Generating a Point Mutation in NF-κB Binding Site of the CD137 Promotor Region using Site-directed Mutagenesis

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Abstract

CD137 is a member of Tumor necrosis factor receptor superfamily 9 (TNFRSF 9). The signaling between CD137 and CD137 ligand will induce apoptotic cell death. Our previous studies discovered that dengue viral infection resulted in the triggering of CD137 gene both upregulation of mRNA and surface protein levels in consistent with the increasing of apoptotic liver cell death. These mechanisms might contribute to the pathogenesis of apoptotic hepatocyte cell death in severe dengue patients. Consequently, our research team realizes the important role of mechanism in triggering the CD137 promoter, which leads to the augmentation of apoptotic cell death in the dengue-infected hepatocytic cell line. The aim of this study is to construct the point mutation of CD137 promoter by using Site-directed mutagenesis technique, which conducted for studying the important position on CD137 promoter triggered the expression of CD137 gene. Screening and DNA sequencing methods demonstrated that alteration from G to C in NF-κB binding site of CD137 promoter region has been successfully constructed and it can be used for further study.

Keywords: *CD137 Promotor, Apoptosis, Site-directed mutagenesis technique, point mutation, Tumor necrosis factor receptor super family 9, Dengue virus*

1. Introduction

Dengue virus (DENV), a positive-strand RNA virus of the *Flavivirus* family with 4 different serotypes (DENV-1, -2, -3, and -4), is transmitted to human by mosquito *Aedes aegypti* (Gubler, 1998). The effect of DENV infection happens worldwide, predominantly in subtropical and tropical regions. All four serotypes of DENV cause human disease with varying degrees of severity. Some patients have asymptomatic infection, while most infected patients develop dengue fever (DF). Unfortunately, some patients reach to severe forms, which are classified into dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Gibbons & Vaughn, 2002).

CD137 is a member of Tumor necrosis factor receptor superfamily 9 (TNFRSF 9) and influences not only the activation state of T cells but also activation, proliferation, survival, apoptosis, and differentiation of several immune and nonimmune cells and the course of immune responses (Alderson, 1994; Shao & Schwarz, 2011).

Site-directed mutagenesis technique employs to study structure and function of interested protein when point mutation was created. Basically, this technique can be used when the wild-type interested gene was constructed in plasmid and one-paired primers were desired to mismatch at 1 position. PCR amplification was generated the two-stranded new mutant plasmid with the two-stranded old wild-type plasmid. In order to get only the new mutant plasmid, *Dnp*I restriction enzymes were utilized to eliminate the old wild-type plasmid containing the methylation (Carter, 1986).

Our previous studies demonstrated the crucial role of CD137 signaling upon DENV infection. Since 2011, we firstly reported the upregulation of CD137 expression both mRNA and surface proteins upon DENV infected-hepatocyte cells line as well as an increase of apoptosis and cytokine production

(Nagila et al., 2011; Nagila et al., 2013). These data implied that CD137 receptor can be inducible upon DENV infection to trigger apoptosis and demonstrated the significant role of CD137 expression in the induction of apoptosis upon DENV infection. Basically, the expression of a certain protein is controlled by its promoter. Therefore, in 2014, we constructed the plasmid containing full CD137 promoter, consisted of several important regions, for instance, NF-*k*B binding site and AP-1 binding site and deletion constructs, in order to examine which regions are important for regulation of CD137 expression. We finally found that NF-*k*B binding site plays an important role in DENV infection to activate apoptosis in hepatocytic cell lines (Netsawang et al., 2014).

However, the precise position of NF-*k*B binding site, along with CD137 promoter, played the crucial role in control the expression of CD137 gene, has not been yet elucidated. Consequently, we employed site-directed mutagenesis technique to initiate only one-point mutation of NF-*k*B binding site in a plasmid containing wild-type CD137 promoter. This selected point mutation, changing from G to C at position -919 of 5′- upstream region of CD137 gene, involved in signal-induced transcriptional activation and enhancer elements necessary for activation responsiveness (Kim, Kim, Baek, & Kang, 2003).

The significance of this research is to generate the point-mutation of the CD137 promoter region in order to further investigate for triggering the expression of CD137 gene and the role of CD137 signaling in DENV –mediated apoptosis in the hepatocytic cell line, thereby leading to disease severity. This knowledge will lead us to better understanding of the pathogenesis of DENV infected hepatic cells and be applied in the utilization of specific drug to block those interactions to minimize the disease severity upon DENV infection.

2. Objectives

To generate a point mutation in NF-*k*B binding site of the CD137 promoter containing plasmid.

3. Materials and Methods

3.1 Plasmid, namely pGL3-basic vector (Promega), containing wild-type CD137 promoter, kindly provided by our previous study (Netsawang et al., 2014), was extracted by QIAGEN plasmid extraction kit, following the manufacturers' recommendation (QIAGEN, Germany).

3.2 The PCR amplification of mutant CD137 promoter, which changed from G to C at position -919 of 5' upstream region of CD137 gene, was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) by using previously described primers (Kim, Kim, Baek, & Kang, 2003), including Mut CD137 PF (5′-TGTGGTGCGAATTTCCCATGAG-3′) and Mut CD137 PR (5′-CTCATGGGAAATTCGCACCACA - 3′), which started with denaturation at 95°C for 30 seconds, followed by 18 cycles of denaturation (95°C for 30 seconds), annealing (55°C for 1 minute) and extension (68°C for 14 minutes), and final extension at 72°C for 10 minutes.

3.3 The elimination of the plasmid containing the wild-type CD137 promoter was performed by using *Dpn*I restriction enzymes with digestion at 37°C for overnight.

3.4 The transformation of the plasmid containing the mutant CD137 promoter into a competent *E. coli* strain DH5α was accomplished by the heat shock method and these mutant plasmids were grown on LB plate containing 100 μg/mL ampicillin.

3.5 The screening method of plasmid containing mutant CD137 promoter was firstly performed by using Colony-PCR matrix technique with primers including Full CD137 PF (5′-CTAGGTACCAATCCCTCCTAGCTCTCAG-3′) and Full CD137 PR

(5′-GTTAAGCTTAGATCTCAGGGCTGCCGG- 3′), which started with denaturation at 94°C for 2 minutes, followed by 25 cycles of denaturation (94°C for 30 seconds), annealing (59°C for 1 minute) and extension (72°C for 40 seconds), and final extension at 72°C for 7 minutes and another one amplified with primers and condition as previously described in method 3.2. The putative positive clones were selected and further screened by using PCR single colony technique with primers and condition as previously described in method 3.2. Lastly, the remaining clones were extracted from competent bacteria and digested by using *Kpn*I and *Hind*III restriction enzymes.

3.6 The positive clones were extracted and confirmed by using direct sequencing method with primers including RVprimer3 (5′-CTAGCAAAATAGGCTGTCCC-3′) and G1 primer2 (5′-CTTTATGTTTTTGGCGTCTTCCA-3′), then aligned with a database from GenBank.

4. Results and Discussion

4.1 Amplification of the plasmid containing the point-mutation of CD137 promoter by using site-directed mutagenesis technique

The 5.6 kb PCR product was performed from pGL3 containing the wild-type CD137 promoter as DNA template (figure 1, lane 1). However, the remaining template was eliminated to minimize false positive by using a restriction enzyme, *Dpn*I (figure 1, lane 2). Transformation of these PCR products was conducted into a competent *E. coli* strain DH5α and then selected by using LB plate containing Ampicillin. We found the several white-rounded colonies grown in selected media (data not shown).

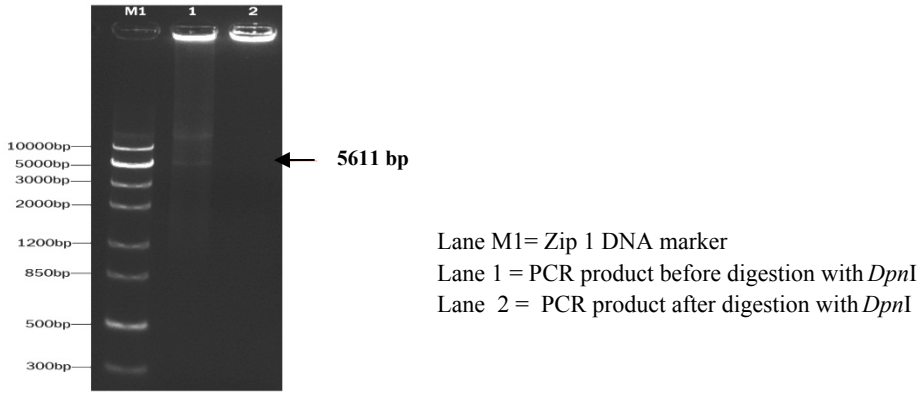

Figure 1 The PCR product of mutant CD137 promoter by using site-directed mutagenesis technique

4.2 Screening of putative positive clones

First, Colony-PCR Matrix technique was employed by using the primers set of full CD137 promoter in order to reduce the number of clones and increased the opportunity to get the positive clones from pooled colonies. The 793 bp PCR products were shown all lanes of pooled colonies of bacteria, in consistent with a positive control (figure 2, lane 1) and no band in two negative controls (figure 2, lane 14-15). The same sets of pooled colonies bacteria were amplified with mutant primers set. As expected, we also found the 132 bp PCR product (figure 3, lane 1-12) as well as amplified with full CD137 primers. These results might be implied that most of colonies bacteria contained the correct size of CD137 promoter with the mutant position. Second, we randomly selected the single clone from pooled colonies bacteria to further examine by Single-Colony PCR technique with mutant primers set. Similar to PCR Matrix results, we discovered that most of the colonies consisted of the mutant CD137 promoter region (figure 4, lane 2-11). Nevertheless, we did not ensure the correct size of insert CD137 promoter along with pGL3 plasmid (figure

2, lane 2-13). Both colony PCR methods were employed to minimize false positive clones. Third, digestion with restriction endonuclease enzymes conferred to increase the specificity. Hence, we utilized the same set of Enzymes, which used in our previous report (Netsawang et al., 2014), namely *Kpn*I and *Hind*III. We finally showed the DNA containing 4818 and 793 bp, which consistent with the size of pGL3 plasmid and full CD137 promoter, respectively (figure 5, lane 5-11). These data represented that these putative positive clones might be composed of the correct size of the CD137 promoter region.

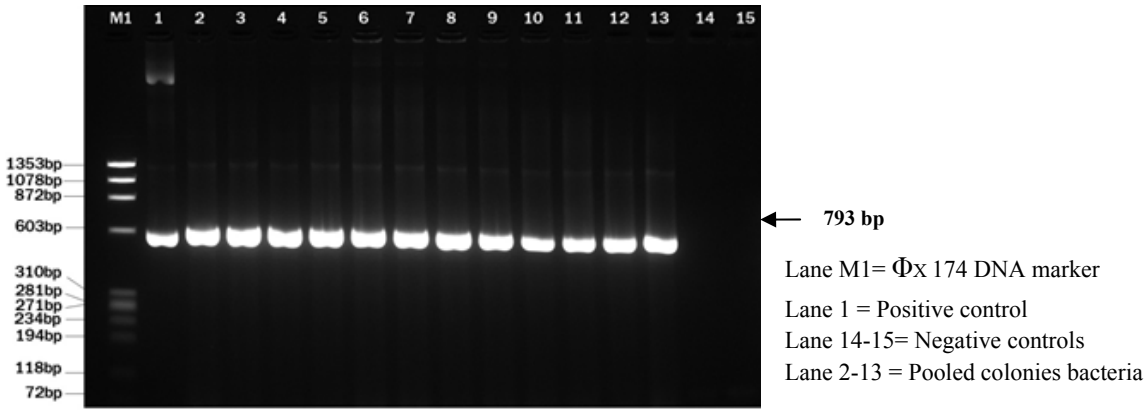

Figure 2 The PCR product of pooled colonies by using Colony-PCR Matrix technique with full CD137 primers

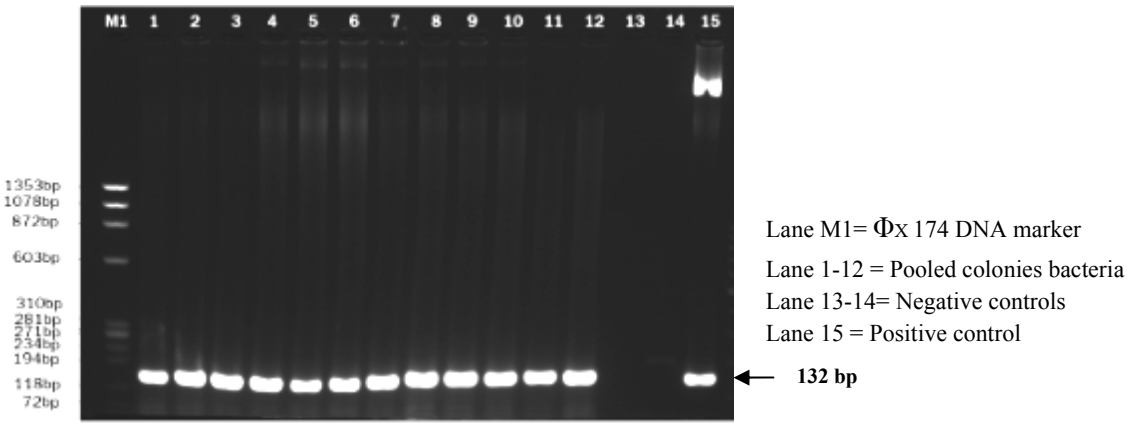

Figure 3 The PCR product of pooled colonies by using Colony-PCR Matrix technique with mutant CD137 primers

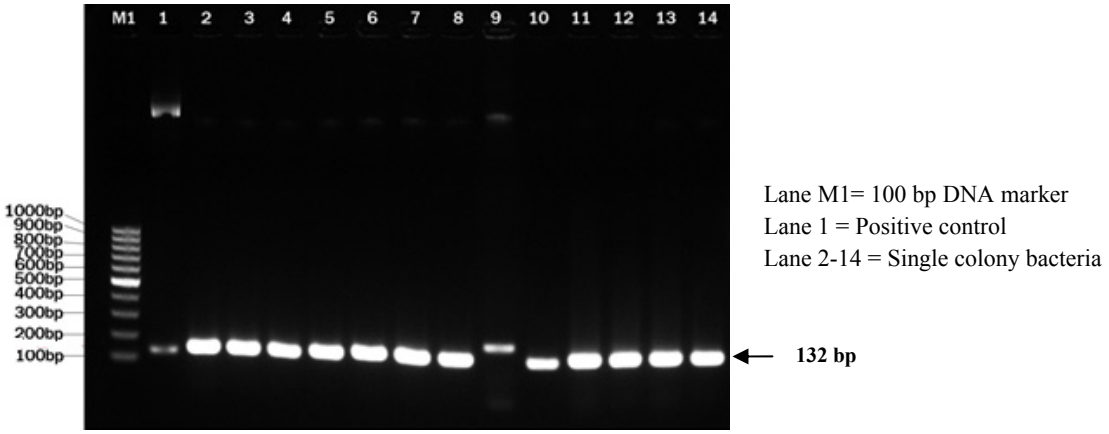

Figure 4 The PCR product of random single colony by using Single-Colony PCR technique with mutant CD137 primers

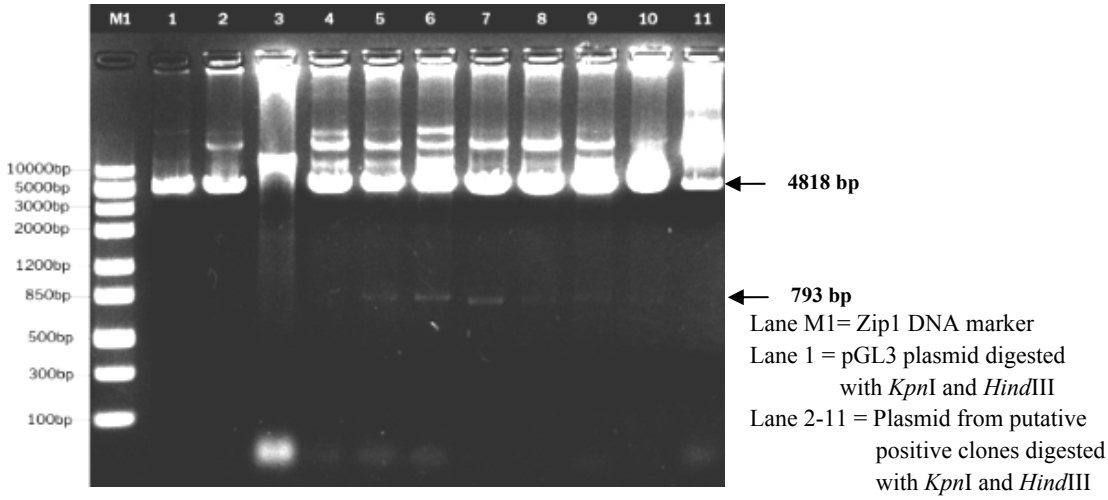

Figure 5 The digestion with restriction enzymes of plasmid from putative positive clones

4.3 Verification of point-mutation CD137 promoter region by using direct sequencing technique

However, we could not discriminate between wild-type and mutant CD137 promoter by using the size of inserted DNA. Consequently, it is necessary to confirm whether the point-mutation initiated along with full CD137 promoter region. Direct sequencing was a definite method to verify point-mutation of these positive clones and then, compared with the database from GenBank by using Pairwise-sequence alignment. Finally, we demonstrated the point-mutation containing the changing from Guanine (G) to Cytosine (C), represented as a square box (figure 6), along with NF-*k*B binding site of the CD137 promoter region in most of the positive clones.

Figure 6 The comparison between positive clone and CD137 template by using Pair-wise alignment

Overall results, including screening and direct sequencing methods, confirmed that site-directed mutagenesis technique provided a large number of positive clones in comparison with other techniques. The advantage of this technique is easily manipulated in single PCR step, no need for digestion of plasmid and insert DNA, inexpensive and time- safer, high sensitivity and specificity, and high yield of mutant clones. Nevertheless, the limitation of this technique is the loss of digestion efficacy by the restriction enzyme, *Dpn*I, to eliminate the wild-type methylated-DNA template, leading to a high rate of false positive clones. Moreover, it requires the wild-type plasmid as a DNA template. The proof-reading activity of DNA polymerase repairs the one base-substitution with the correct strand versus one mismatched primer. Human error occurs when pools a lot of colonies from bacteria in a single tube. The intensity of the band could not determine the chance to get positive clones. Direct sequencing is very expensive and required the expertise to analyze the results. However, direct sequencing is our definite method to determine which clone is a mutant vector that why we have to minimize the number of putative positive clones by using several screening methods. The efficacy of double digestion with restriction enzymes relied on the amount of DNA template and Enzyme, buffer and condition, and incubation time and temperature.

This selected point mutation along with NF-*k*B binding site contained the changing from G to C at position -919 of 5′- upstream region of CD137 gene, had been previously observed by Kim and his colleagues that this element was critical for regulation of CD137 gene expression in activated T lymphocyte (Kim, Kim, Baek, & Kang, 2003). We expected that a similar result will be obtained in DENV infected-hepatocyte cells line as well as the effect of this element with the NF-*k*B deletion construct.

5. Conclusion

Site-directed mutagenesis technique is usefully generated the point-mutation in the plasmid in order to study the relationship between structure and function of an interested protein. This technique applies the amplification of DNA by PCR with a single pair of one mismatched-primers. The wild-type DNA template was removed by the restriction enzyme, *Dpn*I, which specifically digested only methylate-DNA. Subsequently, then transformation of the remaining DNA into *E. coli* strain DH5α was achieved and then, selected the positive clones by using Ampicillin containing LB media. Several screening methods were employed to verify the efficacy of this technique and finally, approved the positive clones by using direct DNA sequencing.

We successfully generated the pGL3 containing the point-mutation of CD137 promoter region with high-yield of positive clones by using site-directed mutagenesis technique. Further investigation, we will employ these mutant plasmids in a functional assay in triggering the expression of CD137 gene with only one-point mutation of CD137 promoter region upon DENV-infected hepatocytic cell line.

6. Acknowledgements

This work was financially supported by Thailand Research Fund–Young Research Scholar (MRG5580152) and Rangsit University. We appreciate the kind assistance from our mentor, Associate Professor Dr. Thawornchai Limjindaporn and all staffs from Faculty of Medicine, Siriraj hospital, Mahidol university.

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