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Construction of a Fusion Gene Carrying E10A and K5 with 2A Peptide-Linked by Using Overlap Extension PCR

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***Abstract*—**E10A is a kind of replication-defective adenovirus which carries the human endostatin gene to inhibit the growth of tumors. Kringle 5(K5) has almost the same function as angiostatin to also inhibit the growth of tumors since they are all the byproduct of the proteolytic cleavage of plasminogen. Tumor size increasing can be suppressed because both of the endostatin and K5 can restrain the angiogenesis process. Therefore, in order to improve the treatment effect on tumor, 2A peptide is used to construct a fusion gene carrying both E10A and K5. Using 2A peptide is an ideal strategy when a fusion gene is expressed because it can avoid many problems during the expression of more than one kind of protein. The overlap extension PCR is also used to connect 2A peptide with E10A and K5. The final construction of fusion gene E10A-2A-K5 can provide a possible new method of the anti-angiogenesis treatment with a better expression performance.

***Keywords*—**E10A, Kringle 5, 2A peptide, overlap extension PCR.

I. Introduction

IN recent years, different kinds of cancers have greatly influenced modern human health and even the treatment exploring. Some certain kinds of cancer like primary liver cancer are very hard to eliminate since they may have no response to normal therapies such as surgery, radiotherapy, and chemotherapy [1], [2]. As a result, it is very important to discover a new strategy for treating the tumor. As the development of modern biology, angiogenesis is considered as a critical process for tumor growing [3]. Based on this new discovery of angiogenesis, it is possible to inhibit the growth of tumors by just blocking tumor angiogenesis since fewer nutrients can reach the tumor to support the further growth. Therefore, developing possible synthetic angiogenesis inhibitors plays a significant role in this new treatment strategy. Endostatin, angiostatin, and Kringle 5 (K5) are all great angiogenesis inhibitors found in recent years. [4] Endostatin is the C-terminal part of the collagen XVIII α1-chain [5] and is able to inhibit the migration and induction of endothelial apoptosis of cells to further suppress the growth of tumor cells [6]. Both obtained from the proteolytic cleavage of plasminogen, angiostatin and Kringle 5 have a strong inhibition of blood vessels growing. At the same time, K5 also maintains the ability to directly influence tumor by gathering neoplastic-associated neutrophils and natural killer T cells [7], which means K5 might have a more powerful effect than angiostatin on inhibiting tumor growth. All the conditions make endostatin and K5 suitable for combination to enhance their treatment effect.

To combine the E10A carrying endostatin and K5 together and express the target protein successfully, a more efficient biological strategy is required. By avoiding the disadvantages of low protein activity or low downstream gene expression when multiple genes are expressed [8]-[10], 2A peptide seems to be suitable to be used in this experiment. Besides, during the fusion gene construction process, overlap extension PCR is an ideal method for gene combination since it can easily bind two or more gene fragments together without having other process [11]. This method just needs to use complementary base sequences from the designed primer to gain the target fusion gene by PCR processes. Consequently, the fusion gene E10A-2A-K5 is linked by 2A peptide using overlap extension PCR technique. The experiment results indicate that this method to combine endostatin and Kringle 5 is possible, which provides new path of constructing angiogenesis inhibitor to suppress the growth of tumor.

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II. Materials and Methods

A. Cell Culture and Reagents

Human embryonic kidney 293T cells HEK293T are provided by Guangzhou Double Bioproduct Co. Ltd. E10A and K5 plasmid are provided by Guangzhou Double Bioproduct Co. Ltd. DEPC water, KOD NEO BUFFER, dNTP, MGSO4, KOD NEO polymerase, and T4-DNA Ligase, T4-DNA Ligase buffer are purchased from NEW ENGLAND BIOLAB. Gel extraction kit and Endo-free plasmid DNA mini kit are purchased from OMEGA Bio-tek, SuperRT One Step RT-PCR Kit is purchased from CWbiotech. All the other materials are provided by Guangzhou Double Bioproduct Co. Ltd.

B. Primer Design

According to the gene sequence of E10A and K5 provided by Guangzhou DoublleBioproduct Co. Ltd., two restriction enzyme cutting sites, EcoR1 and Sal 1, will be added to the 5' end of E10A and the 3’ end of K5, respectively, in the experiment. EcoR1 is inserted into the primer “endo-62-e1”, and Sal 1 is inserted into the primer K5-R-S1. In order to have a greater performance of shear efficiency by 2A peptide, a certain kind of 2A peptide which is called porcineteschovirus-1 2A(P2A) is used in the primer design of 2AK5-F-60 because P2A has the highest shear efficiency among all kinds of 2A peptides (P2A, T2A, E2A and F2A) in human cultured cell

lines, zebrafish embryos and mouse livers [12]. To simplify the procedure of the experiment during overlap extension PCR, P2A sequence is directly included in the primer 2AK5-F-60 to amplify the fusion fragment of P2A linked with K5. The primers endo2a-F-56 and endo2a-R-57 are designed to have 15 bp gene overlapped with the primer 2AK5-F-60 to conduct the overlap extension PCR to bind and amplify E10A with the 2A peptide and K5 gene.

The primers 2AK5-F-60 and endo-62-e1 are synthesized by Beijing RuiBiotech; and the primers endo2a-F-56, endo2a-R-57, and K5-R-S1 are synthesized by Shanghai Sangon Biotech.

Primer sequences are given in Table I.

TABLE I
PRIMER SEQUENCES

Primer name	Sequence (5'-3')	Remark
2AK5-F-60	GGAAGCGGAGCTACTAAC TTCAGCCTGCTGAAGCAGG CTGGAGACGTGGAGAACC CTGGACCTATGGTGGCACC ACCACCT	To amplify 2A-K5
endo-62-e1	CGGAATTCGACAGCCACC GGGA	To amplify E10A, endo2a -K5, and E10A-2A-K5
endo2a-F-56	AGCTTTATGACAGCCTCCA AGGGAAGCGGAGCTACTA ACTTC	To amplify E10A, endo2a -K5, and E10A-2A-K5
endo2a-R-57	GAAGTTAGTAGCTCCGCTT CCCTTGGAGGCTGTCATAA AGCT	To amplify E10A
K5-R-S1	ACGCGTCGACTCAATCGAA GCTAGGGGCGGC	To amplify 2A-K5, endo2a -K5, and E10A-2A-K5

C. Methods

1) The Amplification of Fusion Fragment of 2A peptide (P2A) and K5

Here, 2AK5-F-60 and K5-R-S1 are primers, and K5 is the template. The normal PCR method is used to amplify the fusion fragment 2A-K5.

PCR amplification system is: DEPC water 33 µl, KOD NEO BUFFER 5 µl, dNTP 5 µl, MGSO4 3 µl, primer 2AK5-F-60 1.5 µl (10 µM), primer K5-R-S 1.5 µl (10 µM), template K5 0.3 µl, and KOD NEO polymerase 1 µl.

PCR-Cycler Settings are: Initial denaturation 94 °C – 2 min, denaturation 98 °C – 10 s, annealing 54 °C – 30 s, extension 68 °C – 45 s, 35 cycles, and extension 68 °C – 2 min.

After the PCR, agarose gel electrophoresis is used to detect the amplified product and then the Gel Extraction is used to recycle the amplified product.

2) Amplification of Fusion Fragment of en2a-R-57 and 2A-K5

Here, endo2a-F-56 and K5-R-S1 are primers, and the amplified product 2A-K5 is the template. The overlap extension PCR is used to amplify the fusion fragment endo2a -K5.

PCR amplification system is: DEPC water 33 µl, KOD NEO BUFFER 5 µl, dNTP 5 µl, MGSO4 3 µl, primer endo2a-F-56 1.5 µl (10 µM), primer K5-R-S 1.5 µl (10 µM), template endo2a -K5 0.5 µl, and KOD NEO polymerase 1 µl.

PCR-Cycler Settings are: Initial denaturation 94 °C – 2 min, denaturation 98 °C – 10 s, annealing 62 °C – 30 s, extension 68 °C – 70 s, 30 cycles, and extension 68 °C – 2 min.

After the PCR, agarose gel electrophoresis is used to detect the amplified product and then the Gel Extraction to recycle the amplified product.

3) Amplification of E10A

Here, endo-62-e1and endo2a-R-57 are primers, and plasmid of E10A is the template. The normal PCR method is used to amplify E10A.

PCR amplification system is: DEPC water 33 µl, KOD NEO BUFFER 5 µl, dNTP 5 µl, MGSO4 3 µl, primer endo-62-e1 1.5 µl (10 µM), primer endo2a-R-57 1.5 µl (10 µM), template endo2a -K5 0.5 µl, and KOD NEO polymerase 1 µl.

PCR-Cycler Settings are: Initial denaturation 94 °C – 2 min, denaturation 98 °C – 10 s, annealing 62 °C – 30 s, extension 72 °C – 1 min, 35 cycles, and extension 72 °C – 2 min.

After the PCR, agarose gel electrophoresis is used to detect the amplified product and then Gel Extraction to recycle the amplified product.

4) Amplification of Fusion Gene of E10A and 2A-K5

Here, endo-62-e1and K5-R-S1 are primers, and the amplified product E10A in 2.3.3 and endo2a -K5 in 2.3.2 are the template. The overlap extension PCR is used to amplify E10A-2A-K5.

PCR amplification system is: DEPC water 33 µl, KOD NEO BUFFER 5 µl, dNTP 5 µl, MGSO4 3 µl, primer endo-62-e1 1.5 µl (10 µM), primer endo2a-R-57 1.5 µl (10 µM), template E10A 0.5 µl, template endo2a -K5 0.5 µl, and KOD NEO polymerase 1 µl.

PCR-Cycler Settings are: Initial denaturation 94 °C – 2 min, denaturation 98 °C – 10 s, annealing 65 °C – 30 s, and extension 68 °C – 1 min, 35 cycles.

After the PCR, agarose gel electrophoresis is used to detect the amplified product and then Gel Extraction to recycle the amplified product.

5) Restriction Enzyme Digestion

Here, EcoR I and Sal I are used to do the restriction enzyme digestion on plasmid PDC316 and the amplified product E10A-2A-K5 in 2.3.4.

E10A-2A-K5 enzyme digestion system is: 10×H Buffer 5 µl, EcoR I 1 µl, Sal I 1 µl, E10A-2A-K5 10 µl, and DEPC water 33 µl.

PDC316 enzyme digestion system is: 10×H Buffer 5 µl, EcoR I 1 µl, Sal I 1 µl, plasmid PDC316 10 µl, and DEPC water 33 µl.

The E10A-2A-K5 enzyme digestion system and PDC316 enzyme digestion system are put into a 37 °C water bath for 2 hours after mixing thoroughly.

After the restriction enzyme digestion, the agarose gel electrophoresis is used to detect the digestion product and then Gel Extraction to recycle the digestion product.

6) Ligation

T4-DNA Ligase is used to ligate the enzyme digested E10A-2A-K5 to the enzyme digested vector PDC316.

Ligation system is: T4-DNA Ligase 1 µl, T4-DNA Ligase

buffer 2 µl, E10A-2A-K5 3.5 µl, PDC316 1 µl, and DEPC water 12.5 µl.

The ligation system is put at room temperature for 1 hour after mixing thoroughly.

7) Transformation

The competent cell DH5α is taken out from the -80 °C refrigerator to melt in ice bath, and the ambenzyl resistance culture plate is put into the incubator at 35 °C for 20 min to dry, the connection is added and the products are mixed well, then put it into the ice bath for 30 min. After the ice bath, the DH5α cell is put in the water bath pot at 42 °C for 1 min to heat, then it is taken to the ice bath for 3-5 min, the cell is plated in the ambenzyl resistance culture plate and it is put in the incubator to make it grow for 16 hours to go overnight.

Six monoclonal colonies are picked from the ambenzyl resistance culture plate last night and each of them is placed into a 1.5 µl EP tube. Next, 100 µl ambenzyl resistance culture medium is added into each of the EP tubes and shake them for 6 hours. After this, 5 µl bacterial solutions are extracted from each of the samples to perform the colony PCR and agarose gel electrophoresis for identification of positive clones, and the plasmids are also sent to Shanghai Sangon Biotech for biological sequencing. Then 8 ml ambenzyl resistance culture medium is added into new LB tubes for those colonies which have an obvious stripe during the agarose gel electrophoresis to shake in the shaker overnight.

The Endo-free Plasmid DNA Mini Kit is used to isolate the recombinant plasmid DNA from E. coli and the extracted plasmid is stored at 4 °C for further usage.

8) Transfecting Fusion Gene into HEK293T cells

The HEK293T cells are seeded into a 24-well plate at a ratio of 1:8 one day in advance. After 12 hours, when the cell growth confluence reaches about 60%, the transfection should be done.

A sample of 1 µl of each target plasmid and 50 µl opti-MEN serum-free culture medium are added into a 1.5 µl EP tube, mixing thoroughly. Then 2 µl PE2 culture medium is added into the EP tube, mixing thoroughly, and wait for 20 minutes. After the 20 minutes, the cell from the incubator is taken out and the culture medium is removed from each well, and then the plasmid mixture is added into the wells very carefully, moving the culture dish back and forth and mixing thoroughly. The culture dish is placed back into the incubator at 37 ℃ for 24 hours for further usage.

III. Results and Discussion

A. Acquisition of Fusion Fragment of 2A Peptide(P2A) and K5

The amplified fusion fragment 2A-K5 is produced by primer 2AK5-F-60, K5-R-S, and template K5. The designed length of 2A-K5 is 381 bp. Based on the result of agarose gel electrophoresis (Fig. 1), the length of 2A-K5 is about 400 bp, and the stripe is clear with no other complex zone, which means the amplification of 2A-K5 is successful.

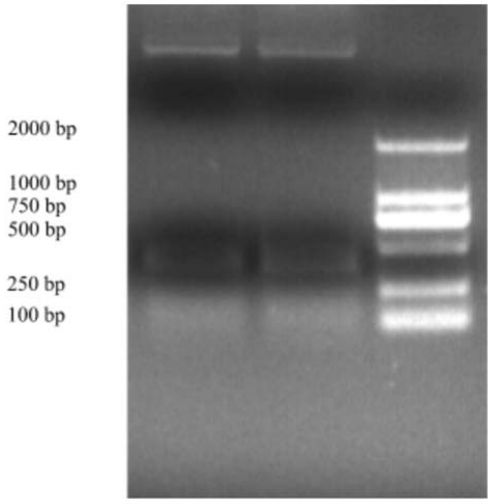

Fig. 1 PCR identification of 2A-K5

B. Acquisition of Fusion Fragment of en2a-R-57 and 2A-K5

The amplified fusion fragment endo2a -K5 is produced by primer endo2a-F-56, K5-R-S1 and template 2A-K5. The designed length of endo2a-K5 is 407 bp. Based on the result of agarose gel electrophoresis (Fig. 2), the length of endo2a-K5 is about 400 bp, and the stripe is clear with no other complex zone, which means the amplification of endo2a-K5 is successful.

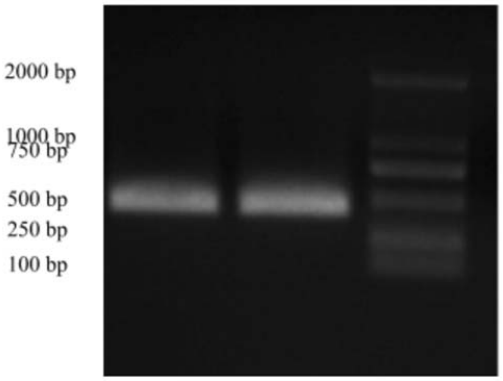

Fig. 2 PCR identification of endo2a-K5

C. Acquisition of E10A

The amplified E10A is by primer endo-62-e1and endo2a-R-57 and template E10A. The designed length of amplified E10A is 573 bp. Based on the result of agarose gel electrophoresis (Fig. 3), the length of amplified E10A is about 550 bp, and the stripe is clear with no other complex zone, which means the amplification of E10A is successful.

D. Acquisition of Fusion Gene of E10A and 2A-K5

The amplified fusion gene E10A-2A-K5 is produced by primer endo2a-F-56, K5-R-S1 and template E10A, endo2a-K5. The designed length of E10A-2A-K5 is 939 bp. Based on the result of agarose gel electrophoresis (Fig. 4), the length of E10A-2A-K5 is about 900 bp, and the stripe is clear, which means the amplification of fusion gene E10A-2A-K5 is successful.

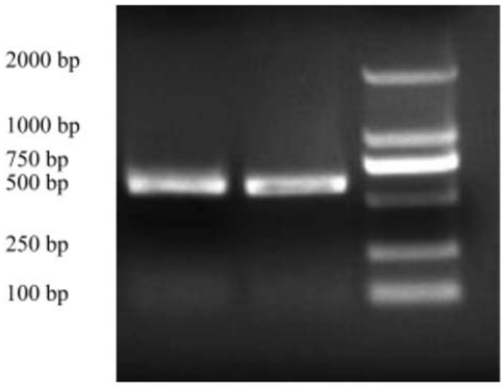

Fig. 3 PCR identification of E10A

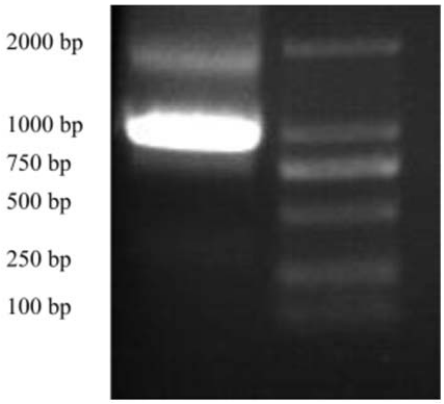

Fig. 4 PCR identification of E10A-2A-K5

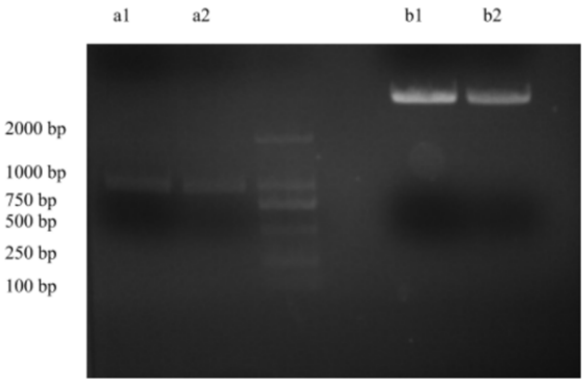

Fig. 5 PCR identification of enzyme digested E10A-2A-K5 and digested vector PDH316 (a1 and a2 are enzyme digested E10A-2A-K5, b1 and b2 are digested vector PDH316)

E. Acquisition of Enzyme Digested E10A-2A-K5 and PDC316

After the restriction enzyme digestion, the product E10A-2A-K5 and PDC316 are tested by agarose gel electrophoresis. Based on the result of agarose gel electrophoresis (Fig. 5), the length of enzyme digested E10A-2A-K5 is about 900 bp, the length of enzyme digested vector plasmid PDC316 is about 4000 bp and the all the corresponding stripes are clear, which means the restriction enzyme digestion is successful.

F. Acquisition of Positive Clones Containing Recombinant Plasmid

The colony PCR is utilized to amplify E10A-2A-K5 to detect the recombinant plasmid after the transformation. The designed length of E10A-2A-K5 is 939 bp. Based on the result of agarose gel electrophoresis (Fig. 6), the length of E10A-2A-K5 is about 950 bp, all the stripes are clear with no other complex zone except sample 3 having no stripe. Therefore, the amplified sample 1, 2, 4, 5, and 6 are sent to Shanghai Sangon Biotech for sequencing. Then the biological sequencing result of the six samples indicates that sample 6 has special structure so that it cannot be identified and used. As a result, there are four colonies that can be used for plasmid extraction to do the transfection into HEK293T cells.

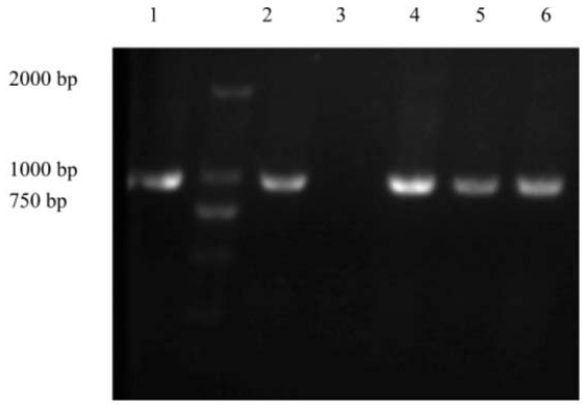

Fig. 6 Colony PCR identification

G. Sequencing and ELISA

HEK293T cells transfected by the recombinant plasmid are used to do RT-PCR and ELISA. RT-PCR and ELISA are used to detect the protein expression of E10A in E10A-2A-K5, the amplified products from RT-PCR are sent to Ruibiotech for sequencing. The sequencing result indicates that the protein expression of E10A-2A-K5 is successful. For ELISA, the result (Fig. 7) indicates the different concentration of protein is very close to the standard concentration line, which means the protein is E10A.

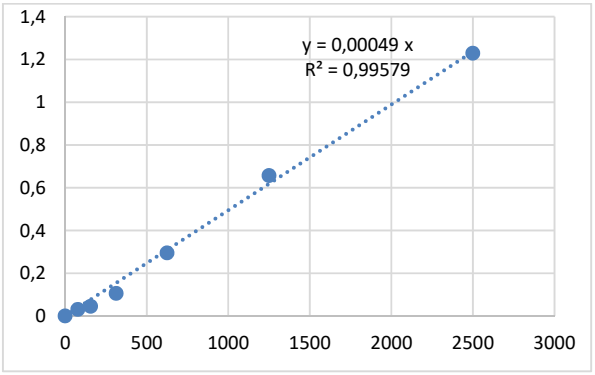

Fig. 7 Human endostatin concentration

IV. Conclusion

In this study, E10A, 2A peptide, and K5 genes were fused with specific primers using overlap extension PCR method, and the vectors were connected by homologous recombination method, the target plasmids are transfected into HEK293T cells.

The fusion gene E10A-2A-K5 was constructed successfully with sequencing results, and the protein expression through HEK293T cells was also successful.

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