Construction of pcDNA3.1(+)/EMC6 Eukaryotic Expression Vector And Its Expression in Human Liver Cell Line L-02

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Abstract: - The present is aim to examine the construction of pcDNA3.1(+)/EMC6 eukaryotic expression vector and its expression in human liver cell line L-02. This will also explain various stages including PCR amplification of target gene fragment, Plasmid Extraction and transfection, Detection of EMC6 mRNA expression by fluorescence quantitative PCR (RT-PCR), and EMC6 protein expression was detected by Western blot (Western-blot). Statistical analysis was performed with the help of SPSS17.0 Software. Results for the study have shown that the autophagy is a highly conserved process evolution; eukaryotic cells by autophagy can remove misfolded proteins and damaged organelles and macromolecules to maintain cellular homeostasis, its basic process, autophagy induction, formation, autophagy and autophagy by soluble hydrolases to lysosomes of the autophagy package enzyme fusion, substance. Also, The eukaryotic expression vector pcDNA3.1(+)/EMC6 is successfully constructed, and highly expressed in L-02 cells. To construct a eukaryotic expression vector pcDNA3.1(+)/Endoplasmic reticulum membrane protein complex subunit 6(EMC6) and to overexpress it in the human liver L-02 cell line. The sequence ofEMC6, gene published in PubMed was analysed, a suitable restriction enzyme cutting site was designed. The Human EMC6 gene was amplified by PCR with the cDNA of L-02 cells. The template and the fragment were combined with plasmid pcDNA3. 1(+) by gene recombination technology. After constructing a eukaryotic expression vector pcDNA3.1(+)/EMC6, the accuracy of it was verified by colony PCR and sequencing. The recombinant expression vector pcDNA3.1(+)/EMC6 was transfected into L-02 cells by lipofectamine. The mRNA and protein expression of EMC6 in L-02 cells was respectively detected by RT-PCR and Western-blot. After identification by Colony PCR and sequencing, the eukaryotic expression vector pcDNA3.1(+)/EMC6 was successfully constructed. The qPCR and Western-blot results showed the overexpression of EMC6 in L-02 cells. The eukaryotic expression vector pcDNA3.1(+)/EMC6 is successfully constructed, and highly expressed in L-02 cells.

Key Words: — ***EMC6, Recombinant plasmid, Vector construction, Transfection, L-02 cells.***

I. INTRODUCTION

The endoplasmic reticulum protein complex subunit - 6 (Endoplasmic reticulum membrane protein complex subunit 6, EMC6) gene is located on human chromosome 17p13.2, gene expression profiling revealed that EMC6 mRNA was widely expressed in all human tissues; EMC6 protein is a Transmembrane protein of the endoplasmic reticulum, the formation of [1] regulation of autophagosome by RAB5A and Beclin1. Autophagy as a lysosome-dependent degradation pathway widely exists in eukaryotic cells, [2-4] plays an important role in cell growth, cellular homeostasis, and cell death in many diseases. Found the liver disease research, improve the level of autophagy can inhibit liver cell death in [5], at the same time, alcoholic fatty liver in patients with liver cell autophagy by [6], however, hepatitis B virus (HBV) and other parts of pathogens can be self-replicating [7] by autophagy. Thus, autophagy is closely related to the occurrence and development of viral hepatitis, non-alcoholic liver, and acute liver injury. EMC6, as a new type of autophagy regulator, has a small amount of expression in liver cells, but there are few

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reports about the relationship between EMC6 and liver disease. The purpose of this study is through the construction of EMC6 eukaryotic expression plasmid, and transfected into the overexpression of human liver cell L-02, for further study using EMC6 gene as a target, to provide the experimental basis to explore the relationship between autophagy and liver diseases.

II. Material And Methods

2.1 Cells with the main reagent L-02 cells purchased from the Chinese Academy of Sciences Shanghai cell bank.

The main reagents: 1640 medium, Opti-MEM medium, trypsin, fetal bovine serum, double-antibody, phosphate buffer (PBS) was purchased from Gibco Company, pcDNA3.1, Trizol, Lipo-fectamineTM-2000 vector was purchased from American Invitrogen company, the competent cells were purchased from Beijing Tiangen Biotechnology Co. Ltd., two, anti-HRP markers restriction enzyme digestion and T4 DNA ligase, Plasmid Extraction Kit, DNA gel extraction kit was purchased from Shanghai beyotime Biotechnology Co. Ltd. Rabbit anti-EMC6 and GAPDH antibodies purchased from the United States Abcam. Construction of eukaryotic expression vector containing

2.2 Construction of Eukaryotic Expression Vector of Pcdna3.1/EMC6

2.2.1 PCR Amplification of Target Gene Fragment

Extraction of total RNA from L-02 cells by Trizol reagent. According to the GeneBank query EMC6 gene sequence, using the software design of EMC6 gene full-length primers (Table 1 sequence), respectively with BamHI and xhol I restriction sites, amplified by long 333bp, GAPDH primer was used (Table 1 sequence), two pairs of primers by the Suzhou gold Weizhi biotech Co. Ltd. synthesis. With the total RNA as template, the first-strand cDNA was synthesized by reverse transcription, and reverse transcription product of PCR amplification of EMC6 gene encoding region of PCR products by 1% agarose gel electrophoresis, gel imager photo analysis, gel Recovery Kit gel cut EMC6 PCR gene product.

2.2.2 Construction of pcDNA3.1/EMC6 recombinant plasmid

The application of BamHI and xhol I restriction endonuclease EMC6 PCR and pcDNA3.1 respectively for the product (+) vector were double digested, digested products by gel extraction and purification, T4 DNA ligase 37 DEG C to connect 2 h, the products were transformed into competent Escherichia coli DH5 alpha cells and plated on LB culture medium screening positive clones were cultured overnight, positive for plasmid identification, the next step.

2.2.3 Identification of recombinant plasmid pcDNA3.1/EMC6

In the selected positive clones were colony PCR identification, T7 forward primer reverse primer RSK2 colony PCR primers (Table 1 sequence). Take 1.2.2 in the night to send the company to identify the bacteria sequencing.

2.3 Plasmid Extraction and transfection

Using plasmid extraction kits to extract granules, the specific methods are as follows. L-02 cells were cultured in a 1640 70%-80% equilibrium humidity incubator with a medium containing 10% fetal bovine serum. The cells were cultured in a 5% of CO2 equilibrium humidity chamber. Cells were seeded in 6 well plates when the cell density reached 60%-80% Lipo-fectamineTM-2000 according to the instructions of transfection, were randomly divided into normal control group (no transfection), negative control group (transfected with pcDNA3.1 empty plasmid), and EMC6 transfection group (transfected with EMC6).

2.4 Detection of EMC6 mRNA expression by fluorescence quantitative PCR (RT-PCR)

The collection of "1.3" in the L-02 cells, each hole with 1 mL Trizol extraction kit, according to the Trizol extraction of total RNA concentration and purity of RNA, determination of trace nucleic acid analyser, application of DEPC water RNA diluted to a final concentration of 1 mu g/ mu L, by reverse transcription kit synthesize cDNA. Detection of the expression of EMC6 gene mRNA in transfected cells by RT-PCR Kit (Table 1).

2.5 EMC6 protein expression was detected by Western blot (Western-blot)

After transfection, cells were collected and grouped with the "1.3" press kit extraction protein, and total protein was extracted, by Elisa quantitative, take 20 mu g protein was transferred to PVDF 10%SDS-PAGE, electric membrane, 5% skim milk at room temperature 2 h closed, EMC6 - (1:500) 4 C closed overnight, TBST fully washed 3 times, each time 10 min, two - (1:1000) at room temperature were incubated for 2 h, 3 TBST, 10 min each time, fully washing, chemical luminescence colour, finally developing chemiluminescence analyser, with GAPDH as control.

2.6 Statistical analysis

All the data were analysed by SPSS17.0 software, and the test method was to test. P<0.05 indicated that the difference was statistically significant, and each experiment was repeated 3 times independently.

III. RESULT

3.1 PCR amplification results of EMC6 gene

The total RNA was extracted as a template and reverse transcribed into cDNA. The cDNA was amplified by PCR. The amplified bands of PCR products were detected by agarose gel electrophoresis at 250 BP - 500 BP, with a target band of 313 BP (see Figure 1).

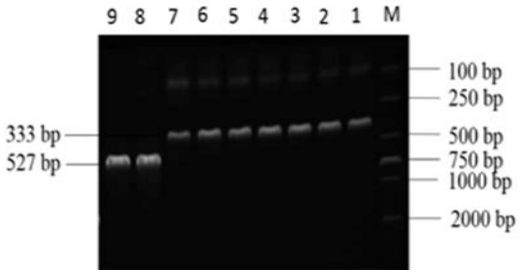

Fig.1. EMC6 Gene

Note: M is marker, 1-7 are target genes, 8 and 9 are internal controls

3.2 *Identification of eukaryotic expression vector of pcDNA3.1-EMC6*

PCR products after gel recovery with pcDNA3.1 (+) BamH and Xhol I respectively through double enzyme digestion and T4 DNA ligase, transformed into Escherichia coli DH5 alpha overnight culture, from single colony PCR and sequencing, with water and empty plasmid as a negative control. The results of PCR and the size of the target gene, bacteria using T7 primer as sequencing primers were sequenced. The sequencing result was consistent with the target sequence (see Figure 2, 3, 4).

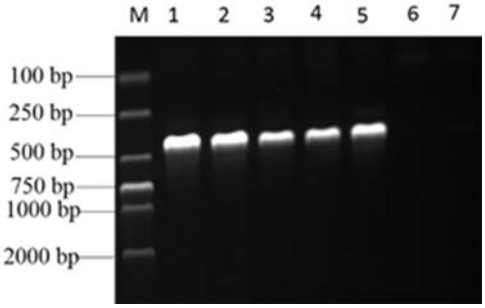

Fig.2. Colony PCR results

Note: M is marker, 1-5 is the target gene, 6 is empty plasmid as the template, 7 is water as the template.

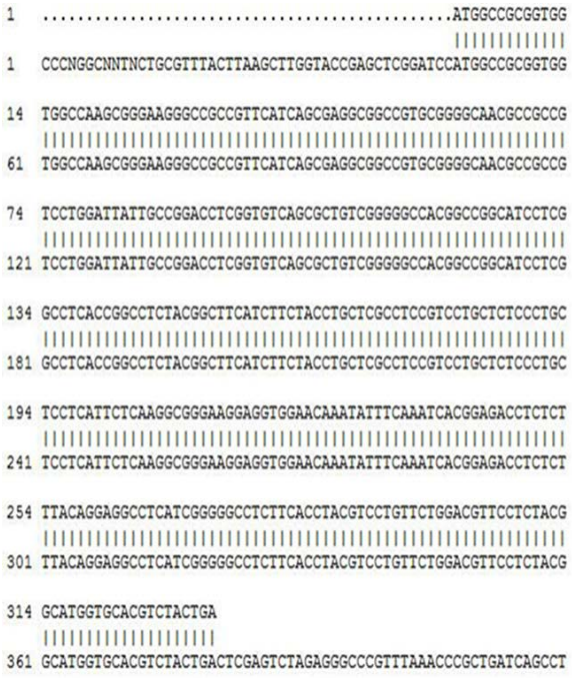

Fig.3. EMC6 colony sequencing comparison results

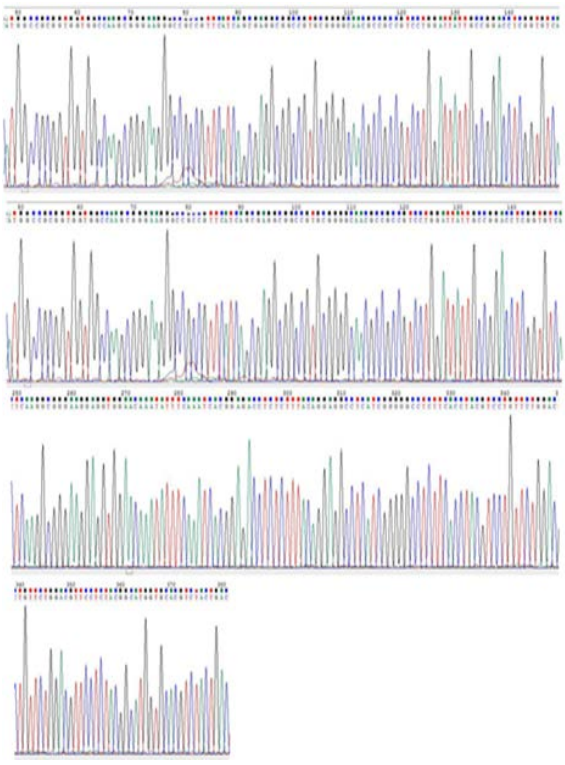

Fig.4. EMC6 Colony sequencing map results

3.3 Detection of EMC6 mRNA expression by RT-PCR

RT-PCR data analysis showed that the expression of pcDNA3.1 (+) EMC6 mRNA cells in -EMC6 group was significantly higher than that of the normal control group and empty plasmid group ($P<0.01$), a normal control group, and empty plasmid group, no statistical significance ($P>0.05$). As Figure 5

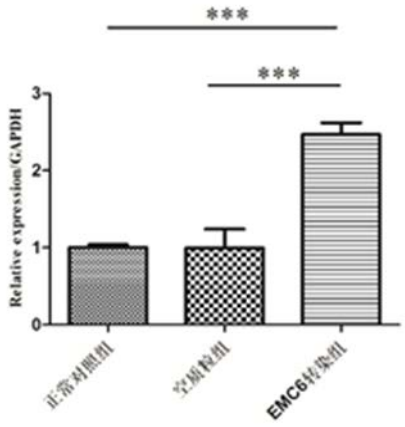

Fig.5. RT-PCR detection of EMC6 mRNA expression

3.4 Detection of EMC6 Protein Expression by Western Blot

Western Blot results showed that the 3 groups of cells were visible GAPDH bands, the transfection group was significantly EMC6 protein band, the normal control group and empty plasmid group EMC6 protein band was weak. These results indicated that the EMC6 gene was overexpressed in L-02 cells. As Figure 6

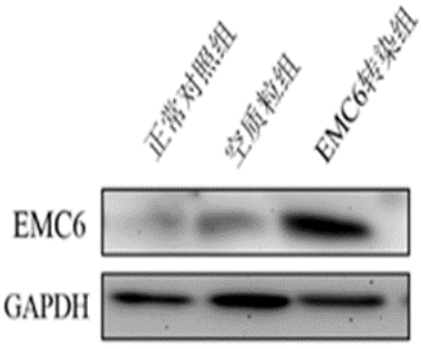

Fig.6. Western Blot detects EMC6 protein expression

IV. DISCUSSION

Autophagy is a highly conserved process evolution, eukaryotic cells by autophagy can remove misfolded proteins and damaged organelles and macromolecules to maintain cellular homeostasis, its basic process, autophagy induction, formation, autophagy and autophagy by soluble hydrolases to lysosomes of the autophagy package enzyme fusion, substance [8]. It is found that autophagy is involved in the development of a variety of diseases, including cancer, immune diseases, degenerative diseases, inflammatory response, [9]. For the study of liver diseases, autophagy can improve insulin resistance, regulate lipid metabolism and reduce the damage of hepatocytes. Data show that by preventing apoptosis and protect the liver [10 cells, autophagy by regulating lipid decomposition inhibiting fat accumulation of 11]; long-term drinking will lead to 5 'adenosine monophosphate-activated protein kinase activity decreased, and down-regulation of autophagy [12, 13]; in addition, increased autophagy activity of HBV and HCV infected patients at the same time, autophagy promotes replication of [14] virus. Thus, autophagy is closely related to the occurrence and development of liver disease. With the exploration of autophagy, new autophagy regulatory genes have been discovered, EMC6 is one of them, and participate in the formation of autophagy.

The process of autophagy by many autophagy-related genes (Autophagy-related Genes, ATG) of the regulation, including 1 Beclin (ATG6) is the specific autophagy gene [15] play an important role in the formation and maturation of autophagosomes. EMC6 protein is a transmembrane protein of the endoplasmic reticulum, widely expressed in various tissues, are highly conserved between different species, analysis of the transmembrane region showed that terminal EMC6 protein C has two conserved transmembrane domain, further research shows that EMC6 and GTP enzyme RAB5A protein interaction, regulation of endocytosis. At the same time, RAB5A can be combined with the Beclin-1-PIK3C3 complex, [1] promotes the formation of autophagosomes. EMC6 interacts with RAB5A and Beclin-1 to promote the localization of RAB5A in the endoplasmic reticulum, and to regulate the formation of autophagy. Studies have shown that over-expression of the EMC6 gene can activate autophagy, whereas EMC6 gene silencing inhibits autophagy levels [1]. A new study reported that up-regulation of the EMC6 gene can inhibit the PIK3CA/AKT/mTOR signalling pathway, promotes the occurrence of autophagy, and inhibit the proliferation of glioma cells [16]. At present, there are few reports about the

relationship between EMC6 and liver disease, which may be a new target for the study of the relationship between autophagy and liver disease.

Autophagy in different liver diseases in different roles can regulate autophagy in prevention and treatment of liver disease, EMC6 as cell autophagy potential, to develop targeted drugs to regulate autophagy function to find the key point of the new. In this experiment, in order to further study the successful construction of the eukaryotic expression vector EMC6, and after PCR and sequencing analysis confirmed successful construction of the recombinant plasmid pcDNA3.1 (+) /EMC6, and successfully expressed in L-02 cells, which laid a good foundation for the next research.

V. CONCLUSION

For the investigation of liver infections, autophagy can improve insulin opposition, control lipid digestion and lessen the harm of hepatocytes. Information show that by forestalling apoptosis and ensure the liver; long haul drinking will prompt 5 'adenosine monophosphate-actuated protein kinase movement diminished, and down-guideline of autophagy; what's more, expanded autophagy action of HBV and HCV contaminated patients simultaneously, autophagy advances replication of virus. The interaction of autophagy by numerous autophagy-related qualities (Autophagy-related Genes, ATG) of the guideline, including 1 Beclin (ATG6) is the particular autophagy quality assume a significant part in the arrangement and development of autophagosomes. EMC6 protein is a transmembrane protein of the endoplasmic reticulum, generally communicated in different tissues, are profoundly saved between various species, examination of the transmembrane locale showed that terminal EMC6 protein C has two saved transmembrane area, further exploration shows that EMC6 and GTP chemical RAB5A protein connection, guideline of endocytosis. Simultaneously, RAB5A can be joined with the Beclin-1-PIK3C3 complex, advances the arrangement of autophagosomes.This can be concluded that the eukaryotic expression vector pcDNA3.1(+)/EMC6 is successfully constructed, and highly expressed in L-02 cells.

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