Expression Profile of ATG16L1 and mTOR Genes in Hepatitis B Virus Infection

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ABSTRACT

Autophagy is an important pathway for host defense against viral infection. In this study, we determined the expression of autophagy 16-like 1 (ATG16L1) and mammalian target of rapamycin (mTOR) genes in HepG2 (human hepatoma cell lines), HepG2.2.15 (human hepatoma cell lines transfected with the hepatitis B virus genome), THLE-2 cell lines (a primary normal liver cells) and liver tissues from patients with hepatocellular carcinoma (HCC). The mRNA expression of these genes was examined by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). Our study found that the ATG16L1 mRNA level significantly increased in HepG2 and HepG2.2.15 cells as compared with THLE-2 cell lines (P = 0.0246 and P < 0.0001, respectively). In addition, the up-regulation of ATG16L1 gene has been found in HepG2.2.15 cells when compared to HepG2 cells (P = 0.0001) as well as mTOR gene (P = 0.0016). In comparison of HepG2 and HepG2.2.15 cells with THLE-2 cell lines of mTOR gene, there was significantly up-regulation in HepG2.2.15 cells (P = 0.0023) but not in HepG2 cells. We also found an increased mRNA expression of mTOR gene in cancer tissues as compared to normal surrounding tissues (P = 0.049). In conclusion, it is suggested that the ATG16L1 and mTOR genes might have a role in hepatitis B virus infection and the development of HCC. However, functional study of these genes is needed to clarify our finding.

Key words: ATG16L1, mTOR, autophagy, hepatitis B virus, expression

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INTRODUCTION

Although implementation of an effective vaccination has resulted in a decrease of the incidence of hepatitis B virus (HBV) infection, there are still more than 350 million HBV chronic carriers worldwide. The chronic HBV (CHB) infection can be developed into liver cirrhosis and hepatocellular carcinoma (HCC), which lead to the cause of death in the next few years (Lavanchy 2004). Autophagy is the basic catabolic mechanism that involves in cell degradation of pathogen or dysfunctional cellular components. It is also the revolutionary pathway occurring in chronic liver diseases including viral hepatitis, alcoholic liver disease and fatty liver disease (Bao et al. 2014). However, some studies suggested that autophagy maintained the liver cells during chronic liver injury. In contrast, many studies reported that the autophagy might play an important role in the replication of HBV, which is the main cause of chronic liver disease and cancer development (Rautou et al. 2010; Rautou et al. 2011; Kotsafti et al. 2012).

ATG16L1 is a member of the autophagy-related protein family, which binds to the Atg12-Atg5 conjugate and forms to a large protein complex for autophagosome formation (Mizushima et al. 2003). There have been reported that the overexpression of ATG16L1 was significantly associated with Crohn's disease, oral squamous cell carcinoma disease (OSCC) and colorectal carcinoma cells (Leal et al. 2012; Zhai et al. 2013; Tang et al. 2014). However, its role in HBV pathogenesis is still not clearly understood. For another interesting gene, mTOR also functions in cellular processes such as cell growth, proliferation, metabolism, migration and apoptosis (Hay and Sonenberg 2004). Recently, a study showed that the mTOR was up-regulated by HBx transfection in HepG2 cells. Nevertheless, the expression study in human liver tissues and cell lines with whole HBV genome are still not investigated.

Therefore, we aim to study the expression of ATG16L1 and mTOR genes in HepG2, HepG2.2.15 cells and THLE-2 cell lines as well as tumor liver tissues vs. normal tissues from patients with HCC.

MATERIALS AND METHODS

1. Subjects

We recruited nine chronic HBV patients with hepatocellular carcinoma from Chulalongkorn Memorial Hospital. The diagnosis of chronic hepatitis B was confirmed by seropositivity for hepatitis B surface antigen (HBsAg) over a 6-month period and did not have any other types of liver diseases such as chronic hepatitis C or alcoholic liver diseases. All patients had increased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. The patients were diagnosed by histopathology and/or a combination of mass lesion in the liver from hepatic imaging and serum alpha fetoprotein level was higher than 400 ng/ml. Two types of liver tissues (liver cancer and
normal tissues) were diagnosed by histology from the incisional biopsy. The ethics committee of the faculty of Medicine, Chulalongkorn University, Bangkok, Thailand approved the study and the subjects gave their informed consent.

2. Cell lines

The human hepatoma cell line (HepG2 (ATCC® HB8065TM, Singapore)) and the HBV-transfected HepG2.2.15 cell line were kindly provided from Professor Antonio Bertoletti (A*Star, Singapore Institute for Clinical Sciences). Both cell lines were cultured in Dulbecco's modified Eagles' medium supplemented with 10 % fetal bovine serum and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml) (Invitrogen, USA). In addition, the HepG2.2.15 cells were supplemented with 150 μg/ml Geneticin® (G418 Sulfate, Gibco, Thermo Fisher Scientific, Inc.) for maintaining HBV plasmid. For autophagy induction, HepG2 and HepG2.2.15 cells were incubated in starvation medium (Earle's Balanced Salt Solution: EBSS; Gibco, Fisher Scientific, Inc.) at 37°C in 5 % CO₂ for 4 h. All cells were collected by trypsinization with 0.25 % trypsin/EDTA (Gibco, Fisher Scientific Inc.) and washed with phosphate-buffered saline (PBS). The cell pellet was re-suspended in RNAlater® Solution (Ambion, USA) and stored at -20 °C until used.

3. RNA isolation and quantitative RT–PCR

Total RNA from three kinds of cell lines (THLE-2, HepG2 and HepG2.2.15) and human liver tissues were extracted with RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's recommendations, followed by a reverse transcription reaction of 50 ng RNA using High Capacity cDNA Reverse Transcription Kits (Invitrogen, USA). Reverse transcription polymerase chain reaction (RT-PCR) was performed with an Applied Biosystem 7500 Real-time PCR System (Applied Biosystems, USA) using the Quantitect SYBR Green PCR Kit (Qiagen, Germany) following the manufacturer's guidelines. The primer sequences were newly designed in this study. The primers for ATG16L1 were forward 5′-AGGGTTCCCTATCTGGCAGT-3′ and reverse 5′-GATTCGGCTTGCAAAATCAT-3′. The mTOR primers were forward 5′-GTTCTGCTGGGTGAGAGA-3′ and reverse 5′-CCGGCTGCTGTAGCTT-3′. PCR conditions were 10 min at 95 °C, followed by 45 cycles of 15 sec. at 95 °C, 1 min. at 60 °C and 2 cycles of 30 sec. at 95 °C and 30 sec. at 60 °C min. for dissociation step. Beta-actin was used as an endogenous reference control. The Beta-actin primers were forward 5′-ACCAACTGGGACGACATGGAGAA-3′ and reverse 5′-GTGGTGAGTGAAGCTGTAGCC-3′. The expression levels were calculated by 2-ΔΔCt method (Livak and Schmittgen 2001).
4. Statistical analysis

Significant differences were determined by t-test with GraphPad Prism, version 5.0 software (San Diego, California, USA). A P-value of <0.05 was considered as statistical significance.

RESULTS

1. ATG16L1 gene expression

Our study demonstrated the ATG16L1 gene expression in HepG2 (human hepatoma cell lines) and HepG2.2.15 (human hepatoma cell transfected with HBV genome) compared with THLE-2 cell lines (primary normal liver cells). The results showed that the levels of ATG16L1 expression in HepG2 cells and HepG2.215 cells were up-regulated when compared with THLE-2 cells ($P = 0.0246$ and $P < 0.0001$, respectively) (figure 1a, 1b). Additionally, the level of ATG16L1 expression in HepG2.215 cells was higher than HepG2 cells ($P = 0.0001$) (figure 1c). However, there was no significant difference between the patient’s liver cancer tissue and normal tissue of the ATG16L1 expression level ($P = 0.804$) (figure 1d).

Figure 1 ATG16L1 expression level in HepG2 cells compared with THLE-2 cells, $P = 0.0246$ (a); The comparison between ATG16L1 expression level in HepG2.2.15 cells and THLE-2 cells, $P<0.0001$ (b); ATG16L1 expression level in HepG2.2.15 cells was higher than HepG2 cells, $P = 0.0001$ (c); ATG16L1 expression level in the patient’s liver cancer tissue compared with normal tissue, $P = 0.804$ (d).
2. **mTOR** gene expression

The mRNA expression of **mTOR** gene was increased in HepG2.2.15 cells as compared with THLE-2 cells and HepG2 cells \( (P = 0.0023 \) and \( P = 0.0016 \), respectively) \( (\text{figure } 2\text{b}, \text{c}) \). However, there was no statistically significant difference of **mTOR** expression between HepG2 cells and THLE-2 cells \( (P = 0.176) \) \( (\text{figure } 2\text{a}) \). The level of **mTOR** gene expression in the cancer tissue was slightly up-regulated when compared to normal surrounding tissue \( (P = 0.049) \) \( (\text{figure } 2\text{d}) \).

![mTOR expression level](image)

**Figure 2** **mTOR** expression level in HepG2 cells compared with THLE-2 cells, \( P = 0.176 \) \( (\text{a}) \); **mTOR** expression level in HepG2.2.15 cells was higher than THLE-2 cells, \( P = 0.0023 \) \( (\text{b}) \); The expression level in HepG2.2.15 cells was higher than in HepG2 cells, \( P = 0.0016 \) \( (\text{c}) \); **mTOR** expression level in the patient’s liver cancer tissue compared with normal tissue; \( P = 0.049 \) \( (\text{d}) \).

**DISCUSSION**

In this study, we found the increased levels of **ATG16L1** gene expression in HepG2 cells and HepG2.215 cells when compared with THLE-2 cells. Moreover, the **ATG16L1** mRNA expression in HepG2.215 cells was higher than in HepG2 cells. The results indicated that **ATG16L1** gene might involve in HBV infection and the development of HCC. A recent study showed that HBV induces autophagy for its DNA replication through the X protein (HBx), which binds to a class III phosphatidylinositol 3-kinase (PIK3C3) \( (\text{Sir et al. } 2010) \). Moreover, a function of HBx in increasing autophagy through the up-regulation of beclin 1 expression was observed in a study of Tang et al.
There has been a report showed that HBx-promoted cell survival depends on the activation of autophagy via up-regulation of autophagy gene beclin-1. Thus, ATG16L1 might be another component of autophagy that functions in HBV replication and/or cell survival. Further study in biological function of ATG16L1 is required to prove this hypothesis. For negative finding in liver tissues, we need more sample sizes to confirm this result.

In mTOR gene expression study, we found the up-regulation of mTOR in HepG2.2.15 cells as compared to THLE-2 cells and HepG2 cells. In addition, the level of mTOR expression was higher in HCC tissue samples than in adjacent normal tissues from the same patients. Our finding suggested that mTOR gene may play a role in HBV pathogenesis including the progression of HCC. A study of Wang et al. (2013) showed that the mTOR gene was increased by HBx transfection in HepG2 cells which was consistent with our finding (Wang et al. 2013), although our study provided the information in another cell line with whole HBV genome (HepG2.215 cell). Furthermore, mTOR has been reported to be a regulator of autophagy pathway. They demonstrated that HBx activates autophagy in hepatoma cell line via the PI3K–Akt–mTOR pathway (Wang et al. 2013). In this study, our results support the importance of mTOR in HBV infection. However, the role of mTOR in other cell lines should be investigated to confirm these finding.

CONCLUSION

We found the up-regulation of ATG16L1 and mTOR genes in cell lines with HBV infection and/or liver cancer tissue from HBV patients with HCC. However, functional significance in HBV pathogenesis of these genes should be further clarified.

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