miR-21 Inhibition Reduces Liver Fibrosis and Prevents Tumor Development by Inducing Apoptosis of CD24+ Progenitor Cells

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Abstract

miR-21 is upregulated in hepatocellular carcinoma and intrahepatic cholangiocarcinoma, where it is associated with poor prognosis. Here, we offer preclinical evidence that miR-21 offers a therapeutic and chemopreventive target in these liver cancers. In mice with hepatic deletion of Pten, anti-miR-21 treatment reduced liver tumor growth and prevented tumor development. These effects were accompanied with a decrease in liver fibrosis and a concomitant reduction of CD24+ liver progenitor cells and S100A4+ cancer-associated stromal cells.

Introduction

miRNA are small noncoding RNA molecules that affect mRNA stability and translation by targeting the 3′-untranslated region (3′-UTR) of various transcripts. Dysregulation of miRNAs affects a wide range of cellular processes, including cell proliferation and differentiation. Interest in miRNA-21 (miR-21) has increased recently, especially in cancer and cardiovascular diseases (1). miR-21 is upregulated in almost all types of cancers, promotes cancer cell proliferation, migration, and survival and therefore was classified as an onco-miR (2, 3). High levels of miR-21 are often associated with aggressive forms of cancer and poor patient survival. miR-21 is significantly upregulated in hepatocellular carcinoma (HCC) and is associated with poor prognosis (4–10). miR-21 was also reported to be overexpressed in intrahepatic cholangiocarcinoma and cholangiocarcinoma, but no correlation with clinicopathological features was found (10, 11). A role of miR-21 in organ fibrosis has also been described (1). miR-21 regulates epithelial-to-mesenchymal transition (EMT), a process in which fibroblasts derive from epithelial or endothelial cells, resulting in organ fibrosis. A direct evidence for a role of miR-21 in organ fibrosis was obtained from studies in mouse models of cardiac and pulmonary fibrosis (12, 13). Upregulation of miR-21 was found in human fibrotic livers, resulting in chronic hepatitis C virus (HCV) infection and intrahepatic miR-21 levels positively correlated with HCV viral load, fibrosis severity, or serum liver transaminase levels (14). Cirrhosis, the result of end-stage fibrosis, is a common preneoplastic condition associated with hepatocarcinogenesis (15). It is therefore highly relevant to study the role of miR-21 in hepatocarcinogenesis. Deletion of the PI3K/PTEN in hepatocytes leads to steatohepatitis, fibrosis, hepatocyte dysplasia, and HCC later in life, reproducing the steps of HCC development observed in human HCC (16, 17). It therefore represents an excellent model to evaluate the potential of targeting miR-21 for the treatment and chemoprevention of HCC.

Materials and Methods

Mice treatment

C57BL/6 mice carrying Pten conditional knockout alleles were crossed with an Albumin (Alb)-Cre-transgenic mouse. For this model, control animals are Pten; Alb-Cre− while the experimental mice are Pten; Alb-Cre+. Only male mice were included in this study as female mice develop tumors at a much lower incidence rate. Two separate sets of treatment were performed. In the first set, 12 x 10.5-month-old male Pten-null mice received anti-miR-21 (25 mg/kg) in PBS carriage medium or placebo by intraperitoneal injection. In the second set, 12 x 7.5-month-old male Pten-null mice received anti-miR-21 (25 mg/kg) or placebo by intraperitoneal injection. The anti-miR-21
used was a high-affinity oligonucleotide complementary to the active site of miR-21 with a phosphorothioate backbone containing modifications (DNA, MOE, cEt, Regulus Therapeutics). The PK of these chimeric phosphorothioate antisense oligonucleotides is independent of the sequence. The compounds PK and pharmaco
dynamics across species (mice, primates, human) together with safety profiles and dose-dependent actions in liver, the major organ of deposition, have been described (18). All mice received eight injections over a period of 6 weeks, three injections in the first week of treatment and one injection per week in the following 5 weeks. C57BL/6 wild-type (wt) and OPN knockout (OPN\textsuperscript{-/}) mice were purchased from The Jackson Laboratory. A total of 7 × 10\textsuperscript{9} 10-week-old male wt mice and 6 × 10\textsuperscript{9} 10-week-old male OPN\textsuperscript{-/} mice were fed with either normal diet or a diet supplemented with 0.1% diethoxycarbonyl-1, 4-dihydro-collidin (DDC; Sigma-Aldrich) for 4 weeks. All animal studies were carried out in strict accordance with institutional regulations and every effort was made to minimize the number of animals required for the study and to minimize the pain and discomfort experienced.

miR-21 in situ hybridization and immunofluorescence costaining

Formalin-fixed paraffin-embedded tissue sections were deparaffinized in xylene, and rehydrated using ethanol dilutions. For miR-21 in situ hybridization, tissue sections were digested with 5 μg/mL proteinase K for 5 minutes at room temperature, then loaded onto Ventana Discovery Ultra for in situ hybridization analysis. The tissue slides were incubated with double-DIG labeled mercury LNA microRNA probe (Exiqon) for 2 hours at 55°C. The digoxigenins were then detected with a polyclonal anti-DIG antibody and alkaline phosphatase conjugated second antibody (Ventana) using NBT-BCIP as the substrate. Negative miRNA probe from Exiqon was used as negative control. Positive control was performed using miRNA U6. For costaining, miRNA probe labeled slides were treated with 3% H₂O₂ to inactivate endogenous peroxidase and blocked with 5% BSA in PBS (w/v). OPN (R&amp;D) primary antibody was used followed by secondary antibody incubation in PBS and tyramine-conjugated fluorochrome.

Apoptosis assays

In vitro cell apoptosis was tested using FITC Annexin V Apoptosis Detection Kit (BD Pharmingen) following transfection with 20 nmol/L oligonucleotide (hsa-miR-21 Anti-miR from Ambion Life Technologies) for 72 hours using 10 μL Lipofectamine RNAiMAX transfection reagent. For OPN rescue assays, 1 μg/mL of recombinant OPN protein (R&amp;D) was added to the culture medium following anti-miR-21 transfection. For ITGaV blocking experiments, 5 μg/mL of ITGaV-blocking antibody (Abcam) was added to the culture medium. Same amount of IgG was used as negative control.

Additional methods are provided in Supplementary Methods.

Results

In mice with hepatocytic deletion of Pten, miR-21 expression is increased in liver tumors, correlates with fibrosis in adjacent liver, and is enriched in progenitor cells

Hepatic deletion of Pten in male mice induced liver steatosis at around 3 months and steatosis severity increased with aging, remaining stable after 6 month (Supplementary Fig. S1A). Mild liver fibrosis was detected in 6-month-old Pten-null mice and gradually increased in severity with aging (Supplementary Fig. S1B). At 9-month-old, around 80% of the Pten-null mice had developed tumors and all 12-month-old mice presented with tumors (Supplementary Fig. S1C). We measured the expression of miR-21 in the liver and tumors of these mice. miR-21 levels were not statistically different in control healthy liver and in steatotic liver from 6-month-old Pten-null mice (median of 3.7 × 10\textsuperscript{9} copies and 2.9 × 10\textsuperscript{9} copies, respectively). miR-21 expression significantly increased in liver of 9- and 12-month-old Pten-null mice (median = 7.99 × 10\textsuperscript{9} copies, P < 0.001) and further increased in tumors (median = 13.0 × 10\textsuperscript{9} copies, P = 0.02; Fig. 1A). Fibrosis and lipid depositions were measured by histology in all mice and the size of the tumors was recorded. Although levels of miR-21 did not correlate with tumor size or steatosis levels, miR-21 expression strongly correlated with fibrosis severity (R = 0.71; Fig. 1B).

By in situ hybridization of miR-21 in liver and tumors of Pten-null mice, we did not detect any miR-21 expression in hepatocytes nor HCC cells. Instead, a strong positive miR-21 signal was detected in ductular reaction areas in the liver, in areas surrounding the tumors and in neoplastic biliary cells (Fig. 1C). More specifically, miR-21 was enriched in cells expressing osteopontin (OPN, Spp-1), a marker of hepatic progenitor cells and biliary cells (Fig. 1D). To further validate the relevance of this expression pattern in human disease, in situ hybridization of miR-21 was performed on six human resected HCCs. As observed in the Pten-null mouse model, miR-21 was mainly expressed in areas surrounding the tumors and in non-neoplastic bile ducts. The tumor areas were negative in five HCCs and partially positive in the sixth one (Fig. 1E).

Anti-miR-21 treatment inhibits tumor growth, modifies the tumor differentiation phenotype, and reduces liver fibrosis

To evaluate the therapeutic potential of targeting miR-21, we treated 10.5-month-old Pten-null mice for 6 weeks, with chemically modified antisense oligonucleotides specific for miR-21. At necropsy, tumors and adjacent tissues were collected and processed for gene expression and histology analyses. We first validated the anti-miR-21 treatment efficiency by measuring miR-21 target genes Sprouty-1 and Sprouty-2 (Spry1 and Spry2). Upon anti-miR-21 treatment, expression of Spry1 and Spry2 significantly increased (2.1-fold; P = 0.032 and 2.0-fold; P = 0.043, respectively), confirming that the anti-miR-21 treatment was effective in reducing miR-21 activity (Fig. 2A). We next evaluated the effect of anti-miR-21 on tumor growth (Fig. 2B–D). Anti-miR-21 treatment decreased the ratio of liver/body weight from 23.4% to 20.1% (P = 0.028; Fig. 2B). Although anti-miR-21 treatment did not affect the average number of tumors per mouse (2.7 and 2.8 for placebo and anti-miR-21 groups, respectively; Fig. 2B), the average tumor burden in anti-miR-21 treated mice was significantly smaller than in placebo-treated mice (891 mm\textsuperscript{3} compared with 2,308 mm\textsuperscript{3}, P = 0.05; Fig. 2C). The average tumor size in anti-miR-21 treated mice was also significantly smaller than that in placebo-treated mice (342 mm\textsuperscript{3} compared with 865 mm\textsuperscript{3}, P = 0.05; Fig. 2C). The reduction in tumor size following anti-miR-21 treatment was further demonstrated by the tumor size distribution per mouse in both groups. While half of the tumors in placebo-treated mice were >500 mm\textsuperscript{3}, only 21.7% of the tumors in anti-miR-21 treated mice were >500 mm\textsuperscript{3} (Fig. 2D).

Blinded evaluation by a pathologist of 14 tumors from the placebo group and 11 tumors from the anti-miR-21-treated
group identified the placebo tumors as displaying multiple morphologic characteristics, including HCC, cholangiocarcinoma, and hepatocellobilangiocellular carcinoma. In contrast, anti-miR-21 treated tumors showed a significant reduction in the incidence of cholangiolar tumors with incidence of cholangiocarcinoma and hepatocellobilangiocellular carcinoma decreasing from 29% and 71%, respectively, in the placebo treated group to 9% and 27%, respectively, in the anti-miR-21 treated group. In addition, anti-miR-21 treatment resulted in a decrease in the grade of tumor malignancy from predominantly pleomorphic and heterogeneous HCC to well-differentiated HCC. The incidence of pleomorphic HCC decreased from 42% in the placebo-treated group to 27% in the anti-miR-21 treated group. The majority of tumors in anti-miR-21 treated group were well differentiated HCCs, suggesting that inhibition of miR-21 resulted not only in reduced tumor growth, but also in histologically less malignant liver tumors. Representative images of hepatocellobilangiocellular carcinoma and pleomorphic HCC in the placebo-treated group and of well-differentiated HCC in the anti-miR-21 treated groups are shown in Supplementary Fig. S2.

Finally, because of the strong correlation we observed between liver fibrosis and miR-21 expression, we also evaluated the effects of anti-miR-21 treatment on fibrosis in adjacent liver tissue. Masson’s trichrome staining showed a significant reduction of fibrosis from 19.0% to 12.0% (P = 0.002) upon anti-miR-21 treatment (Fig. 2E).

Anti-miR-21 treatment results in a significant reduction of progenitor cells and S100A4+ cancer-associated stromal cells

To determine the mechanisms by which anti-miR-21 inhibits tumor growth and liver fibrosis, we first evaluated the effects of
anti-miR21 treatment on OPN-expressing progenitor cells. A strong reduction in OPN\(^+\) cell population was observed following anti-miR-21 treatment. EPCAM, another marker of hepatic progenitor cells and biliary cells showed similar reduction. In addition, S100A4, a marker of cancer-associated stromal cells was also tested. Anti-miR-21 treatment resulted in a dramatic decrease of S100A4\(^+\) cells (Fig. 3A). These results were further validated by real-time PCR confirming a significant decrease in Opn and Epcam.
mRNA expression (−2.7-fold; \( P = 0.004 \) and −2.9-fold; \( P = 0.012 \) respectively). The expression of other hepatic progenitor markers \( Krt7, Krt19 \) and of the stem cell marker \( Prom1 \) was also significantly reduced upon anti-miR-21 treatment (−2.7-fold; \( P = 0.019 \); −2.1-fold; \( P = 0.027 \) and −2.3-fold; \( P = 0.028 \), respectively). Finally, anti-miR-21 treatment resulted in a significant reduction of \( S100a4 \) mRNA expression (−2.1-fold; \( P = 0.034 \); Fig. 3B). Together, these data showed that anti-miR-21 treatment results in a decrease in progenitor cell population, an effect accompanied with a decrease in \( S100A4^{+} \) cancer-associated stromal cells.

MiR-21 is required for the survival of CD24\(^+\) cells: a mechanism mediated by osteopontin and integrin \( \alpha\nu \)

To determine the mechanisms by which anti-miR-21 results in a decrease in progenitor cell population, we treated the human HepaRG liver progenitor cells with 20 nmol/L anti-miR-21 oligonucleotide. miR-21 level was 3.5-fold higher in HepaRG cells compared with healthy liver and hepatoma cell lines Huh7 and PLC/PRF5 (Fig. 4A) and anti-miR-21 treatment resulted in increased expression of miR-21 targets, \( SPRY1 \) and \( SPRY2 \) (2.2-fold; \( P = 0.015 \) and 1.9-fold; \( P = 0.039 \), respectively), validating the efficiency of the anti-miR-21 treatment in HepaRG cells (Fig. 4B). After 72 hours of anti-miR-21 treatment, an average of 38% of the HepaRG cells underwent apoptosis as shown by Annexin V/PI staining (Fig. 4C, top). The same treatment in the hepatoma Huh7 cells did not induce any apoptosis (Fig. 4C, bottom). To further identify the liver progenitor cell subpopulation undergoing apoptosis upon miR-21 inhibition, we stained HepaRG cells with antibodies directed against two commonly used liver stem cell surface markers CD44 and CD24. Approximately 54% of HepaRG cells expressed CD24, whereas 99% of HepaRG cells expressed CD44 (Fig. 4D). Upon miR-21 inhibition, \( CD24 \) mRNA expression in HepaRG cells decreased (−2.13 fold; \( P < 0.001 \)), whereas \( CD44 \) mRNA expression slightly increased, suggesting a specific effect of miR-21 on \( CD24^{+} \) cells (Fig. 4E). C oataining of CD24
Figure 5. Modulation of CD24+ cell survival by OPN and ITGAV. A, HepaRG cells were costained with CD24 and ITGAV antibodies and the positive populations were characterized by flow cytometry. B, apoptosis of CD24+ cell population upon treatment with neutralizing ITGAV antibody. Right, quantification data from three independent experiments, including percentage of apoptotic cells induced by ITGAV-neutralizing antibody treatment and the distribution of these apoptotic cells between CD24+ and CD24− population. C, CD24 mRNA expression upon ITGAV-neutralizing antibody treatment measured by qRT-PCR. D, HepaRG cells were treated with or without recombinant OPN protein (1 μg/mL) following anti-miR-21 transfection. Cell apoptosis was analyzed at 72 hours by costaining with Annexin V and PI. The figure shows the quantification data from three independent experiments. E, CD24 mRNA expression in liver from wt and OPN−/− mice treated for 4 weeks with DDC.

and Annexin V further showed that the large majority (75%) of apoptotic cells resulting from miR-21 inhibition were CD24+ cells (Fig. 4F). The specific effect of miR-21 inhibition on the CD24+ subpopulation of liver progenitor cells was further confirmed in vivo, by immunostaining showing a significant reduction of CD24+ cells in Pten-null liver treated with anti-miR-21 from 12.9% to 4.1% (P = 0.013; Fig. 4G). In conclusion, miR-21 is required for the survival of CD24+ liver stem cells. To further evaluate whether CD24 is indeed a stem-like marker, we separated HepaRG cells into CD24+ and CD24− populations by FACS and subjected them to sphere formation assays. CD24+ cells formed significantly larger spheres and at a significantly higher frequency as compared with CD24− cells (Fig. 4H).

We wished to identify the mediators of anti-miR-21 induced apoptosis of CD24+ cells. We failed to identify candidate targets of miR-21 with apoptotic functions and preferential effects on CD24+ cells. We therefore investigated the possible involvement of external signaling effects. We measured the expression of integrin αv (ITGAV), a receptor for OPN. Over 90% of HepaRG cells expressed ITGAV (Fig. 5A). To evaluate whether ITGAV signaling is required for CD24+ cell survival, HepaRG cells were treated with 5 μg/mL ITGAV-neutralizing antibody for 72 hours. This treatment resulted in the apoptosis of 34.5% HepaRG cells. Among those apoptotic cells, 77% were CD24+ cells (Fig. 5B). Real-time PCR also showed that ITGAV-neutralizing antibody treatment caused a strong (~3.7-fold; P < 0.001) decrease in CD24 mRNA expression (Fig. 5C). We then added 1 μg/mL of recombinant OPN protein to the cell culture medium following anti-miR-21 transfection. Addition of OPN partially blocked the anti-miR-21 induced apoptosis of HepaRG cells, decreasing apoptosis from 29.87% to 20.25% (P = 0.046; Fig. 5D).

Inhibition of Notch2 in Pten-null tumors upon anti-miR-21 treatment

To further identify the upstream events leading to OPN inhibition by anti-miR-21, we investigated whether Notch expression and activity were increased in Pten-null liver and tumors and whether anti-miR-21 treatment affected Notch expression and activity. The Notch family members have been associated with biliary differentiation of hepatoblast, liver carcinogenesis, and liver fibrosis. In addition, Opn expression is directly regulated by the transcriptional factor RUNX2, a Notch target. Runx2 was significantly increased in Pten-null tumors compared with adjacent liver (1.5-fold, P = 0.011) and anti-miR-21 treatment resulted in a ~1.5-fold reduction of Runx2 mRNA in tumors (P = 0.009). Similarly, the expression of another Notch target, Hes1 was increased in Pten-null tumor compared with adjacent liver (1.7-fold; P = 0.019) and strongly reduced upon anti-miR-21 treatment (~3.3-fold; P < 0.001; Fig. 6A). These results are indicative of increased Notch activity in Pten-null tumors that can be inhibited by anti-miR-21 treatment. We then measured the
Anti-miR-21 treatment prevents tumor development

Because of the effects of miR-21 inhibition on CD24+ cell survival, OPN levels, expansion of S100A4+ stromal cells, and Notch activity, we investigated whether targeting miR-21 could prevent tumor development in vivo in Pten-null mice. We treated 7.5-month-old Pten-null mice with anti-miR-21 for 6 weeks. Ultrasound did not detect any tumor in these mice at the start of treatment. At the end of treatment, the incidence of histologically confirmed tumors was 67% in the placebo-treated group and 33% in the anti-miR-21 treated group (Fig. 7A). The average tumor burden in anti-miR-21-treated mice was also significantly smaller than in mice from the placebo-treated group (23 mm³ compared with 107 mm³, P = 0.039; Fig. 7B). While the average volume of the largest tumor in mice from the placebo group was 92 mm³, the average volume of the largest tumor in mice from the anti-miR-21 group was only 18 mm³ (P = 0.05; Fig. 7C). Overall, only one tumor in the anti-miR-21 treated group was over 20 mm³. Ultrasound analysis also showed that anti-miR-21 treatment significantly reduced the tumor growth rate from 2.24-fold to 1.25-fold over 14 days (P = 0.01; Fig. 7D). As observed in the first set of Pten-null–treated mice, fibrosis was significantly reduced from 14.3% to 7.2% (P = 0.014) upon anti-miR-21 treatment (Fig. 7E).

Discussion

MiR-21 has been identified as an onco-miR associated with many types of cancers, including HCC and intrahepatic cholangiocarcinoma (10, 19). MiR-21 promotes cancer cell proliferation and invasion, and prevents apoptosis through the regulation of its target genes (8, 20, 21). The study presented here is the first evaluation of miR-21 as a therapeutic and preventive target in a genetically engineered mouse model of cancer. We found miR-21 expression in liver to be enriched in progenitor cells. Furthermore, we showed that a subpopulation of these progenitor cells, CD24+ cells, is dependent on miR-21 for their survival, an effect mediated by OPN-ITGAV signaling, and that anti-miR-21 treatment reduced CD24+ progenitor cell population in vivo by inhibiting RUNX2-mediated transcriptional regulation of OPN expression. CD24+ cells have been identified as liver tumor-initiating cells and have been associated with higher risk of tumor recurrence (22). CD24 is an important molecule for stem cell self-renewal and tumor initiation ability through regulation of STAT3 phosphorlation and NANOG expression (22, 23). STAT3 can promote miR-21 expression by direct binding to the promoter region of miR-21 (24–27). It is therefore likely that the high expression of miR-21 in CD24+ cells we observed is mediated by STAT3.

The reduction in tumor incidence and growth following anti-miR-21 treatment in vivo may be the direct consequence of the depletion of CD24+ cell population. miR-21 may mediate the
early events in liver tumor initiation by maintaining the survival of CD24\(^{+}\) liver tumor-initiating cells. This reduction in stem cell population may also explain the reduced malignancy of the tumors and concomitant increased differentiated phenotype following anti-miR-21 treatment. The increased differentiated phenotype following anti-miR-21 treatment may also result from the inhibition of Notch activity we observed. Notch signaling regulates biliary differentiation of hepatoblast in the developing and adult liver (28), drives cholangiocarcinoma development through trans-differentiation of hepatocytes (28–30), and contributes to liver carcinogenesis (31–33). Notch signaling has also been reported to modulate hepatic fibrosis (34). We found that Notch2 is the main Notch family member expressed in Pten-null liver tumors. This preferential expression of Notch2 may be due to the predominant expression of Notch2 in CD24\(^{+}\) progenitor cells. While anti-miR-21 treatment decreased Notch1 expression in adjacent liver, Notch2 expression decreased in tumors following anti-miR-21 treatment. This observation suggests that the different members of Notch have different cell distribution and non-redundant functions in liver.

The reduction in tumor incidence and growth following anti-miR-21 treatment in vivo may also be the direct consequence of a reduction in fibrosis and changes in the stroma largely associated with depletion of S100A4\(^{+}\) and of CD24\(^{+}\) cells, and reduction in OPN levels. A role for miR-21 in renal and cardiac fibrosis has been previously reported (1, 12, 35). Although miR-21 has been reported increased upon profibrogenic stimulation in liver (36), this is the first report demonstrating a direct role of miR-21 in liver fibrosis with a strong positive correlation between miR-21 levels and fibrosis severity in the Pten-null liver and a strong antifibrotic effect of anti-miR-21 treatment. In this model, we did not observe any correlation between \(\alpha\)-SMA levels, a stellate cell marker and the effect of anti-miR-21 on fibrosis. Instead the expression of S100A4 decreased following anti-miR-21 treatment. This decrease was associated with a strong reduction of osteopontin, an extracellular matrix protein known to be involved in liver fibrosis and early stages of liver tumor development (37, 38). Crosstalk between CD24\(^{+}\) cells and S100A4\(^{-}\) cells may contribute to the extension of fibrosis in the liver and anti-miR-21 treatment may reduce liver fibrosis by blocking this crosstalk.

In summary, this study provides in vivo evidence of a role for miR-21 in maintaining the survival of CD24\(^{+}\) progenitor cells and of a crosstalk between progenitor cells and cancer-associated stromal cells. It also suggests that anti-miR-21 may be effective at targeting tumor initiating cells as well as the tumor microenvironment, and therefore shows great promise for clinical studies of liver cancer prevention and treatment. The results of the study are summarized in a model diagram (Supplementary Fig. S3). Further study is warranted to determine whether anti-miR-21 treatment can be effective as a companion therapeutic agent to drugs killing the tumor cells or as a therapeutic target for the prevention of liver tumor in patients at risk and the prevention of recurrence.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: J. Zhang, S. Zabludoff, R. Kalluri, L. Beretta
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