The Construction of Recombinant D6 Clone for *in Vitro* Breast Cancer Study

Tan Wee Yee, Khoo Boon Yin, and Chew Ai Lan

***Abstract*—D6, which is also known as CCBP 2, is one of the decoy chemokine receptors. It was recently found to play a role in the progression of breast cancer cells. In this study, the existence of D6 in invasive breast cancer cells, MDA-MB-231 was investigated by One-step RT-PCR with additional Pfu DNA polymerase in the reaction. The amplicons were then sequenced and compared with the reference sequence from GenBank database. Nucleotide sequence analysis showed that the amplicon sequence matches the reference sequence. Thus, it is confirmed that full length D6 sequence had been amplified from MDA-MB-231.**

***Index Terms*—Cloning, D6, DNA sequencing, MDA-MB-231.**

I. Introduction

Breast cancer is the most common cancer in women in most parts of the world. Majority of the breast cancer patients were found to surrender to this disease due to cancer invasion and metastasis [1]. In Oncology study, breast cancer research had become one of the most progressively evolving fields [2]. To date, with the advanced understanding of key molecular features, breast cancer is no longer considered a single disease but a combination of different subtypes with different biological behaviors and clinical outcomes [3]. Novel molecules and new diagnostic methods are being discovered and developed constantly, globally. Recently, the identification of various signaling pathways implicated in the cellular process of breast cancer cells has drawn the attention of researchers worldwide. The involvement of growth factors or signaling molecules in breast cancer cell proliferation and invasion were reported worldwide [4]-[7]. For examples, chemokines and chemokine receptors were reported to be involved in cancer growth and metastasis [8]-[13].

D6 (or CCBP2) was originally known as a binding molecule of CCL3 and its' expression was found in murine hemopoietic stem cells [14]. However the expression of D6 in human cells was found to be atypical, in which it will not lead to the activation of signaling pathway upon binding with the respective target proteins [1]-[16]. The findings engrossed the attention of oncologist and researchers to investigate the roles of D6 in cancer networks. Recent findings showed that D6 is plentiful on different types of human cells such as leukocytes, lymphatic endothelial cells of the gut and lungs, malignant vascular tumors, to name a few [1]-[18]. Besides, the involvement of D6 in breast cancer has been described in recent years too [8]-[16].

In the present study, the presence of D6 in invasive breast cancer cells, MDA-MB-231 was investigated. The nucleotide sequence of D6 was amplified and recombinant clone of D6 was constructed.

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II. Methodology

A. Maintenance of MDA-MB-231 Cell Line

MDA-MB-231 (ATCC number: HTB-26), which is a highly invasive human breast cancer cell line, was routinely maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cells were grown and maintained in a humidified 5% carbon dioxide incubator. The cells were then used for total RNA extraction.

B. Total RNA Extraction

The 80% confluent cultured cells were trypsinized and the cell pellet was obtained by centrifugation using a table top centrifuge. Then, total RNA was purified from the cultured cells using commercially available RNA extraction kit (AxyPrep Multisource Total RNA Miniprep kit, Axygen). The purification of total RNA was carried out according to the user manual provided by the kit's manufacturer.

C. Generation of Full Length D6 Sequence

Total RNA purified from the previous step was used as a template in the One-step RT-PCR. To perform this, Maxime RT-PCR Premix kit (Intron) was used to convert total RNA to cDNA and then PCR, continuously in a single tube reaction. For proof-reading purpose, appropriate amount of Pfu DNA polymerase (BioAtlas) was manually added into the premix reaction mix prior to the experiment. Gene-specific primers as mentioned below (Table I) were used in the PCR reaction. Published sequence of D6 with GenBank accession number NM_001296.4 was used as reference sequence for the generation of gene specific primers. Different restriction sites were incorporated in the primer sequences, to allow the ligation of PCR product into desired expression vector. Forward primer contains *Eco*RI restriction site (bolded) whereas reverse primer contains restriction site for *Sac*II (bolded). One-step RT-PCR was started with 30 minutes hot start at 45℃ in a 20 μl reaction. Then, cDNA samples were denatured at 94℃ for 5 minutes. After that, the reaction was cycled 35 times at 94℃ for 30 seconds, annealing at 61℃ for 30 seconds and elongation at

72℃ for 2 minutes. Finally, the reaction was extended at 72℃ for 10 minutes towards the end of PCR. Negative control reaction consisted of everything as mentioned in the sample reaction except total RNA purified from the cell lines. The PCR products were electrophoresed on 1% agarose gel stained with ethidium bromide, and visualized under UV light. DNA Ladder, 100 bp Plus (Fermentas) was used as a marker.

TABLE I: THE GENE-SPECIFIC PRIMERS USED IN THIS STUDY

Primer	Sequence (5'-3')	Tm
Forward	GC**GAATTC**GCATTTCCTTCCAACATGGCC	63.04
Reverse	GC**CCGCGG**AGGCTGATTTATTCCCCACATCC	67.27

D. TA Cloning and DNA Sequencing

Gel purification kit (Fermentas) was used to purify PCR products from the agarose gel after 60 minutes of 100V gel electrophoresis. Ligation of gel purified sample into TA vector, pTZ57R/T (Fermentas), was performed at 20℃ using T4 Ligase (Fermentas). The ligation process was terminated by heat inactivating the samples at 65℃ for 10 minutes, after 2 hours of incubation. Upon ligation, recombinant vectors were transformed into electrocompetent bacterial cells, TOP 10 (Invitrogen). The electrocompetent cells with 2 µl of recombinant vectors were pulsed in an electroporation cuvette (0.2cm) using an electroporator (Bio-Rad). Cells were revived immediately by the addition of 1 ml LB broth after pulsing. Transformed cells were agitated for 30 minutes at 37℃, 750 rpm using a thermoblock shaker before plating on a low salt LB agar (with 25 µg/ml Zeocin, Invitrogen) and incubated at 37℃, overnight to select positive clones. Selected positive clones were propagated overnight in a Zeocin containing low salt LB broth. Plasmid extraction kit (Fermentas) was used to purify plasmid DNA from the clones. Then, *Eco*RI and *Sac*II (Fermentas) were used in the double digestion analysis. The presence of full length sequence of interest gene in the clones was confirmed on 1% agarose gel stained with ethidium bromide after double digestion process. Positive clones carrying the gene of interest were sent for sequencing. Primers used for the sequencing were universal primers, M13F_20 and M13R_pUC_26. Nucleotide sequence from GenBank with the accession number of NM_001296.4 was used as a reference and the sequencing result was analysed by ClustalW.

E. Cloning of D6 Nucleotide Sequence into Yeast Expression Vector

Positive TA clones carrying the gene of interest were restricted at *Eco*RI and *Sac*II sites based on the double digestion protocol from Fermentas. The digested recombinant TA vectors were gel purified prior to further experiments. Yeast expression vector, pPICZ alpha A (Invitrogen) was used as eukaryote expression vector in this study. The agarose gel purified gene of interest was ligated into pPICZ alpha A by T4 Ligase (Fermentas) based on the ligation protocol as described above. The recombinant yeast expression vectors were then electro-transformed into electrocompetent TOP 10 for propagation purpose. The transformed TOP 10 were screened again by plate selection method, and positive clones were selected for plasmid purification. The positive recombinant plasmid DNA was sent for sequencing and analysed again by ClustalW. Sequencing of recombinant yeast expression vector was performed using forward AOX1 primer and reverse AOX1 primer.

III. RESULTS AND DISCUSSION

The presence of D6 in invasive breast cancer cells was an attractive topic since atypical chemokine receptors had started to gain attention and interest in recent breast cancer studies [16]-[20]. Hence, MDA-MB-231, which is an invasive breast cancer cell line, was chosen in this study, and the existence of D6 in the cell line was investigated.

A. Total RNA Extraction

The total RNA was extracted using Axygen kit and separated by agarose gel electrophoresis. Fig. 1 showed the total RNA extracted from MDA-MB-231 which consisted of 28s rRNA, 18s rRNA, 5s rRNA and tRNA.

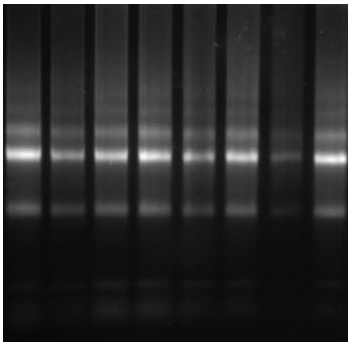

Fig. 1. The gel picture of total RNA extracted from MDA-MB-231. The distinct bands of 28s rRNA, 18s rRNA, 5s rRNA and tRNA in the total RNA were clearly shown.

B. Generation of Full Length D6 Sequence

Total RNA from MDA-MBA-231 was reverse-transcribed and full length D6 sequence was amplified in a single tube reaction using gene-specific primer pairs. Forward primer was built to hook the nucleotides from 165 bp to 185 bp on the reference sequence (NM_001296.4), whereas reverse primer was built to flank the nucleotides from 1310 bp until 1331 bp on the same sequence. Reverse primer was designed to allow in frame cloning of interest gene (insert) without stop codon, with myc and His tag at the C terminal to be cloned in the yeast expression vector. Based on the position of gene-specific primer pairs on reference sequence from GenBank, the amplified PCR product of One-step RT-PCR was expected to be in the range of 1185-1300 bp with the additional of restriction enzymes (*Eco*RI and *Sac*II) recognition sites and multiple A nucleotides at the C terminal. Fig. 2 showed the amplified full length D6 sequence on ethidium bromide stained 1% agarose gel. The amplicon band was observed to be approximately 1200 bp based on 100 bp Plus DNA Ladder (Fermentas). The location of the bands on gel picture was correspondent to the estimated size of full length D6 sequence, indicating existence of D6 in MDA-MB-231.

C. TA Cloning and DNA Sequencing

Upon TA cloning, double digestion was performed to

confirm the presence of interest gene in the TA vector. Full length D6 sequence with the size approximately 1200 bp was shown on 1% agarose gel after the overnight incubation of TA clones with specific restriction enzymes (Fig. 3). The band appeared within the range of 2000-3000 bp was correspondent to the size of empty TA vector, which is 2886 bp (Fermentas); while another band appeared above 3000 bp was expected to be undigested recombinant TA vector, which harbours the full length sequence of D6. The result showed the success of TA cloning and the positive clone was then sent for DNA sequencing.

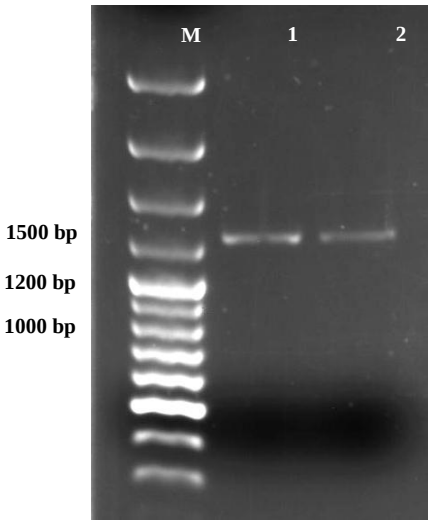

Fig. 2. The amplicons at the size of approximately 1200 bp, were shown on ethidium bromide stained 1% agarose gel. M lane refers to 100 bp Plus DNA ladder (Fermentas). Lanes 1 and 2 refer to One-Step RT-PCR products, with total RNA extract from MDA-MB-231.

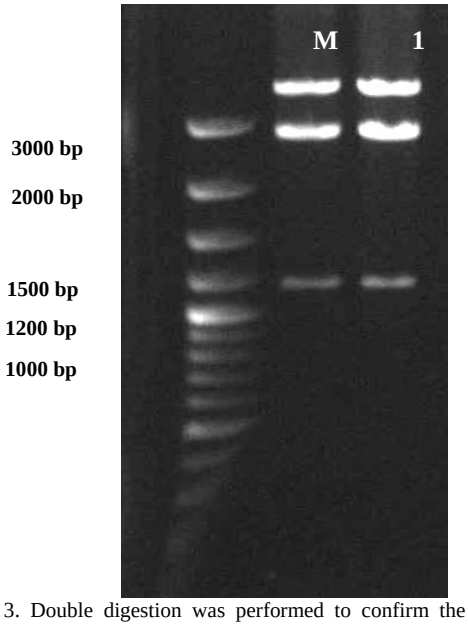

Fig. 3. Double digestion was performed to confirm the ligation of full length D6 sequence in TA vector. M refers to 100 bp Plus DNA Ladder (Fermentas), whereas Lanes 1 and 2 refer to recombinant TA vectors which were double digested with *Eco*RI and *Sac*II.

Sanger sequencing was known to be accurate in DNA sequencing compared to other method such as Maxam Gilbert method [21]. It is a DNA sequencing method with the use of chain terminating inhibitors [22]. However, to sequence the full length of sample DNA, two reactions were performed under Sanger sequencing method. Forward reaction with forward primer M13F_20 and reverse reaction with reverse primer M13R_pUC_26 were carried out. ClustalW analysis was performed by aligning sample sequence with the reference sequence, D6 (NM_001296.4) from GenBank. The analysis showed no mismatch, deletion or insertion in amplified sequence compared to the reference sequence (Result not shown). DNA sequence of amplicon matched reference sequence from GenBank database. Hence, the finding deduced that a complete full length D6 sequence of MDA-MB-231 had been amplified using One-step RT-PCR.

D. Cloning of D6 Nucleotide Sequence into Yeast Expression Vector

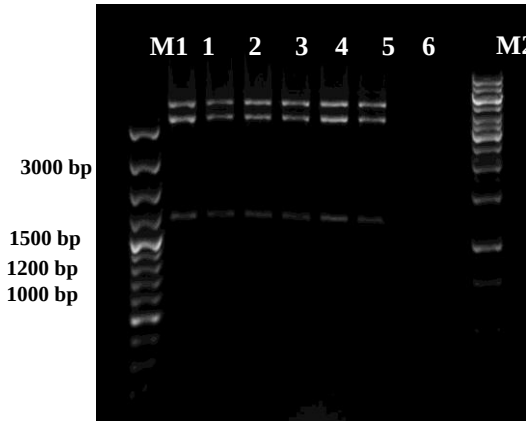

Fig. 4. The double digestion of recombinant pPICZ alpha A vector. M1 refers to 100 bp Plus DNA Ladder whereas M2 refers to 1kb DNA Ladder (Fermentas). Lanes 1 to lane 6 refer to the digestion of recombinant pPICZ alpha A vector.

Yeast expression vector (Invitrogen), pPICZ alpha A is 3593 bp in length. Agarose gel picture showed that the full length sequence of D6 was cloned into the expression vector (Fig. 4). Gel picture showed double digestion yielded three distinct bands. The band of 1200 bp was the gene of interest, whereas another two bands appeared above 3000 bp were digested and undigested pPICZ alpha A, respectively.

wo reactions of Sanger DNA sequencing were performed again to read the full length sequence of the insert; one with forward AOX1 primer while another with reverse AOX1 primer. ClustalW analysis showed a complete matching of insert sequence from the recombinant yeast expression vector to the reference sequence from GenBank database. Besides, the sequencing reactions with forward AOX1 and reverse AOX1 primers also showed that the gene of interest was embedded in-frame and downstream of alpha factor signal sequence and in-frame with *c-myc* and polyhistidine tag in yeast expression vector. Hence, the gene can be expressed as a recombinant fusion protein.

IV. Conclusion

Current study showed that One-step RT-PCR with the aid of Pfu DNA polymerase amplified potential D6 sequence from total RNA of MDA-MB-231. The DNA sequencing with the aid of two different sequencing primers further proved the presence and also confirmed the nucleotide sequence of full length D6 in MDA-MB-231 cell lines. The generated recombinant D6 clones will be used in-house for further experiments in breast cancer studies. The research

outcome will serve as a catalyst to channel rapid and innovative advances of our research findings to the biotechnology industry.

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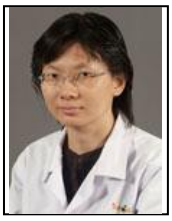

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Chew Ai Lan obtained her BSc Hons majoring in microbiology from Universiti Sains Malaysia (USM) in 1997 and PhD in Biotechnology (USM) in 2005.

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