Cloning Of Human CD137 Receptor Gene Using Recombinant DNA Technology

Janjuree Netsawang*, Nichaboon Sawakphon, Rattiya Chuthong, Thirapat Pancharoen, Patcharin Soontorn, Ruchirapron Sirirungthip and Atippong Boonyakit

Faculty of Medical Technology, Rangsit University, Pathum Thani, Thailand
*Corresponding author, E-mail: janjuree.n@rsu.ac.th

Abstract

CD137 receptor, belonging to Tumor necrosis factor receptor superfamily 9, classifies as a CD marker resting on the surface of the cell. Its function involves both nonimmune and immune cells, such as cellular apoptosis, survival, differentiation, proliferation, and also activation. We postulated that the interaction between the CD137 receptor and CD137 ligand leads to cellular apoptosis in particular cells. Most of the severe patients with Dengue virus infection demonstrated the apoptotic body containing hepatocytes. In our earlier reports, we revealed that infection of Dengue virus in hepatocyte cell line stimulated the expression of CD137 receptor in both mRNA and surface protein expression levels, which related to enhanced apoptosis in Dengue-infected hepatocytes. This mechanism might be the pathogenesis of apoptosis in the hepatocytes of the patient with severe Dengue infection and still has not been elucidated. Nevertheless, we noticed the pivotal role of the mechanisms of the CD137 receptor that leads to apoptosis in Dengue-infected hepatocytes. The purpose of this research is to produce the expression recombinant plasmid containing CD137 receptor gene by using Recombinant DNA technology to test its function upon Dengue-infected hepatocytes later. Recombinant DNA technology is usually employed with the advantage of easy handling and low cost. Amplification of insert CD137 receptor gene was done by using Nested PCR. Plasmid and insert DNA were double-digested with restriction endonuclease enzymes and subjected to ligate. Then, transformation into competent bacteria was performed and selected the white colonies on Ampicillin-containing media. Screening methods and direct sequencing were utilized to verify the correct base pairing sequence. Our result demonstrated the accomplishment in the construction of recombinant plasmid comprising CD137 receptor with completely matched to its template in GenBank and it will be employed in further study in surface CD137 receptor expression in protein level and functional level during DENV-infected hepatocytic cell line.

Keywords**:** *CD137 receptor, Apoptosis, Dengue virus, Recombinant DNA technology*

1. Introduction

CD137 receptor, also named ILA, CDw137, MGC2172, and FLJ43501, is classified as a CD marker and belongs to Tumor necrosis factor receptor superfamily 9 (TNFRSF 9). It is generally expressed in immune cells, activated T lymphocytes, and some monocytes and inducible primary cells (Schwarz, Valbracht, Tuckwell, von Kempis, & Lotz, 1995). This gene resides on chromosome 1p36 and this region was associated with mutations in several malignancies (Schwarz, Arden, & Lotz, 1997), however, its soluble form was found to increase in Rheumatoid arthritis disease (Michel, Langstein, Hofstadter, & Schwarz, 1998). Additionally, CD137 receptor function involves both nonimmune and immune cells, such as cellular apoptosis, survival, differentiation, proliferation, and also activation (Shao & Schwarz, 2011).

Dengue virus (DENV) encloses a positive-stranded RNA genome and comprises 4 serotypes (DENV-1, -2, -3, -4) along with the family of Flavivirus. It can be transmitted through the biting of mosquitoes, namely *Aedes aegypti*, which are predominantly discovered in tropical and subtropical areas (Gubler, 1998). The wide ranges of disease severity initiate from Dengue fever (DF), Dengue hemorrhagic fever (DHF), and Dengue shock syndrome (DSS). Most of the patients develop an asymptomatic infection, whereas others generate a severe form of the disease, leading to shock and death (Gibbons & Vaughn, 2002).

Our earlier reports established that CD137 receptor signaling plays an important role during DENV infection. We originally revealed the augmentation of mRNA and surface proteins expression during the infection of DENV in hepatocyte cells line along with the rising in the synthesizing of cytokine and cellular

apoptosis (Nagila et al., 2011; Nagila et al., 2013). Our results indicated that the inducible surface CD137 receptor during infection of DENV activates the cellular apoptosis in the hepatocyte cell line. Later on, the full CD137 promoter-comprised plasmids, which contained particular areas such as binding sites of NF-*k*B, AP-1, and deletion, were generated to test which areas are significant in controlling the expression of CD137 receptor. Lastly, we discovered that the binding region of NF-*k*B is involved in the regulation of cellular apoptosis during the infection of DENV in the hepatocytic cell line (Netsawang et al., 2014).

Nevertheless, the particular role of the CD137 receptor upon DENV infected-hepatocyte cells line in the context of apoptosis induction and intracellular mechanism has not been elucidated. Consequently, this research was conducted to produce the CD137 receptor gene for future study in the context of surface protein expression of the CD137 receptor on the hepatocyte cell line. Moreover, the interplay between CD137 receptors and its ligands might provide the signaling, which involves hepatocellular apoptosis during the infection of DENV, contributing to the severity of the disease. Overall, our novel data will assist to improve the knowledge, involved in the etiology of hepatocellular apoptosis during the infection of DENV and will be employed in drug targeting CD137 receptor to obstruct its interaction, leading to reducing in the severity of disease during the infection of DENV.

2. Objectives

To produce the expression recombinant plasmid containing CD137 receptor gene in order to test its function upon Dengue-infected hepatocytes later.

3. Materials and Methods

3.1 The plasmid, namely pCDNA3.1/His C, was obtained from Invitrogen, Carlsbad, CA, USA. It is a eukaryotic expression vector for the mammalian cell line. G418-resistance gene, which contains in the structure, is employed for the selection of stable cells. The extraction of this plasmid was done by a QIAGEN plasmid extraction kit, following the manufacturers' recommendation (QIAGEN, Hilden, Germany).

3.2 RNA was extracted from a human monocyte cell line, namely THP-1, and then synthesized into cDNA as a template with first-strand cDNA synthesis following the manufacturers' recommendation (Invitrogen, Carlsbad, CA, USA). Insert DNA was amplified by Nested PCR with newly designed primers, including outer primers, namely Out CD137 RF (5′-TGAAGACCAAGGAGTGGAAAG-3′) and Out CD137 RR (5 ′-TATGTAGGATGGTGTTCTTGC-3′), and inner primers, namely CD137 RF (5′-GGTACCATGGGAAACAGCTG-3′) and CD137 RR (5′- GAATTCTCACAGTTCACATCC-3′). The Gene Amp PCR System 9700 (Applied Biosystems, Waltham, MA, USA) was utilized for the reaction of PCR. The first round of PCR reaction was started with a hot start at 95°C for 2 minutes, followed by 30 cycles of denaturation (95°C for 30 seconds), annealing (55°C for 1 minute), and extension (72°C for 2 minutes), and final extension at 72°C for 7 minutes. The second round of PCR reaction was begun with a hot start at 95°C for 2 minutes, followed by 30 cycles of denaturation (95°C for 30 seconds), annealing (55°C for 1 minute), and extension (72°C for 3 minutes), and final extension at 72°C for 7 minutes.

3.3 Insert DNA containing CD137 receptor and pCDNA3.1/His C were double-digested with restriction enzymes, *Eco*RI, and KpnI at 37°C for overnight. Then, ligated with the T4 DNA ligase enzyme (New England Biolabs, Ipswich, MA, USA).

3.4 A competent *E. coli* strain DH5α was employed for the transformation of plasmid comprising the CD137 by using the heat shock method. Then, the selection of these recombinant plasmids was done by using the growth of colonies on an LB plate containing 100 µg/mL of ampicillin.

3.5 The putative positive clones were firstly screened by using the Colony-PCR matrix technique, in which colonies were polled following the matrix tables, PCR reactions were done with inner primers and PCR conditions, as described above. The intersect colony, which conferred the high probability for

recombinant plasmid containing the gene of interest, was selected in the matrix table. Subsequently, these clones were subjected to extraction of plasmids from competent bacteria. Finally, the restriction enzymes, *Eco*RI and *Kpn*I, were used for digestion at 37°C overnight.

3.6 Direct DNA sequencing method was utilized to confirm the correct base-pairing along with the CD137 receptor gene. The CD137 receptor gene-containing plasmids were extracted by QIAGEN plasmid extraction kit, as mentioned above, and then, performed PCR by using universal primers including T7 promoter (5′-TAATACGACTCACTATAGGG-3′) and BGH-R (5′- TAGAAGGCACAGTCGAGG -3′). Finally, these sequencing data were analyzed by using the Pairwise alignment program and aligned with a database from GenBank with accession number, NM_001561.6. The completely matched CD137 receptor containing plasmids were selected to further test for protein expression and surface localization in the hepatocytic cells line.

4. Results and Discussion

4.1 Generation of the insert CD137 gene by using Nested PCR

The 780 bp PCR product was performed by using Nested PCR similar to the size of the CD137 receptor gene (figure 1, lane 2-3).

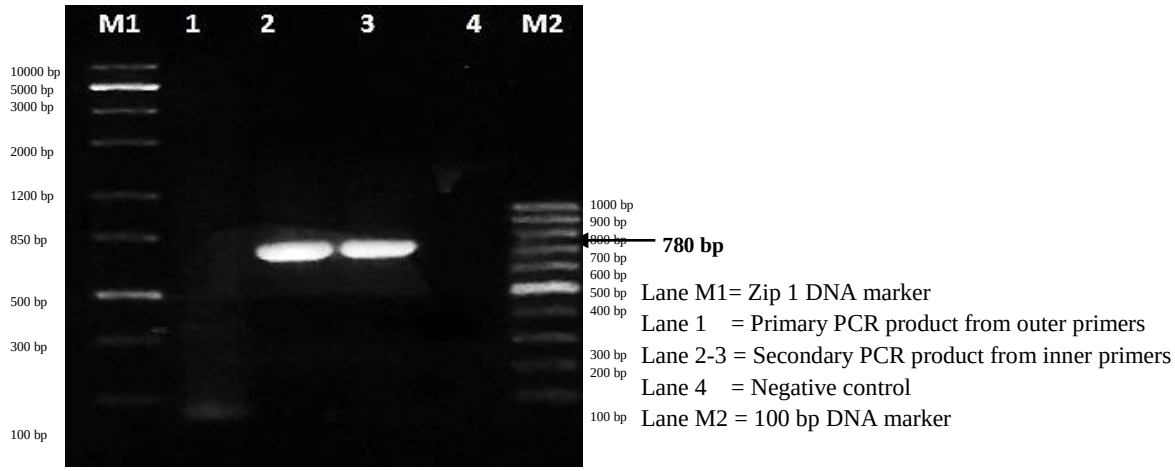

Figure 1 The production of insert CD137 receptor gene by Nested-PCR

4.2 Digestion of plasmid and insert CD137 receptor gene by using restriction enzymes

The 5.5 Kbp and 780 bp represented the result of pCDNA 3.1/His C and insert CD137 receptor, which double-digested with *Eco*RI and *Kpn*I (figure 2, lane 2 and 3), respectively. Linearized plasmid and insert DNA were ligated and then, a competent *E. coli* strain DH5α was subjected to transformation. The LB plate containing Ampicillin was used to selection of recombinant plasmids.

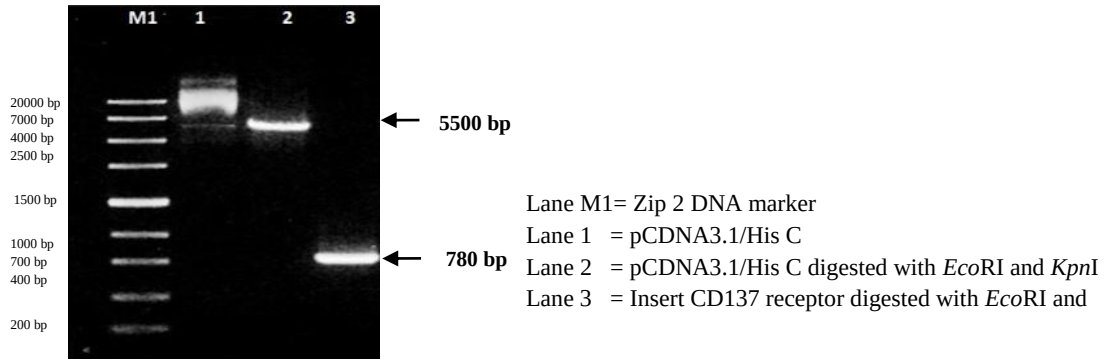

Figure 2 The digestion of insert CD137 receptor and plasmid DNA

4.3 Screening methods for positive clones

The first screening technique, which aimed to decrease the number of clones and improve the chance to receive the putative positive clones, was subjected to the Colony-PCR Matrix technique with the inner primers set of CD137 receptor. Pooled colonies were a mixture of colonies in a single tube along with a matrix table. Most lanes of pooled colonies of bacteria demonstrated the PCR products with 780 bp (figure 3, lane 2, 3, 5, 6, 9-12), as well as a positive control (figure 3, lane 1), whereas negative control did not show any precise band (figure 3, lane 13). These data indicated that the accurate size of the CD137 receptor was mostly found in our pool colonies of bacteria. However, the precise position of the insert CD137 receptor and pCDNA3.1/His C plasmid was not confirmed. In order to increase the specificity, restriction endonuclease enzymes were employed. Therefore, similar enzymes, namely *Eco*RI and *Kpn*I, were subjected to use in this study. Here, the band of DNA is composed of 5500 and 780 bp, as same as the correct size of pCDNA3.1/His C plasmid and CD137 receptor, respectively (figure 4, lane 2, 6, 7, 9, 10). Overall results indicated that the CD137 receptor was inserted into these putative positive clones.

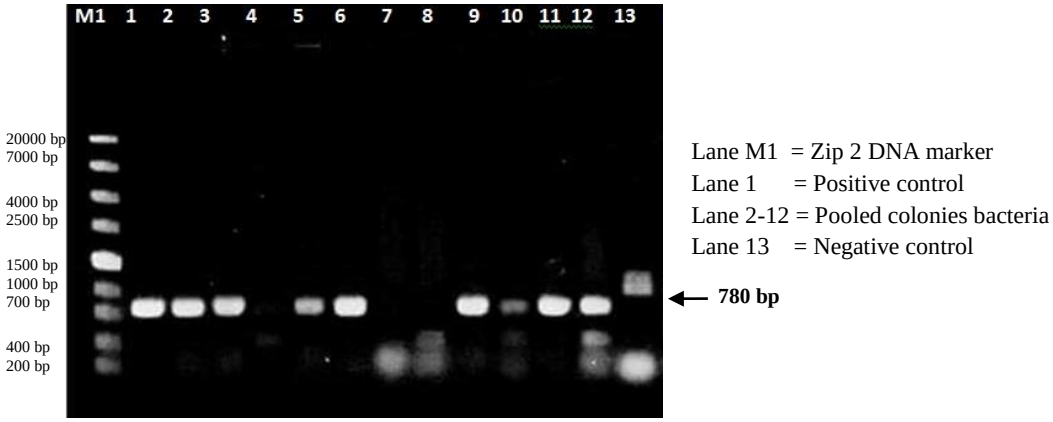

Figure 3 The production of insert CD137 receptor from pooled colonies by using Colony-PCR Matrix technique with inner primers

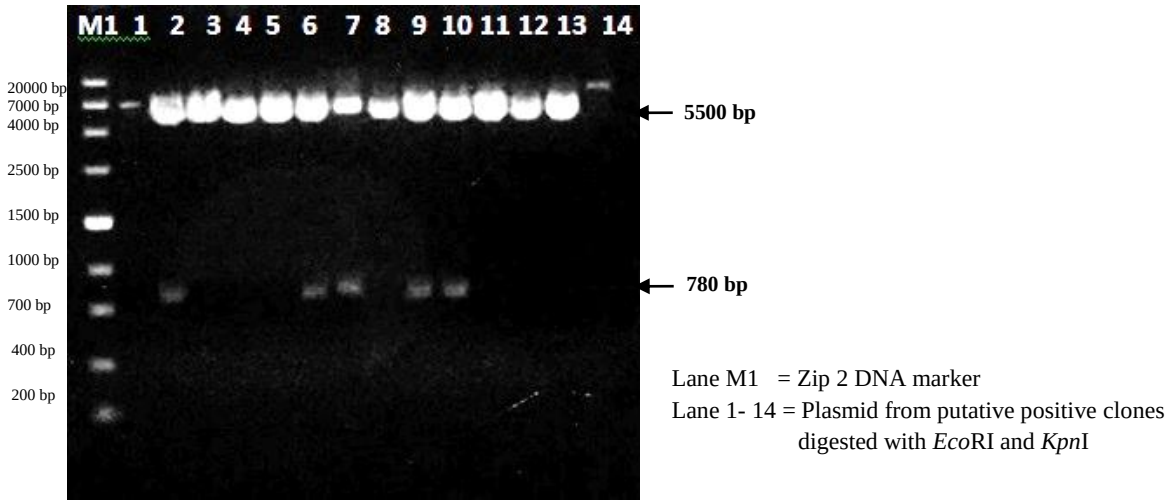

Figure 4 The restriction enzymes digestion of plasmid from putative positive clones

4.4 Confirmation of recombinant plasmid containing CD137 receptor with direct sequencing

Generally, colony-PCR ensures whether the insert DNA contain in the plasmid while digestion with restriction enzymes determines whether the insert DNA locates at the correct position and has no mutation along with the restriction site along with the plasmid. In order to examine the expression in protein level and the function, the correct base pairs along with insert DNA are crucial. Thus, the mutation in the CD137 receptor gene should be validated. In order to prove any mutations of these positive clones, direct sequencing was conducted in this study. The sequencing results were analyzed by using Pairwise-sequence alignment with the GenBank database with accession number, NM_001561.6. Lastly, we showed the completely matched recombinant clone, when compared with the CD137 receptor template (figure 5), in most of the positive clones.

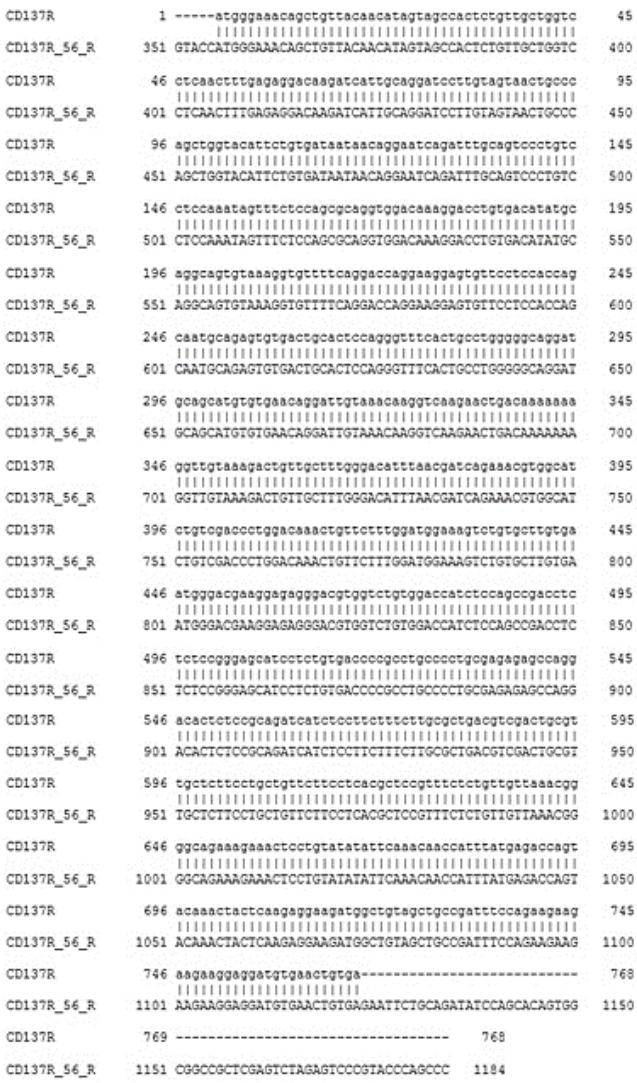

Figure 5 The comparison between positive clone and CD137 template by using Pair-wise alignment

Generally, our results established that recombinant DNA technology produced a high amount of positive clones of the plasmid containing the CD137 receptor. This technique is widely employed with the advantage of being easily to handle and low cost. The specificity of this technique dues to the utilization of restriction enzymes to digest both plasmid and insert a gene. However, the unsuccessful cloning might occur from the loss of restriction enzymes activities, such as incompletely cutting, leading to a lot of bacteria containing a plasmid with no insert gene, which can grow in ampicillin composing media, and the wrong direction of insert gene when ligated to the linearized plasmid, affecting the expression of protein later. Direct sequencing is the best way to approve the real and correct recombinant plasmid but it is very expensive. Hence, we generate the screening techniques to select the putative positive clones, which confer the high opportunity rates of an insert containing the plasmid. The colony-PCR matrix was created to screen a lot of pool colonies of bacteria by using the specific primer against the inserted gene. However, a false positive can be found from this technique. The more specificity technique was conducted by using the digestion of restriction enzymes, which are used in cloning.

Here, we synthesized the recombinant plasmid containing the human CD137 receptor along with the histidine epitope-tagged vector, which helps to track the interested protein later, in comparison with other, commercially available recombinant plasmids. Previous reports demonstrated the cloning of rat, swine, monkey, and human CD137 receptors and examined the functional studies (Alderson et al., 1994; Chen et al., 2008; Dong et al., 2005; Zhao et al., 2013). Our previous reports demonstrated the upregulation of CD137 during the infection of DENV in hepatocyte cells line along with the rising in the synthesizing of cytokine and cellular apoptosis (Nagila et al., 2011; Nagila et al., 2013). Monocytes are well known for CD137 receptor signaling, which induces the production of proinflammatory cytokine and apoptosis. Bidirectional signaling regulates the induction of apoptosis between immune and nonimmune cells, especially hepatocytes. However, little is known about the apoptosis-mediated by CD137 receptor signaling in monocytes and CD137 receptor /CD137 ligand interaction between hepatocytes and monocytes during DENV infection.

5. Conclusion

Recombinant DNA technology is usefully employed to construct the recombinant plasmid, which further tests more in functional relevance of protein with the advantage of easily in handling and low cost. Nested-PCR was applied in this study for the generation of the inserted gene of interest, followed by the digestion with the restriction enzymes, together with plasmid. Linearized plasmids were ligated to digested insert DNA. Subsequently, competent bacteria were subjected to the transformation process and the white colonies, which grew on ampicillin-containing media, were selected for further screening. Colony-PCR matrix and restriction enzyme digestion were utilized in this study to increase more opportunities for putative positive clones. Lastly, direct sequencing was used to confirm the correct sequence and site of insert DNA.

We accomplished to generate the CD137 receptor gene-containing plasmid with 100% homology with the CD137 receptor template in GenBank and obtained a high yield of positive clones by using recombinant DNA technology. The future study will examine the surface protein expression of the CD137 receptor on the hepatocyte cell line, especially the apoptosis event dunging DENV infection.

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7. References

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