The Primary Study of VEGF –C Gene Therapy of Lymphedema

Yushun Bi, Ping Dong, Yunhai Fang, Zhiyu Liu

Department of Human Anatomy, School of Medicine, Shandong University, Jinan, 250012,China

Abstract **Objective** To construct eukaryotic expression vector of human vascular endothelial growth factor C (VEGF-C) gene in order to explore new stratogies in lymphedema therapy. **Methods** According to human VEGF-C cDNA sequence, we designed a pair of specific primers which contained digestion site of EcoR Ⅰ and BamH Ⅰ on the 5' end respectively. Then revert transcript polymorase chain reaction(RT-PCR) was employed to amplify VEGF-C cDNA from human breast cancer cell MDA-MB-231. After being purified, the product of RT-PCR was inserted into a clone vector Pumt-18. The recombinant plasmids pUMT-18, first propagated in Esherichia coli DH5α, then extracted, purified and digested with EcoR Ⅰ and BamH Ⅰ. Agarose gelanalysis and DNA sequence analysis showed that it contained full length of VEGF-C cDNA. The obtained VEGF-C cDNA was inserted into eukaryotic expression vector pcDNA3.1(-). The pcDNA3.1(-)/VEGF-C, digested with EcoR Ⅰ and BamH Ⅰ, was found to contain the VEGF-C cDNA sequence by agarose gel electrophoresis. **Results** The product of RT-PCR contained the human VEGF-C cDNA. The recombinant pUMT-18 contained correct nucleotide sequence of full length of human VEGF-C cDNA fragment. The VEGF-C cDNA fragment had been inserted into the eukaryotic expression vector pcDNA3.1(-). **Conclusion** The pcDNA3.1(-)/VEGF-C, eukaryotic expression vector for human VEGF-C, is constructed successfully.

Key words VEGF-C; Gene therapy; eukaryotic expression vecton; lymphedma

Lymphedema is characterized by the progressive accumulation of protein-rich fuild in the intestinal spaces that results from the dysfunction of lymphatic system[1]. It is, especially secondary lympedema, common worldwide disease [2]. The treatment of the lymphedema is difficulty and is a troublesome problem for clinic physicians. In view of pathophysiology, the restoration of the lymph-transporting path would be the optimal treatment method.

Vascular endothelial growth factor C(VEGF-C) is a new member of VEGF family, whose receptors are VEGFR-2 and VEGFR-3. As VEGFR-3 is mainly expressed in lymphatic endothelial cells, VEGF-C is believed to be the chief specific regulating factor of lymphatic endothelial cells. Experimental results with the VEGF-C transgenic mice have shown that the over expression of VEGF-C may induce the proliferation of lymphatic vessels [3,4]. In order to study the role of VEGF-C in lymphangiogensis and explore new strategies in lymphedema therapy, we constructed eukaryotic expression vector pcDNA3.1 (-)/VEGF-C containing VEGF-C cDNA fragment by the method of molecular biology.

Correspondence: Yushun Bi, Proffessor, school of Medicine, shandong clniversity, Jinan 250012
Email: byshun@sdu.edu.cn

MATERIALS AND METHODS

Materials

Cell and bacterium lines Human breast cancer cell line MDA-MB-231 was purchased from Basic Medical Research Department of the Chinese Academy of Medical Sciences. Esherichia coli DH5α was gift from Molecular Biology Department of our school.

Primers According to human VEGF-C cDNA sequence, we designed a pair of specific primers, which containing digestion site of EcoR Ⅰ and BamH Ⅰ respectively. The sequence of upstream primer was 5'-CCGAATTCCACCATG-CACTTGCTGGGCTTC-3', the sequence of downstream primer was 5'-CGCGGATCCT-TAGCTCATTTGTGGTCTTTTCC-3', both of them were constructed by Shanghai Sangon Biotechnology Co. Ltd.

Vectors PMD18-T was a kind of specific vector for clonal PCR products. Eukaryotic expression vector pcDNA3.1(-) was purchased from Invitrogen Co. Ltd.

Other agents Trizol agent kit and cDNA the first chain construction kit were purchased from Giboco Laboratories/Life Technologies. PCR agent kit was purchased from Dalian TaKaRa Biotechnology Co. Ltd. Gel retrieve agent kit and plasmid extraction agent kit were purchased from Qiagen of Germany. Restriction enzyme, DNA molecule mass standard DNA Marker DL2000 and λEcot14 Ⅰ digest were purchased from Dalian TaKaRa Biotechnology Co. Ltd. λ-DNA/EcoR Ⅰ + Hind Ⅲ was purchased from Shanghai Sangon Biotechnology Co. Ltd.

Methods

The culture of cells The cell culture liquid is composed of L-15 (purchased from Giboco Laboratories/Life Technologies), 10% fetal calf serum, 100U/ml penicillin and 100U/ml streptomycin. Cells were cultured in incubator containing 5% CO2 and saturation humidity at 37℃, digested by 0.25% trypsin.

Extract total RNA Collect MDA-MB-231 cells in logarithmic growth period. The amount of cells was about(5~6)×106, Extract total RNA according to the illustration of Trizol agent kit.

RT-PCR Five microliters total RNA were reverse transcribed according to the illustration of cDNA first chain construction agent kit with random primers Oligo dT. Two microliters of 20μl to tal RT reaction volume was used as the template DNA for the PCR. PCR was performed with specific primers for 35 cycles with cycle times of 5min at 94℃, 30s at 60%℃ and 1min at 72℃, the final elongation time was 7min at 72℃. Ten microliters of 50μl total PCR mix were electrophoresed on 1% agarose gel. Human β-actin was used as internal contrast.

Retrieve PCR products 60μl PCR products electrophoresed on 1% agarose gel. Using Utraviolet-light analysis apparatus as monitor, cut down the gel containing objective gene by clean scalpel. Then retrieve DNA by gel according to the illustration of agent kit. Identified by electrophoresed on 1% agarose gel.

Link up PCR products and pMD18-T vector Ligating DNA and vector overnight at 16℃, then putp-MD18-T to 200μl DH5α cells with $CaCl_2$, plated at LA flat plate and incubated 16h at 37℃.

Screening and identify of positive recombined plasmid Select several single clone randomly, then inoculated in 5ml LA liquid culture medium respectively, culture at 37℃ 180 rpm overnight. Extract plasmid according to illustration of plasmid extraction agent kit and digested by restriction enzyme EcoR Ⅰ and BamH Ⅰ respectively. The total volume was 50μl plasmid 20μl, EcoR Ⅰ 1μl, BamH Ⅰ 1μl, 10×K Buffer 5μl, dH_2O 23μl, digested 2.5h at 37℃ in water bath, then inactivated the activity in water bath for 30min at 65℃, Ten microliters reaction products electrophoresed on 1% agarose gel to screen positive recombined plasmid and retrieve objective gene fragment. Carried out sequence analysis with 1.5ml liquid containing positive recombined plasmid by Shanghai Sangon Biotechnology Co. Ltd.

Digest and retrieve pcDNA 3.1(-) Use EcoR Ⅰ and BamH Ⅰ to digest pcDNA3.1 (-). Digest volume was 50μl: plasmid 20μl, EcoR Ⅰ 1μl, BamH Ⅰ 1μl, 10×K Buffer 5μl, dH_2O 23μl, Digested 2.5h at 37℃ in water bath, then inactivated in water bath for 30min at 65℃. Ten microliters reaction products electrophoresed on 1% agarose gel to identify.

Ligation of VEGF-C DNA and pcDNA3.1 (-) Volume was 10μl:VEGF-cDNA fragment 1μl, retrieved pcDNA3.1(-) fragment 1μl, T4 DNA ligase 1μl, 10×K Buffer 1μl, dH_2O 6μl, ligated at 16℃ overnight. Put pcDNA3.1 (-) to 200μl DH5α cells with $CaCl_2$, plated at LA flat plate and incubated 16h at 37℃.

Identification of pcDNA3.1(-)/VEGF-C Select several single clone randomly, then inoculated in5ml LA liquid culture medium respectively, cultured at 37℃ 180 rpm overnight. Extracted plasmid according to illustration of plasmid extraction agent kit and digested respectively by restriction enzyme EcoR Ⅰ and BamH Ⅰ. The volume was 50μl: plasmid 20μl, EcoR Ⅰ and BamH Ⅰ 1μl, 10×K Buffer 5μl, dH_2O 23μl, reacted 2.5h at 37℃ in water bath, then put out the activity in water bath for 30min at 65℃. Ten microliters reaction products electrophoresed on 1% agarose gel to screen positive reformed plasmid.

RESULTS

Identification of RT-PCR products According to the background reference of human VEGF-C cDNA record by Genebank and the trait of the designed specific primers, the whole length of RT-PCR products should be 1.28kb. The length of RT-PCR produced fragments i-

dentified by electophoresed on agarose gel was accord with the analysis of background reference(Fig.1).

Screen of reformed pMD18-T Choose six reformed pMD18-T clone by random, extract plasmid,digested by EcoR Ⅰ and BamH Ⅰ, then electrophorsesed on agarose gel. One clone presented of 2.6kb was accord with pMD18-T, another fragment of 1.27kb was accord with RT-PCR products digested by enzyme(Fig.2).

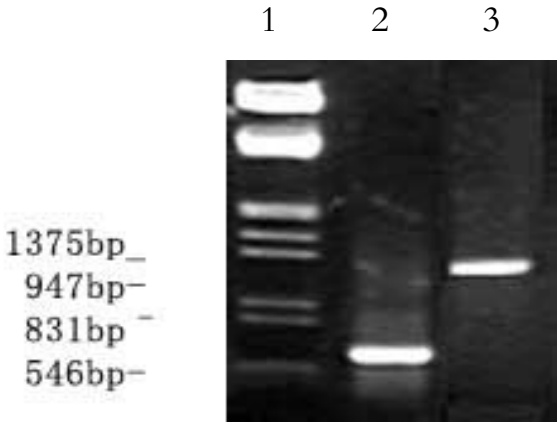

Fig.1 Agarose gel electrophroesis of products of RT-PCR
1. Marker λDNA/EcoR Ⅰ+Hind Ⅲ;
2. Human β-actin; 3. Human VEGF-C cDNA

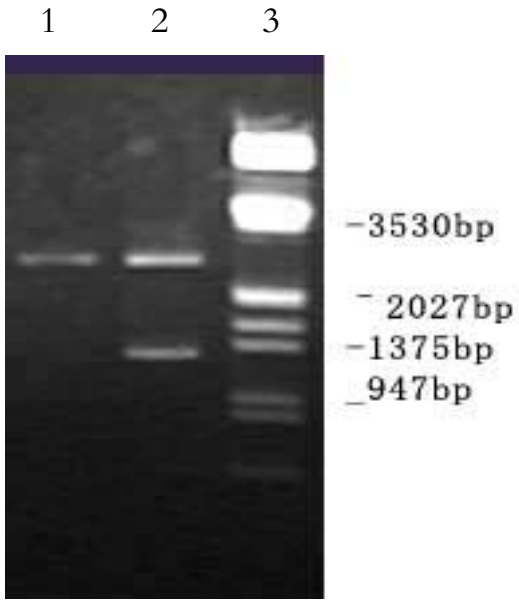

Fig. 2 Results of recombinant plasmid pMD 18-T digested by EcoR Ⅰ and BamH Ⅰ
1. Nagative clone; 2. Positive clone;
3. Marker DNA/EcoR Ⅰ + Hind Ⅲ

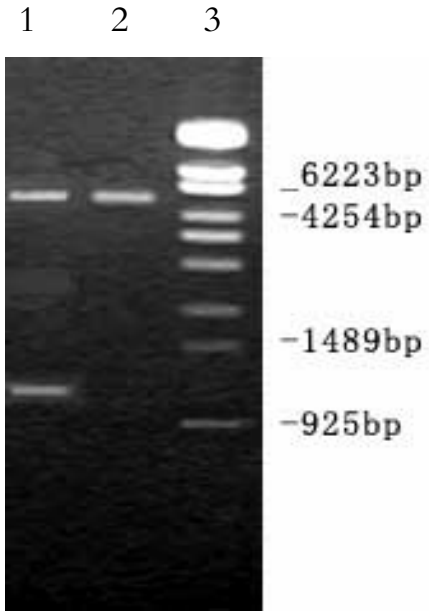

Fig.3 Digestion of pcDNA3.1(-)/VEGF-C with EcoR Ⅰ and BamH Ⅰ
1. Marker λDNA/EcoT14 Ⅰ; 2. pcDNA3.1(-);
3. pcDNA3.1(-)VEGF-C

Identification of reformed pcDNA3.1 (-)/VEGF-C There were two fragments of 5.4kb and 1.27kb when pcDNA3.1 (-)/VEGF-C was digested by EcoR Ⅰ and BamH Ⅰ and electrophoresed on agarose gel. The fragment of 5.4kb was accord with pcDNA3.1 (-), another fragment of 1.27kb was accord with human VEGF-C cDNA fragment produced by RT-PCR. This demonstrated there was VEGF-C cDNA been inserted to pcDNA3.1(-)/VEGF-C(Fig.3)

DISCUSSION

The mechanism of lymphangiogenesis has been a long-standing controversy. Some researchers believe lymphatic vessels are sprout from veins, others believe that lymphatic vessels have their independent origin just like blood vessels, neither of them have exact evidence. With the development of modern biology and more attention was paid to the function of lymphatic system, people further developed the study on lymphangiogenesis. The relationship between lymphangiogenesis in the tumor and the lymphegenic metastasis is becoming the hot point of study. Recent findings show lymphangiogenesis is affected and influenced by many factors, among which VEGF-C is believed to be the chief specific regulating factor on lymphatic endothelial cells[5,6].

VEGF-C was separated and purified in 1996, it be-

long to VEGF family. Researches showed that VEGF-C can regulating the development of lymphatic system in embryo by VEGFR-3[4], VEGF-C plays a significant role in the lymphangiogenesis of tumor[7,8], Furthermore, the overexpression of VEGF-C can induce the hyperplasia of lymphatic vessels, but its precise mechanism was still unclear. As a member of VEGF family, VEGF has been used on the study of gene therapy and obtained remarkable results. According to the trait of VEGF-C, it is conceivable that VEGF-C can be used on gene therapy of lymphatic defect diseases, for instance, lymphatic dropsy.

To study the role of VEGF-C in lymphangiogenesis and carry out corresponding study on gene therapy of lymphangiogenesis defect diseases, the key material foundation is the construction of eukaryotic expression vector of VEGF-C gene. Vectors of eukaryotic expression used in the study for external gene were divided into virus vectors and nonvirus vectors. Virus vectors have the advantage of efficiency in transfection and stabllity in expression, but they are poor in security. Most of the nonvirus vectors were various plasmids. They exist in target cells as additional body; they have the advantage of good in security and large in capacity. In this experiment pcDNA3.1(-) have the below traits in structure: ① containing SV40 ori reproduce component, can reproduce with host cells, this ensure the stable transfer of object gene in some degree; ② there are T7 and pCMV in the upstream of polyclone site which can express the inserted objectgene fragment efficiently; ③ there is neo gene, object cells containing this plasmid can be screened by G418.

In short, we constructed pcDNA3.1(-)/VEGF-C eukaryotic expression vector successfully by molecular clone technology. We also used this plasmid on gene therapy of animal model of lymphedena. With the development of this study. We believe that the study will create a base of our exploring new strategies in lymphedema therapy, and help to understand the machanism of VEGF-C playing in lymphangiogenesis.

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