Screening of Sensitive Antisense Target in ODC mRNA

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Abstract **Objective:** To screen the sensitivity antisense target in the ODC mRNA. **Methods:** Four parts of ODC gene--extron 2, extron 3, extron 6-10, extron 11-12--were amplified from hepatic cancer cells by RT-PCR and then reversely subcloned into pcDNA3.1 vector. Their inserted directors were identified by sequencing. After these recombinant vectors and empty vector pcDNA3.1 were transfected into the hepatic cancer cell line MMC7721, we adopted Western-blot and MTT assay to identify and compare these vectorseffects on the expression of ODC and cell growth. **Results:** Three best inhibitive sites of ODC recombinant vectors were successfully selected . **Conclusion:** Extron 2, extron 3, extron 6-10 were found to be the best antisense sites of inhibition.

Key Words Ornithine decarboxylase; Cell proliferation; Antisense RNA; Eukaryotic expression vector; Western-blot.

The ornithine decarboxylase(ODC) catalyzes the first step in the polyamine biosynthetic pathway forming putrescine, which is then converted into the polyamines spermidine and spermine. Polyamines can increase the synthesis of DNA, transcription of RNA and synthesis of proteins. They are important materials in the regulation of cell proliferation[1,2]. As the first rate-limiting enzyme in the polyamine biosynthetic pathway, ODC regulates the proliferation and transformation of normal cells and plays an important role in the development of cancer [2,3]. Many studies have indicated that ODC was overexpression in tumor tissues[4,5]; transgenic animal experiments have also demonstrated that elevated ODC activity can induce tumorigenesis [1]. Therefore, ODC had became an important target in tumor therapy. The antisense RNA technology, is a technique that can specifically suppress corresponding gene at the levels of replication、transcription and translation [6], has been one of the main methods in gene therapy and gene function research. The key point of antisense RNA technology is to improve the stability and inhibit efficiency of antisen RNA. Therefore, the sensitive antisense targets should be screened out first. In this paper, we cloned the cDNAs of four commonly used antisense inhibit regions. Then the cDNA clones were reversely inserted into eukaryotic expression vector pcDNA 3.1 and the recombinant vectors were named pcDNA-ODCr I to IV. The results of Western Blot and MTT assay demonstrated that pcDNA-ODCrI-IV inhibited the ODC gene expression in hepatic cancer cell line MMC7721 and arrested the cells growth. Consequently, extron 2, extron 3, extron 6-10 were suggested to be the best antisense sites of inhibition.

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MATERIALS AND METHODS

Plasmid, strain, cell line and reagents

The pcDNA3.1/Myc-his (-)A, Lipofetamine2000, RPMI 1640 medium were purchased from Invitrogen (USA). MMC7721 was conserved in our lab. QIAgen Plasmid Midi kit, QIAquick DNA Gel Extraction kit were purchased from Qiagen (Germany).The cDNA synthesis kit, Taq DNA polymerase, T4 DNA ligase, pMD 18-T Vector, miniBEST plasmid DNA purification kit、restriction enzyme Xbal, HindIII, BamHI, KpnI were purchased from Takara (Japan).MTT was purchased from Sigma (USA). Brandford protein assay kit was purchased from Shanghai Sangon Biotechnology

Co., Ltd. The ODC antibody was prepared in our lab. The rabbit anti goat antibody and the goat anti mouse antibody were purchased from Beijing Zhongshan Biotechnology Co., Ltd. The actin polyclonal antibody and Western Blot Reagent were purchased form Santa-cruz. And the pMD-ODCII vector was constructed in our lab.

Primer design and synthesis

The primers were designed by the Primer Express software program (PE Applied Biosystems)and synthesized by Shanghai Sangon Biotechnology Co., Ltd. The four sequences are listed as follow: pcDNA-ODCrI(the up stream primer of PCR P1 is 5` GAC GGT ACC ATT GTC ACT GCT GTT CCA AG 3` and the down stream primer P2 is 5` GAC GGA TCC TGT TCC TAT GGA AAA CTA AGA 3`); pcDNA-ODCrII(the up stream primer P3 is 5`GGT CGACCA GGA TCC AAT GAA CAA CTT TGG T 3` and the down stream primer P4 is 5`GCC GAG ATC TCA GAA GAA ACT TC 3`); pcDNA-ODCrIII (the up stream primer P5 is 5` GAG GTA CCA TGC TGC TAA TAA TGG AGT C 3` and the down stream primer P6 is 5` GAG GAT CCC ATG TGC GTG GTC ATA GAG T 3`); pcDNA-ODCrIV (the up stream primer P7 is 5` GAG GTA CCG GAT TGT TGA GCG CTG TGA C 3` and the down stream primer P8 is 5` GAG GAT CCA ATA CTA GCC GAA GCA CAG G 3`). The 5end of P1, P5, P7 contained KpnI restriction site; the 5end of P2, P3, P6, P8 contained BamHI restriction site; the 5end of P4 contained SalI restriction site.

Amplification of the four ODC gene fragments

Total RNA was extracted from hepatic cancer tissue, RNA templates were used to reversely transcribed the first strand of cDNA with cDNA synthesis kit. The synthesized cDNAs were then used as the templates to perform polymerase chain reaction (PCR) for special amplification of ODC gene fragments. The reaction system contained: cDNA 2μl, $MgCl_2$ (25mmol/L) 4μl, Buffer 5μl, dNTP (2.5mmol/L) 3μl, Taq DNA polymerase 0.25μl, up/down stream primer (2pmol/L) 5μl each. The Tm of ODC cDNAI-IV were 61℃, 56℃, 60℃, 63℃.

Construction and identification of TA Clone

150μl of the ODC cDNAI-IV PCR products were electrophoresed in agarose gel, then the desired bands were cut out from the gel and extracted with QIAquick DNA Gel Extraction kit. After the extracted PCR products were quantified with the ultraviolet spectrophotometer, they were ligated with pMD18-T (10:1 mol). The ligation products were then transformed into the DH5a cells. After 16 hours, the colonies which resisted to ampicillin were selected and multiplied. Then these bacteria were used to extract plasmid DNA, which were then digested by XbaI, Hind III, BamHI, KpnI. Then sequencing was performed to identify the correct clones, which were named pMD-ODCI-IV.

Construction of the recombination vector pcDNA-ODCrI-IV

pMD-ODC I、III、IV were digested by BamHI, KpnI and pMD-ODCII were digested by XbaII, HindIII, the desired ODC fragments were extracted and purified after the digested products were separated with electrophoresis. Then ligated them with eukaryotic expression vector pcDNA3.1 (3:1 mol), reversely inserted the four ODC gene fragments into pcDNA3.1 vectors. The final clones were identified by enzyme digestion and sequencing, and named them pcDNA-ODCrI-IV. High-purity plasmids of pcDNA-ODCrI-IV and pcDNA3.1 were then prepared by QIAgen Plasmid Midi Kit following the protocol of the kit.

Measurement of ODC protein expression by Western Blot analysis

MMC7721 cells were cultured in 6 wells plate (1×10^6/well) for 24 hours, then transfected the plasmid pcDNA3.1 and pcDNA-ODCrI-IV into the cells (5μg/well) with Lipofectamine 2000, collected The total cell lysates (200μl/well) 72 hours later, and then qualified the proteins from these samples by BCA method. After the partes aequales of lysates were electrophoresed by SDS-PAGE, the proteins were transferred onto the cellulose-acetafolic membranes and sequentially probed with monoclonal ODC antibody (1:

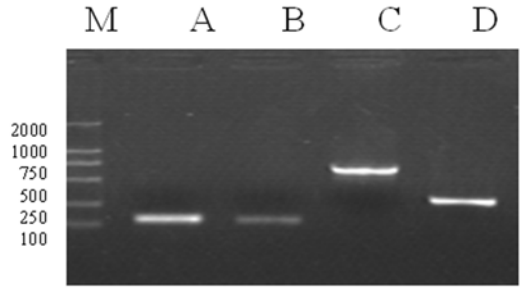

Fig. 1: The RT-PCR result of the four ODC fragments
M: DNA Marker DL-2000
A: PCR product of ODCI
B: PCR product of ODCII
C: PCR product of ODCIII
D: PCR product of ODCIV

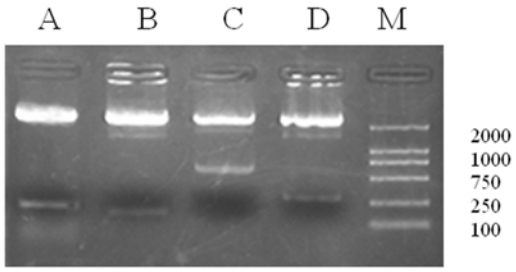

Fig. 2: Restriction digest analysis of pMD-ODCI-IV
M: DNA Marker DL2000
A: Digested product of pMD-ODCI
B: Digested product of pMD-ODCII
C: Digested product of pMD-ODCIII
D: Digested product of pMD-ODCIV

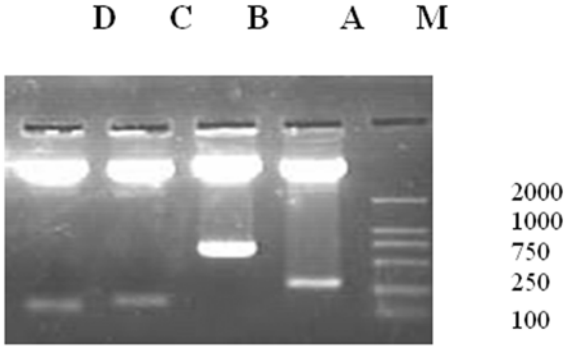

Fig. 3: Restriction digest analysis of pcDNA-ODCrI-IV
M: DNA Marker DL2000
A: Digested product of pcDNA-ODCrI
B: Digested product of pcDNA-ODCrII
C: Digested product of pcDNA-ODCrIII
D: Digested product of pcDNA-ODCrIV

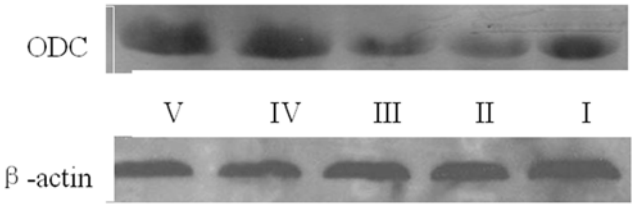

Fig. 4: Western-blot analysis of ODC andβ-actin expression
I : pcDNA-ODCr I transfected group
II: pcDNA-ODCr II transfected group
III: pcDNA-ODCr III transfected group
IV: pcDNA-ODCr IV transfected group
V: pcDNA3.1 transfected group

1000) or actin antibody. The membranes were washed with PBS and TBS three times and reached with a goat anti mouse or rabbit anti goat antibody (1:1000) for 2 hours. Then the membranes were washed 3 times with TBS and reacted with Western-Blot Luminol Reagent for 1 minute. Then they were exposed and developed in the dark room.

Measured the effects of pcODCrI-IV on MMC7721 cell

The cells were cultured in 96 wells plate(5×10^3/well) for 24 hours, then transfected pcDNA3.1 and pcDNA-ODCrI-IV into the cells (0.2μg/well) with Lipofectamine 2000. After 0、24、48、72hours, added 20μl MTT solution(5mg/ml) into each well and maintained at 37℃ for 4 hours, then the medium was diacarded and 100μl dimethyl sulphoxide was added to release the blue formazan reduction product and shaken for 10 minutes. Then the optical density of the samples was measured by the Bio-rad microplate reader at 490nm. The relative inhibition ratio was calculated as follow:

RESULTS

RT-PCR and identification of TA clone

The four cDNA products, which were amplified

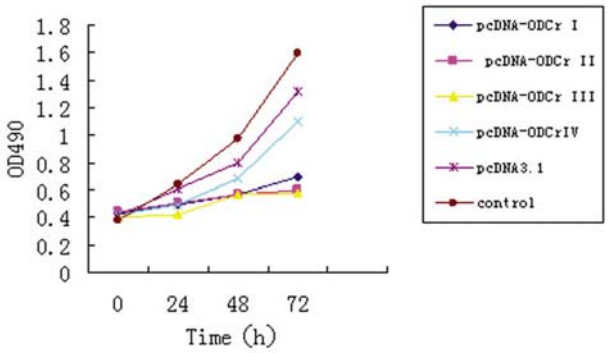

Fig.5: Detect pcDNA－ODCrI－IV′s effects on the activity of MMC7721 by MTT

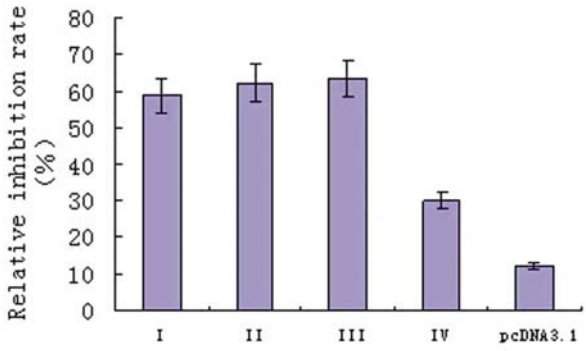

Fig. 6: The relative inhibition rate 72 hours after transfection

from the hepatic cancer tissue by reverse transcription PCR, were detected by 1.5% agarose gel electrophoresis, the molecular weight of them is: 35bp, 20bp, 37bp, 81bp (Fig 1)respectively. The extracted PCR products were inserted into pMD18－T vector and formed pMD－ODC I－IV. pMD18ODC－I, III, IV were digested by BamHI and KpnI, pMD18－ODC II was digested by XbaI and HindIII, they were all at the desired positions (Fig 2).The sequencing showed that they were the four ODC cDNA fragments that we needed.

Identification of pcDNA–ODCrI–IV

After pMD－ODCI－IV were digested by their corresponding enzymes, the ODC fragments were extracted from the gel and inserted into pcDNA3.1 reversely, then the ODC antisense eukaryotic expression vector pcDNA－ODCrI－IV were constructed. pcDNA－ODCrI, III, IV were digested by BamHI and KpnI, pcDNA－ODCrII was digested by XbaI and HindIII, obtained 4 bands: 135bp,120bp,637bp,281bp (Fig 3). The sequencing results verified their sequences.

Result of Western–blot analysis

After pcDNA－ODCrI－IV were transfected into MMC7721 by transient transfection for 72 hours, actin and ODC protein expression were detected by Western－blot analysis (Fig 4). The expressions of －actin among the groups were not obviously different. However, the pcDNA－ODCrI－III groups showed noticeable decrease in ODC expression compared with the pcDNA－ODCrIV group and pcDNA3.1 empty vector group. No significant distinction in ODC expression was observed between pcDNA－ODCrIV group and pcDNA3.1 empty vector group.

Result of MTT assay

Compared with the control and pcDNA3.1 transfected group, the proliferation of the pcDNA－ODCrI－III transfected MMC7721 cells were inhibited significantly after 72 hours (P<0.05) (Fig 5).Their ratios of fractional inhibition on 3 days were 59%、62%、64% respectively (Fig 6) and no conspicuous distinction was observed among pcDNA－ODCrI－III (P>0.05). The survival rate of the pcDNA－ODCrIV transfected group on 3 days was 70%, lower than the pcDNA3.1 group, but higher than the pcDNA－ODCrI－III tansfected groups(P<0.05).

DISCUSSION

The ornithine decarboxylase has attracted peoples interest as the first key enzyme in polyamine biosynthetic pathway for a long time. The ODC, which is degraded mainly by antienzyme[7],has a short half－life and plays an important role in the development of cancer. The ODC level in G_0 stage cells is very low, but its activity can be enhanced when treated with carcinogen phorbol ester and oncogene product C－Ha－Ras. Overexpression of ODC is able to induce the malignant transformation from normal cell (such as NIH3T3). Several researches had reported that ODC expresses increasingly in most cancer cells. The latest researches indicated that the elevated of ODC level is correlated with the metabasis、recurrence and angiogenesis of cancer [8,9].

Therefore, ODC has become an important cancer-therapeutic target. A number of researches demonstrated that ODC inhibitors can inhibit the growth of cancer, but their adverse reactions are also obvious. Accordingly, to find a novel method which can inhibit ODC with high efficiency and low toxicity is highly requested.

Antisense technology is a method that inhibits gene expression selectively and specifically. It mainly includes antisense RNA technology and antisense oligonucleotide technology. Antisense technology is commonly used in gene therapy by inhibiting the expression of special gene, Now it is not clear how antisense RNA technology works, the possible mechanisms is: pairing with noncoding regions of mRNA, affecting translation in coding regions directly or indirectly, impacting the joining regions between extrons and introns, hampering pre-mRNA splicing [10,11], etc. The inhibition effect of this technology is due to the selection of antisense target sequence. Generally speaking, in prokaryotic cells, the antisense RNAs aim at the target mRNA SD sequences and AUG regions have much stronger effects than the ones aim at a coding region. It is more complicated in eukaryotic cells and no precise conclusion and screening method have been reported. However, the selection of antisense RNAs target sequence focus on the following 4 aspects[12,13]: (1) Paring with the 5` end of pre-mRNA and hampers the addition of "cap" and translation; (2) Paring with the upstream of AUG translation initial point and interrupting the combination of ribosome and mRNA; (3) Paring with the coding regions and hampering translation by preventing the elongation of ribosome; (4) Paring with the 3` end of mRNA and preventing the addition of poly "A" tail and its transportation from nucleus to cytoplasm.

Based on the conclusions mentioned above, we adopted reverse transcription PCR to clone extron 2, 3, 6-10, 11-12 of ODC gene. The four extrons correspond to the upstream of translation initial point、the translation initial point、coding region and the 3` end of the coding region, then reversely inserting them into the eukaryotic expression vector pcDNA3.1 and tansfected into the MMC7721. After 72 hours, we adopted Western-blot to analyze the ODC expression in the MMC7721 and found that pcDNA-ODCrI-III inhibited ODC expression significantly. The amounts of ODC protein in these groups were lower than pcDNA-ODCrIV and pcDNA3.1 group, but no obvious distinction was observed between pcDNA-ODCrIV and pcDNA3.1. Consequently, pcDNA-ODCrI-III were initially proved to have evident inhibitory action on the expression of ODC expression, and their corresponding sitesthe untranslated region, the initiation point and the coding region of ODC mRNAwere suggested to be the best antisense sites for inhibition. Their favorable effects may relate to their simple secondary structures of RNA and they are able to match with the antisense fragments easily. Especially at the translation initial regions, mRNA always forms single strand to allow ribosome in, which may facilitate for the hybridization of the antisense fragments[14].

To further prove whether the antisense expression vectors can inhibit the proliferation of hepatic cancer cells, and to reversely verify these antisense vectorsinhibition effects on ODC from the cell proliferation angle, we used MTT assay to compare the pcDNA3.1 and pcDNA-ODCrI-IVs effects on the cellsviability and proliferation. The results demonstrated that pcDNA-ODCI-III was able to inhibit the proliferation of caner cell; and the pcDNA-ODCrIV, which did not have an obvious effect on ODC, was also not able to inhibit the cancer cells proliferation. In another aspect, these results illustrated that the antisense expression vectors can inhibit ODC specifically, and the development of the cancer can be suppressed by inhibiting the expression of ODC gene.

To sum up, we screened out three sensitive inhibition sites of the ODC extron 2, 3, 6-10, and proved the antisense expression vectors that target at the three sites can effectively inhibited the growth of the hepatic cancer cells. Consequently, it may provide a solid foundation for antisense gene therapy of hepatic cancer or other tumors from the aspect of blocking the polyamine biosynthetic pathway.

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