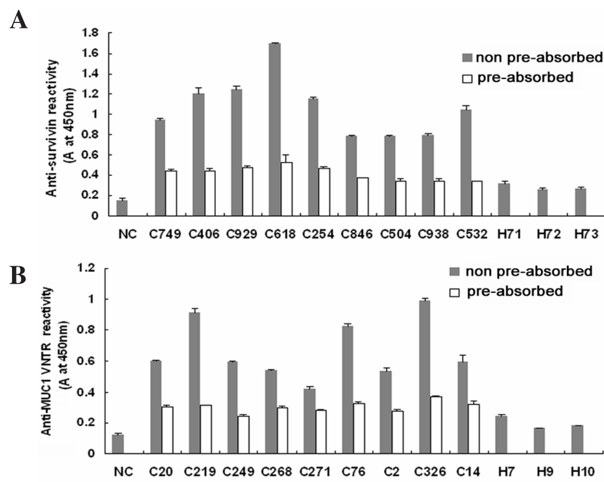

Figure 3. Pre-absorption of Sera from CRC Patients with Recombinant Survivin or MUC1 VNTR. (a) Sera from nine CRC patients (cases 20, 219, 249, 268, 271, 76, 2,245 and 14) and three healthy donors (H 7, 9 and 10) were tested in the survivin ELISA after pre-absorption of sera with recombinant survivin. (b) Sera from nine CRC patients (cases 749, 406, 929, 618, 846, 504, 938, 326 and 532) and three healthy donors (H 71, 72 and 73) were tested in the MUC1 VNTR ELISA after pre-absorption of sera with recombinant MUC1 VNTR protein. NC indicates negative control, which is the ELISA carried out with no protein incorporated into the assay

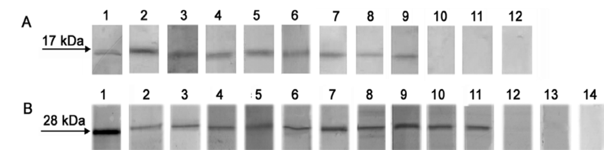

Figure 4. Detection of Antibodies Against Recombinant Survivin and MUC1 VNTR in ELISA-positive Serum Samples from CRC Patients Using Western Blot Analysis. (a) Detection of antibodies against recombinant survivin: lane 1, positive control with survivin MAb; lanes 2-9, sera from CRC patients; lanes 10-12, sera from healthy controls. (b) Detection of antibodies against recombinant MUC1 VNTR: lane 1, positive control with MUC1 VNTR MAb; lanes 2-11, sera from CRC patients; lanes 12-14, sera from healthy controls

for antibody reactivity against recombinant survivin and MUC1 VNTR in Western blots, with MAb against survivin and MUC1 VNTR as positive controls. Serum samples from 3 healthy controls were also examined. To illustrate the results of Western blotting, Figure 4 showed the staining pattern of sera from several patients with CRC and healthy volunteers. Antibodies in 8 out of 20 ELISA-positive serum samples (40.0%) recognized survivin (~17 kDa) (Figure 4A), while 10 out of 15 ELISA-positive serum samples (66.7%) recognized MUC1 VNTR (~28 kDa) (Figure 4B).

Correlation between auto-antibodies against survivin and MUC1 VNTR in CRC patients

The auto-antibody levels of survivin and MUC1 VNTR were analyzed for correlation and compared between the 135 CRC patients (Figure 5A) and healthy controls (Figure 5B). The intensity of anti-survivin antibody responses was significantly correlated with intensity of anti-MUC1 antibody responses (r = 0.3652, P < 0.0001). These results suggest that vaccines targeting both survivin and MUC1

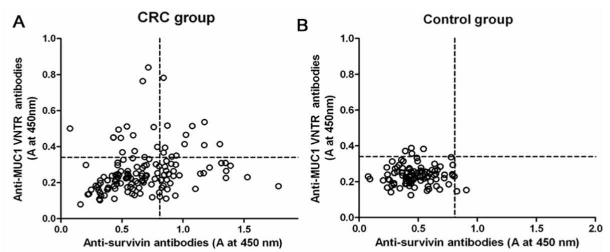

Figure 5. Correlation Between Anti-survivin and Anti-MUC1 VNTR Antibodies. (a) There was a significant correlation between intensities of anti-MUC1 antibodies and anti-survivin antibodies in sera from CRC patients. Spectrophotometric absorbance for the anti-survivin antibody ELISA was plotted against that for anti-MUC1 VNTR antibody (r = 0.3652, P < 0.0001). (b) No healthy donors were positive for both anti-survivin and anti-MUC1 antibodies

VNTR would elicit immune responses more effectively than those targeting either protein alone. Among the 135 sera, 49 (36.3%) were positive for antibodies to survivin and/or MUC1 VNTR by ELISA, with a specificity of 95.8%. In the CRC group, 10 (7.4%) were positive in both ELISAs, whereas none were positive among the healthy controls (Figure 5B).

Discussion

Our effort in this work was inspired by the finding that vaccines targeting survivin or MUC1 VNTR have elicited detectable immune responses in pre-clinical stages and was undertaken to clarify the inconsistent results from previous studies examining antibody responses in CRC (Rohayem et al., 2000; Zhang et al., 2003; Chen et al., 2010). This study further explored the correlation of auto-antibodies against survivin and MUC1 VNTR in the serum of CRC patients, which we believe is important for cancer vaccine design.

In examining the presence of IgG1 against survivin and MUC1 VNTR in sera from CRC patients, both types of antibodies were increased significantly compared with those in sera from the healthy donors (P < 0.01), consistent with previous reports (Nakamura et al., 1998; Silk et al., 2009). By using ELISA, an anti-survivin serum-positive response was found in 31.1% of CRC patients and in only 1.05% of healthy donors. Meanwhile, anti-MUC1 VNTR antibodies were detected in 18.5% (25/135) of CRC patients and in 3.2% of healthy controls. In addition, a combined 36.3% (49/135) of the serum samples from CRC patients versus only 4.2% in the controls were ELISA-positive against recombinant survivin protein, recombinant MUC1-VNTR protein, or both. Thus, our detection method showed significantly better specificity, although with a relatively lower sensitivity, than that described in previous reports (Nakamura et al., 1998; Chen et al., 2010). Such discordant data may result from different patient demographics, recombinant antigens or statistical methods. Nevertheless, a statistically significant correlation was found between the two types of antibodies (r = 0.3652; P < 0.0001), suggesting that the vaccines targeting both survivin and MUC1 VNTR would effectively elicit immune responses. Although the anti-survivin and anti-MUC1 VNTR antibodies did not

simultaneously appear in some patients, a tumor vaccine designed to elicit responses to both antigens will expand the scope of protection in the population as a whole. As survivin is an intracellular immunogen, and MUC1 VNTR is an extracellular antigen, a vaccine targeting both proteins will likely to be potent for CRC therapy. We propose that future vaccines should target more than one TAA in order to better elicit broad immune responses. Competitive ELISA and Western blotting were also used to confirm the specificity of the auto-antibodies. Several ELISA-positive serum samples showed specific bands in the Western blot, suggesting that Western blotting had lower sensitivity than ELISA, consistent with previous studies (Megliorino et al., 2005; Chen et al., 2010).

Auto-antibody responses associated with cancer have attracted limited attention despite the fact that they may provide useful information about helper T cell responses against TAAs. Common properties emerging from the study of naturally occurring antibody responses give clues to the immunogenicity of some TAAs. Naturally occurring antibodies are frontier soldiers that act as the first line of defense in the battle against cancer (Toubi et al., 2007). They can mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (Howat et al., 1979), a cell-mediated immune defense mechanism, whereby an effector cell actively lyses a target cell that has been bound by specific antibodies. The generation of natural protective auto-antibodies against TAAs indicates that specific humoral immune responses can be produced, which is important for the ability of cancer vaccines to break immune tolerance. On this theoretical basis, tumor vaccines which target the same TAAs could boost immune response significantly. Thus, we believe that the presence of pre-existing auto-antibodies is predictive of the effectiveness of a vaccine to elicit effective anti-tumor immune responses. Although there is still a lack of consensus on the correlates of immune protection, there is increasing evidence showing that cancer vaccines which target survivin and MUC1 VNTR could elicit both humoral and cellular immune responses (Dermime et al., 2002; DiFronzo et al., 2002; Chung et al., 2003; Coronella-Wood et al., 2003; Lladser et al., 2006).

We also evaluated the relationship between serum survivin and MUC1 VNTR auto-antibody concentrations and clinicopathological variables. However, the survivin and MUC1 antibody titers did not correlate with age, gender, TNM staging, lymph node status, distant metastasis or degree of differentiation (data not shown). Nevertheless, this finding suggests that vaccines targeting survivin and MUC1 VNTR can be broadly applied to the population of CRC patients.

In conclusion: The results clearly showed that anti-survivin and anti-MUC1 VNTR antibodies could be detected in the serum of CRC patients. We propose that vaccines targeting both survivin and MUC1 VNTR would elicit immune responses more effectively in light of the significant correlation between pre-existing antibodies to those antigens in CRC patients. Thus, our study provides valuable information for the design of vaccines for CRC immunotherapy. However, the functional role of humoral immune responses against tumor antigens needs to be further analyzed in the context of cellular immune responses and especially in cancer immunotherapy settings.

Acknowledgements

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