Construction and Expression of Transmembrane and Secretory Recombinant Human CD33 Extracellular Domain in HEK293F Cell

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Abstract

The CD33 molecule is a transmembrane receptor with a molecular weight of 67KD. it is mainly exist in blood bone and lymphocytes. It belongs to immunoglobulin super family and is also a member of sialic acid-binding immunoglobulin-like lectin family .The extracellular domain of CD33 protein contains a lot of epitopes, which is more potent than that of antigen peptide molecules. In this study, we engineered and constructed an extracellular domain of CD33protein ($CD33_{ECD}$) and an $CD33_{ECD}$-His fusion protein, the recombinant vectors were transfected into HEK293F cells, and then expressed and purified. Functional assay showed the $CD33_{ECD}$ was successfully expressed on the cell membrane, and the $CD33_{ECD}$-His fusion was obtained in cell supernatant. Therefore, we successfully expressed transmembrane and secretory $CD33_{ECD}$ proteins.

Keywords

Transmembrane CD33ECD, secretory CD33ECD, HEK293T cell, expression and identification.

1. Introduction

The CD33 molecule is a transmembrane receptor with a molecular weight of 67KD. The CD33 molecule contains an immunoglobulin domain of the N-terminal binding to V-set and a variable C2-set immunoglobulin domain[1-3], they are linked to cytoplasm through the transmembrane domain. These two immunoglobulin domains usually contain one or more immune receptor tyrosine related inhibitory motif (ITIM)[4]. It mainly exists in the cytoplasmic tail of the CD33 molecule.

The CD33 molecules of ITIM can induce the rapid phosphorylation of the tyrosine motif, when the protein kinase is stimulated by a foreign protein kinase, tyrosine phosphorylation occurs in the tail of the cytoplasm. at the same time, the immunoreceptor tyrosine-based inhibitory motifs is used as a cell membrane molecule to transmit the inhibitory signal to the cell[5] .The 340 loci of tyrosine, 343 loci of leucine and 358 tyrosine of TIM are conserved and functional areas. When the amino acids of these sites change, ITIM can not complete phosphorylation and dephosphorylation, and the whole signal pathway is closed[6].

CD33 can expression of B-cells and activated T- and natural killer (NK) cells[7-14] .When CD33 is combined with bivalent antibody, internalization occurs on the surface of the cell, this process is slower than other cell surface antigens such as transferrin receptor[15-23], through the research mechanism, the researchers found endocytosis determined by the intracellular domain of CD33, the reason may be that have tyrosine phosphorylation and ubiquitination of the cytoplasmic tail. although some internalization of CD33/antibody complexes occurs in a phosphorylation-independent manner[21-23], engagement of CD33 with bivalent will decrease of cell surface levels.although the new CD33 will expression, but the reduction of available target binding sites that can be will result in reduce the efficacy of CD33-directed therapeutics.

Acute myeloid leukemia is one of the major diseases that threaten human life and health., it is also the main type of adult leukemia.n the United States, 2.7 of the 100000 adults suffer from AML each year and 14.1 in adults over 65 years of age, and about 7000 people die of the disease each year[24-26]. recent advances in molecular biology have opened up a new field for the treatment of acute myeloid leukemia., it is found that the expression of CD33 exists on the surface of most acute myeloid leukemia cells, but there is no glycoprotein in normal hematopoietic pluripotent stem cells and non hematopoietic cells[27]. Therefore, CD33 is the most suitable target antigen for immunotherapy. At present, monoclonal antibodies have made great achievements in the treatment of acute myeloid leukemia.for example, Gemtuzumab ozogamicin is a monoclonal antibody against CD33 targets[28]

2. Materials and Methods

2.1 Reagents

1.Kpn, Xho, Bg1, Pst Ⅰ Fast Cutase purchased from Fementas. 2.Prime STAR Max DNA Polymerase was purchased from Takara.

3.HER293T cells were supplied by our laboratory.

4.Anti-His mouse antibody

5.anti-human CD33 mouse antibody

6.goat anti-mouse IgG-HRP antibody

2.2 Construction of pDisplay-CD33ECD and pCMV-CD33ECD-Fc Coding Sequence

The gene of CD33ECD was amplified by PCR using cCD33ECDF-1

(ATAAAAAGATCTGATCCAAATTTCTGGCTGC)andcCD33ECDR-1 (ATAAAACTGCAGATGAACCACTCCTGCTCTG) primers. The CD33 gene was cloned into pDisplay plasmid (InvivoGen, USA) .The recombinant vector was

transformed to DH5α and the sequence of plasmid pDisplay-CD33ECDwas confirmed by colony PCR and sequencing.

The gene of CD33ECD and His fragment was amplified by PCR using

cCD33ECDF-2 (ATAAAAGGTACCGATCCAAATTTCTGGCTGC) and

cCD33ECDR-2(ATAAAACTCGAGTCAGTGGTGGTGGTGGTGGTGATGAACCACTCCTGCT).The recombinant vector was transformed to DH5α and the sequence of plasmid pCMV-CD33ECD-Fc was confirmed by colony PCR and sequencing.

2.3 Bacterial Strains, Media and Culture Conditions

The E.coli strain DH5a was stored in our laboratory, and used as the host strain for cloning and maintenance of plasmids throughout the experiments. The pDisplay and pCMV (CHENDU TRANSVECTOR BIOTECHNOLOGY CO. Lid) were used as expression vectors for protein expression. The HEK293F cell was maintained at 37 C in 5% CO2 and 95% air.

2.4 Protein Expression

The vectors(pDisplay-CD33ECD / pCMV-CD33ECD-His) were amplified in E.coli cells and DNA was purified using Endo Free Plasmid Maxi Kit E.(Qiagen). HEK293 cells in the exponential growth phase were grown in Gibco FreeStyle 293 Expression Medium (Invitrogen) until they reached a cell density of 1×106cells/ml. The cells were transiently transfected using FreeStyleMAX Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 120μg DNA diluted in Gibco OptiPro SFM(Invitrogen) were gently mixed with 120 μl FreeStyle MAX Reagent diluted in OptiPro SFM and incubated for 10 min. The mixture was added drop-wise to a flask containing 150ml HEK293F cells. The transfected cells were allowed to grow in suspension for six days at 37° C in a humidified atmosphere of 5% CO2 on an orbital shaker platform rotating at 135 rpm. the blank control group without transfection was set.

2.5 SDS-PAGE and Western Blot Analysis

To evaluate the protein expression of the transmembrane and secretory CD33ECD proteins, a reducing SDS-PAGE and western blotting were performed on the samples. The collected cells were digested with 1% SDS at 100° C for 10 min, and the supernatant was used to detect the total protein concentration by BCA in order to adjust the same protein concentration in the samples.

For western blotting, the proteins were transferred from the second gels to a nitrocellulose membrane (GE Healthcare, UK), where residual protein binding sites were subsequently blocked with 5% BSA. In order to detect the human CD33ECD region of the protein, the membrane was incubated with anti-human CD33 HRP antibody (Sigma, Germany) at 4 C overnight, and goat anti-mouse IgG-HRP antibody was made as secondary antibody. Finally, the bands were revealed by 3, 30-Diaminobenzidine (DAB) solution (Sigma,Germany).

2.6 Flow Fluorescence Analysis

After 48h, 5×106 cells transfected by pDisplay-CD33ECD were washed with 1×PBS, fixed with 5% paraformaldehyde. Anti-human CD33 mouse antibody was used as primary antibody, goat anti-mouse IgG-FITC antibody was made as secondary antibody. The cells of blank control group were set the fluorescence domain values, then the expression of CD33ECD on HEK293F cell membrane was detected by Guava micro-capillary cell analyzer.

2.7 Purification and Identification of CD33ECD-His

The cell supernatant with CD33ECD-His was filterred with 0.45 filter and purified by protein A Ni-NTA affinity chromatography column. The mixture of column was equilibrated with 1×PBS（pH 7.4). Then the targeted protein was eluted with 250 mM imidazole, the purity was analyzed by SDS-PAGE

3. Results

3.1 Construction of Display-CD33ECD and pCMV-CD33ECD-His

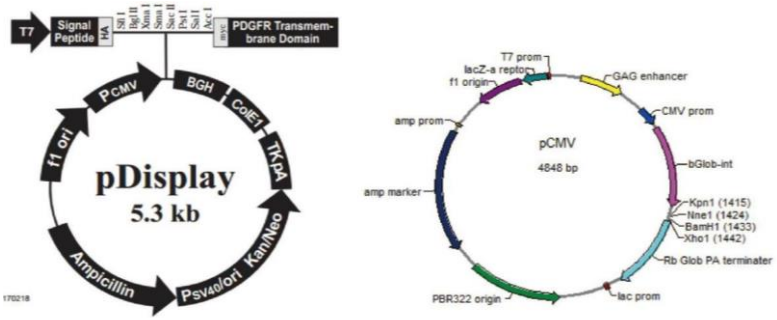


Fig. 1 The map of pDisplay and pCMV

The gene fragment CD33ECD and His were amplified by PCR, and detected by agarose gel electrophoresis. As shown in Fig.2A , the amplified CD33ECD fragment was about 777bp, the amplified His fragment was about 16bp, Colonies PCR verifying whether the target gene is inserted into the pCMV plasmid, As shown in Fig.2B the amplified CD33ECD fragment was about 777bp,As shown in Fig.2C Colonies PCR verifying whether the target gene is inserted into the pDisplay plasmid. As shown in Fig.2D.

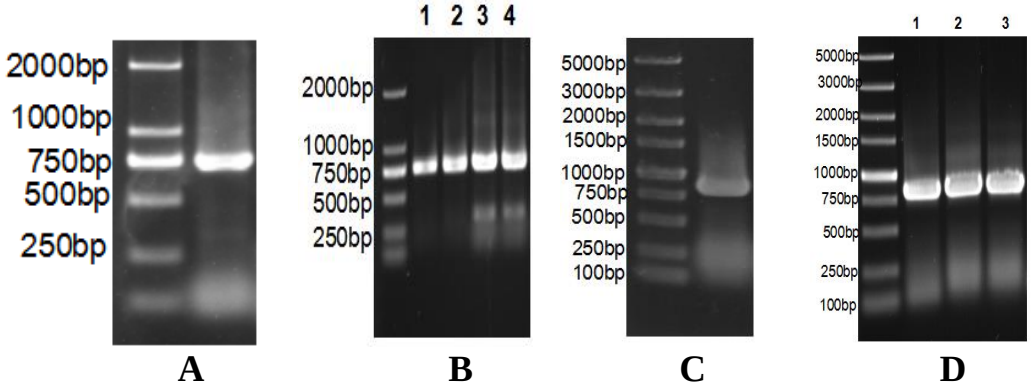


Fig. 2 Construction of pDisplay-CD33ECD and pCMV-CD33ECD-His

A: Overlapping PCR of CD33ECD and His B: Colony PCR of pCMV-CD33ECD-His

C: Amplification of CD33ECD D: Colony PCR of pDisplay-CD33ECD

3.2 Western Botting Test of CD33ECD and CD33ECD-His

The supernatants of cell transfected by pDisplay-CD33ECD and pCMV-CD33ECD-His were analyzed by Western Blotting. The results of CD33ECD were shown in Fig. 3A. There were only one targeted band with a size of 60 kDa of CD33ECD. Further measureent of the internal β-actin were carried out with a size of 43 kDa in line with its expected size. Fig. 3B showed the result of CD33ECD-His that only one targeted band with a size of 50 kDa consistent with predicted protein size. The above results confirmed the successful experssing of CD33ECD and CD33ECD-His.

Fig. 3 Western Botting Test of CD33ECD and CD33ECD-His

3.3 Flow fluorescence analysis of expression of CD4ECD on HEK293F cell membrane

The cells in the blank control group and experimental groups were analyzed by flow cytometry. As shown in Fig. 4, the three experimental groups (Fig. 4B-C) showed positive results compared with the blank control group (Fig. 4A). The positive rate respectively were 50.5%, 51%, indicating CD33ECD was successfully expressed in the cell membrane.

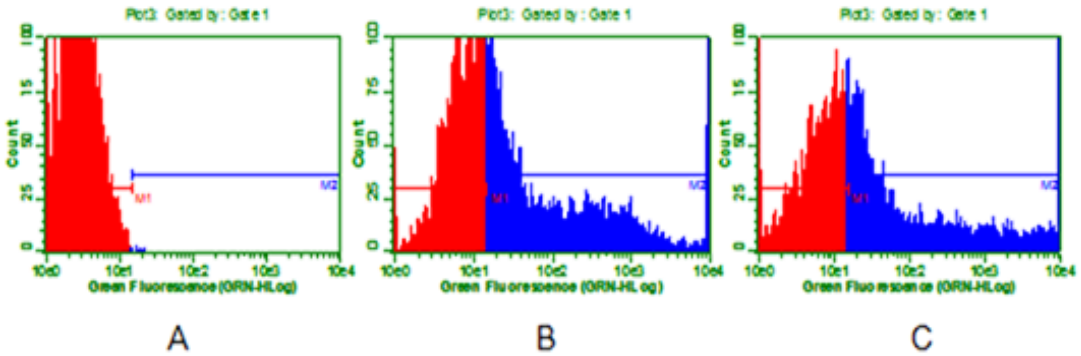
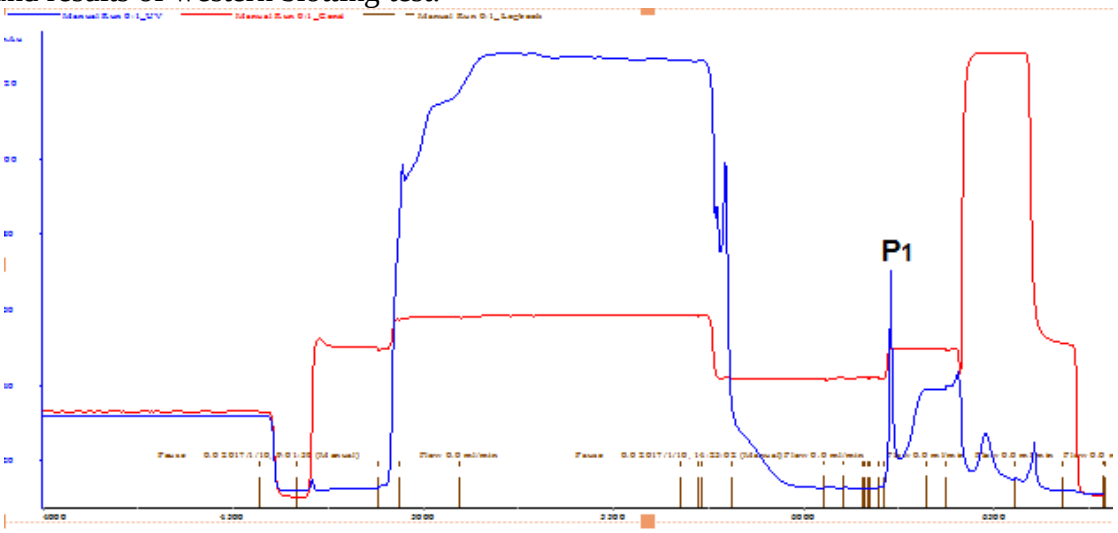


Fig. 4 Flow fluorescence analysis of expression of CD33ECD on cell membrane

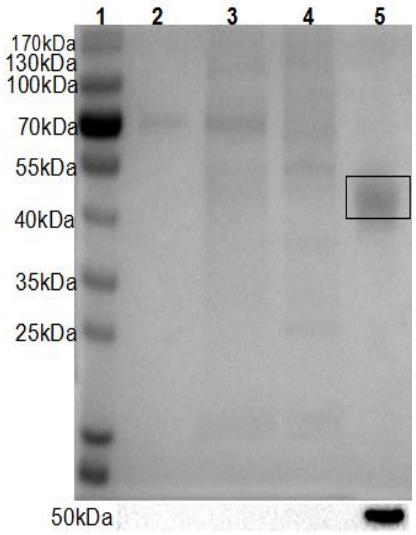
3.4 Identification and Purification of CD33ECD-His

After 120h of cell transfection, the supernatant was collected to purify by protein A Ni-NTA affinity column. The mixture of column was equilibrated with 1×PBS (pH 7.4). Then the targeted protein was

eluted with 250 mM imidazole.Fig.5A is the purification chromatography chart .The P1 refers to the elution peak of the targeted protein. The purified protein was subjected to SDS-PAGE electrophoresis. As shown in Fig.5B the five lanes is target band, the size of 50kDa, consistent with the theoretical value and results of western blotting test.



A



B

Fig. 5 Identification and purification of CD33ECD-His

A: Chromatography of CD33ECD-His purified by Ni-NTA .

B:SDS-PAGE and WB of CD33ECD-His.

3.5 Detection of Biological Activity of CD33ECD-His by Elisa Method

The amount of antigen coated in the Elisa plate is 1ug/ml. The milk with a concentration of 2% diluted the CD33 antibody to 5000 times, which was the initial concentration, and then diluted three times. Determine the size of the absorption value and make the correlation curve between the concentration of antibody and the value of absorption Fig.6

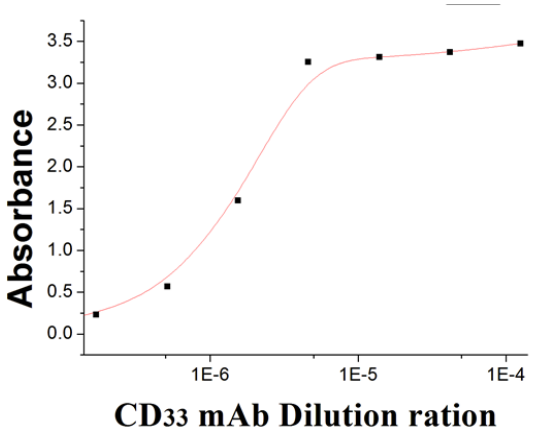


Fig. 6 Detection of biological activity of CD33ECD-His by Elisa method

4. Discussion

At present, the treatment of tumor is chemotherapy, radiotherapy,therapy, etc. Target treatment is a new method for treating tumors, CD33 is an targetson acute myeloid leukemia cells, In recent years, In recent years, there have been many marketed drugs targeting CD33 as a target for the treatment of acute myeloid leukemia. For example, Gemtuzumab ozogamicin is a monoclonal antibody against CD33 targets. The antibody of CD33 binds to the antigen on the cell, and the cell carries the drug into the cytoplasm through endocytosis.Gentetuzumab is a good medicine for the treatment of acute myeloid leukemia, but it also has side effects.

In this study, the transmembrane and secretory CD33ECD were made, which was the first step of obtaining the high biological activity anti-CD33 antibody through immune response. pDisplay and pCMV were used as vectors for the expression of transmembrane and secretory CD33ECD, respectively. There is a PDGFR Transmenbrance Domain in pDisplay. The PCR products of CD33ECD and CD33ECD-His were respectively cloned to pDisplay and pCMV to form the recombinant plasmids of pDisplay-CD33ECD and pCMV-CD33ECD-His. At present, researchers have expressed CD33 protein in E. coli. However, the protein expressed by Escherichia coli is present as an inclusion body, which needs to be purified and renatured to become a bioactive soluble protein. Therefore, in this study we use HEK293F eukaryotic expression system. The HEK293F cells transfected by pDisplay-CD33ECD were detected by Western Blotting, flow cytometry, and the results showed that CD33ECD was successfully expressed on the membrane. The supernatant of HEK293F cells transfected by pCMV-CD33ECD-His was confirmed by Western Blotting, using protein A Ni-NTA affinity chromatography to purify the protein. Protein purity was verified by SDS-PAGE and Western Blotting.

At present, there are many problems with CD33 monoclonal antibodies such as side effects. Nanobodies have good stability and high affinity, which overcome the shortcomings of small molecule functional antibodies. It also has monoclonal antibodies and polyclonal antibodies that have low molecular weight, low immunogenicity, and strong tissue penetration. With the advantages, there is a great application prospect in disease diagnosis and treatment, and it has great potential to become a tumor means. We can use 293F cells to express the extracellular domain of CD33, and then to immunize the camel, using phage display technology to screen out specific nanobodies against CD33 antigen.

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