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The establishment of CD47 deficient cholangiocarcinoma cell line by CRISPR/Cas9 technique

Phattarin Pothipan[1], Ryusho Kariya[2], Paweena Dana[1], Kanha Muisuk[3], Kanlayanee Sawanyawisuth[1], Chaisiri Wongkham[1], Sopit Wongkham[1], Seiji Okada[2], Kulthida Vaeteewoottacharn[1,*]

[1]Department of Biochemistry, Faculty of Medicine, and Cholangiocarcinoma Research Institute, Khon Kaen University, Khon Kaen, Thailand
[2]Center for AIDS Research, Kumamoto University, Kumamoto, Japan
[3]Department of Forensic Medicine, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
*E-mail: kulthidava@kku.ac.th

Abstract

The CRISPR/Cas9 technology is a powerful genome-editing tool. By using sgRNA-directed complementation, Cas9 introduces DNA double-strand break (DSB) to the target sequence, which will be subsequently repaired by an endogenous DSB repairing system. Nucleotide insertion, deletion, or substitution is commonly observed because of error-prone non-homologous end joining (NHEJ) repair. It is a useful tool for a mutation study. Cholangiocarcinoma (CCA) is an aggressive bile duct cancer that is a major health problem in the northeast Thailand. The unique "chronic inflammation-related" characteristic, such as increased tumor-associated macrophage (TAM), is reported in CCA. However, the mechanism by which CCA escape the macrophage recognition and phagocytosis has never been demonstrated. Cluster of differentiation 47 (CD47) or "don't eat me" signaling molecule is a membrane phagocytic inhibiting molecule. We hypothesized that CCA expresses CD47 to modulate phagocytic activity of macrophage and this might contribute to disease progression. Therefore, the CD47 deficient CCA cell line was established in the current study by CRISPR/Cas9-mediated mutagenesis. The loss of membrane CD47 were confirmed by flow cytometry. The CD47 deficient clones created in the current study might be a useful tool for CD47 functional study in CCA.

Introduction

Genome editing or gene editing is a technique for nucleotide sequence modification by adding, removing or replacing the cell endogenous double-strand breaks (DSBs) repair system. Several approaches for editing the genome have been developed such as z*inc finger nucleases* (ZFNs) (1), transcription activator–like effector nucleases (TALENs) (2) or *clustered regularly interspaced short palindromic repeats* (CRISPR)-associated9 (CRISPR/Cas9) (3). Among them, the CRISPR/Cas9 system is widely used because of its simplicity, and high effectiveness. The CRISPR/Cas9 consists of 2 components: a single guide RNA (sgRNA) and a Cas9 endonuclease. The sgRNA contains 20 nucleotide guide RNA (gRNA) that are complementary with the target sequence and is immediately adjacent to the protospacer adjacent motif (PAM), an important component for Cas9 digestion (4). Once the Cas9 introduces DSBs to the target DNAs, the damage DNAs are commonly repaired by non-homologous end joining. As a consequence, the insertion-deletion (indel) mutations and the

loss of function products are yielded. Therefore, this system is very useful for the functional studies of interesting genes (5).

Cholangiocarcinoma (CCA) is an aggressive bile duct cancer. CCA is a rare cancer worldwide, but a high incidence is reported in Thailand, especially in the Northeastern part (6). The pathogenesis of CCA in Thailand is tightly related to the liver fluke (*Opisthorchis viverrini, Ov*) infection and the carcinogen consumption (7, 8). An involvement of chronic inflammation and monocyte/tumor-associated macrophage (TAM) infiltration in CCA progression has been reported (9, 10). However, the interaction between monocytes/macrophages and cancer cells, and the significance of immune surveillance in CCA remains unclear. Acquiring immune destruction evasion is an essential characteristic of cancer cells to avoid the immune recognition and promotion of cancer progression (11). Cluster of differentiation 47 (CD47) is a surface glycoprotein that is widely expressed on the human cells, particularly the hematologic cells (12). It plays a protective role in anti-phagocytosis to avoid the recognition and destruction of self-cells. It is known as a "don't eat me" signal.

An increase of CD47 expression is frequently observed in cancers to support immune escape (13, 14). However, little is known regarding the role of CD47 in CCA. To clarify the functions of CD47, CD47-null CCA cells are required. The aim of this study was to establish a CD47 deficient CCA cell line by CRISPR/Cas9 technology. CD47 gRNA was introduced to KKU-213 CCA cells by a lentivirus system. CD47 deficient KKU-213 clones were selected by fluorescence-activated cell sorting (FACS). Confirmation of loss-of-CD47 was performed by flow cytometry. The molecular characterizations of CD47 genetic alteration in CD47 deficient clones are ongoing. These CD47 deficient clones will be a useful tool for the functional characterization of CD47 in cholangiocarcinoma.

Methodology

CCA Cell line

KKU-213 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic and 25 mM HEPES. Cells were maintained in a humidified incubator at 37°C and 5% carbon dioxide. All culture reagents were purchased from Gibco BRL. KKU-213 was established by Prof. Banchop Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University as previously described (15) and was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, Japan.

Design of CD47 gRNA and generation of CD47-CRISPR/Cas9 plasmid

The CD47 gRNA sequences were designed by CRISPRdirect software (https://crispr.dbcls.jp/)(16). The targeting sequences were 5'-CCTGGTAGCGGCGCTGTTGC-3'. A pair of oligonucleotides for gRNA and complementary strands containing the adapter sequences were as followed; 5'-CACCGCCTGGTAGCGGCGCTGTTGC-3'and 5'-AAACGCAACAGCGCCGCTACCAGGC-3'. This pair was designed to target exon 1 of CD47, which contains signal sequence for CD47 localization. Location of gRNA, predicted cut site and PAM sequences are shown in figure 1

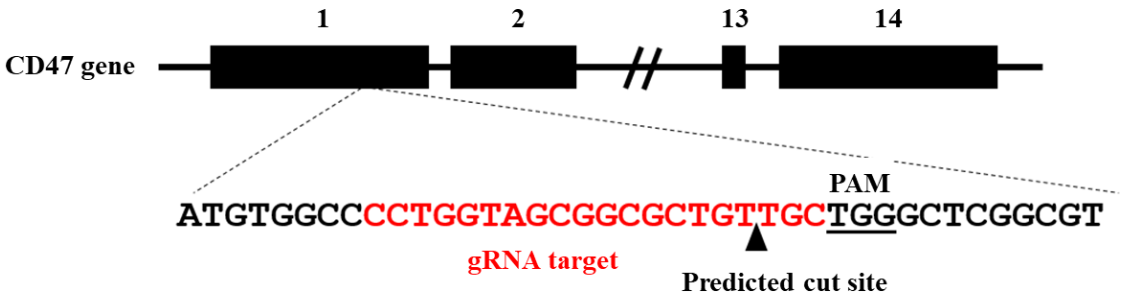

Figure 1 CD47 gene. CD47 gene contains 14 exons. Designed gRNA is targeting the first exon as indicated in red characters. Predicted Cas9 digestion site and PAM sequences were shown.

A pair of oligonucleotides was annealed to generate the double-stranded oligonucleotides and ligated into the pL-CRISPR.EFS.GFP (Addgene, #57818), which was previously digested with *Bsm*BI enzyme (NEB). After transformation and propagation, pL-CRISPR.EFS.GFP containing gRNA targeting CD47 (CD47 CRISPR/Cas9 plasmid) was confirmed by *Bsm*BI and *Eco*RV (TOYOBO) double digestion.

Establishment of CD47 knockout CCA cells

Lentiviral particles were generated in HEK293T packaging cells by the introduction of pCMVR8. 74 (Addgene, #22036), pMD2. G (Addgene, #12259) and CD47 CRISPR/Cas9 plasmids using a HilyMax transfection reagent (Dojindo Laboratories). pCMVR8.74 and pMD2.G were gifts from Professor Didier Trono, School of Life Sciences, Swiss Institutes of Technology, Lausanne, Switzerland. Forty-eight hours after transfection, lentiviral particles were collected by centrifugation and were transduced into KKU-213 cells using polybrene (Sigma-Aldrich).

CD47 deficient KKU-213 cells were sorted by FACS (JSAN) and CD47 deficient clones were generated by limiting dilution technique.

Flow cytometry analysis

The expression of CD47 was monitored using LSRII flow cytometer (BD Bioscience). Cells were trypsinized and were incubated with anti-human CD47 antibody conjugated with PerCP/Cy5.5 (clone CC2C6, BioLegend). After staining, cells were analysed using flow cytometry and data were analysed by FlowJo software (Tree Star Inc.).

Results and Discussion

Establishment of CD47 knockout KKU-213 cells

KKU-213 cell line was transduced with lentivirus containing CD47 CRISPR/Cas9 plasmid. Forty-eight hours after transduction, Green fluorescent protein (GFP) expressing cells (CD47 CRISPR/Cas9 containing cells) were observed (Figure 2, lower panel) as compared to the KKU-213 parental cells (Figure 2, upper panel). Five to ten percentage of KKU-213 cells were successfully transduced and expressed GFP.

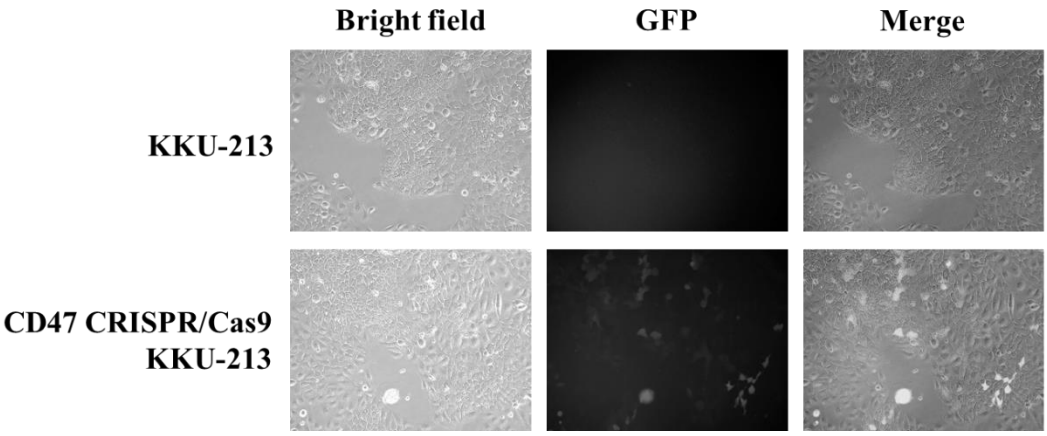

Figure 2 KKU-213 containing CD47 CRISPR/Cas9. Forty-eight hours after lentiviral transduction, green fluorescent signal from pL-CRISPR.EFS.GFP plasmid was observed. Comparison of cell morphology (bright field), green fluorescent signals (GFP) and merged signals (Merge) are demonstrated.

Eight days after transduction, CD47 expression was determined in CRISPR/Cas9 transduced KKU-213 pooled cells. Cells were stained with anti-human CD47 antibody. Only 0.2% of pooled clones was CD47-negative and GFP-positive ($CD47^-/GFP^+$). These cells were sorted out by FACS and then were continued cultured until the cell reached 70-80% confluence. $CD47^-/GFP^+$ cells from first isolation were re-sorted by FACS. Figure 3 showed GFP (Y-axis) and CD47 (X-axis) expressions in CD47 CRISPR/Cas9 KKU-213 pooled cells after second sorting (right panel) when compared to the KKU-213 parental cells (left panel). The results showed 85.7% of CD47 CRISPR/Cas9 KKU-213 pooled cells were expressed GFP but not CD47, 5.28% of cells were expressed both GFP and CD47, 2.41% of cells were expressed only CD47 and 6.65% were not expressed neither GFP or CD47.

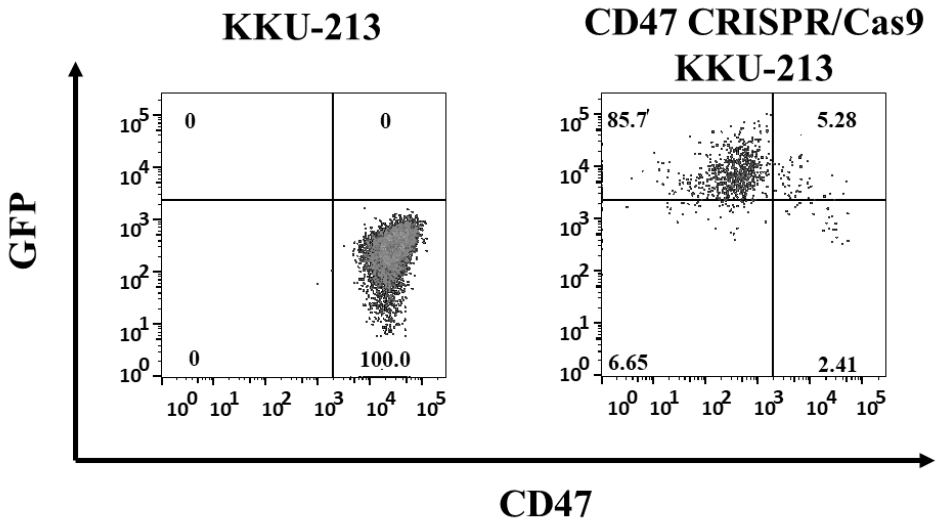

Figure 3 CD47 and GFP expression on the KKU-213 parental (KKU-213) and CD47 CRISPR/Cas9 transduced KKU-213 (CD47 CRISPR/Cas9 KKU-213) cells. CD47 CRISPR/Cas9 transduced KKU-213 cells were stained with the anti-human CD47 antibody and the expressions of CD47 and GFP were compared with KKU-213. Due to the low transduction efficiency, $CD47^-/GFP^+$ cells were sorted twice and $CD47^-/GFP^+$ cells from the second sorting were demonstrated in upper left quadrant of the right graph. These cells were used for single cell cloning by limiting dilution.

Single cell clones were generated by limiting dilution. Sorted cells were separated into a single cell per well. Fifteen clones were randomly selected and the expression of CD47 on cell surface was determined. The result showed 6 clones expressed a lower level of CD47 while CD47 expression was not detectable in 9 clones. Representative clones of CD47 suppressed clones (clone #4 and #11) and CD47 deficient clones (clone #19, #23 and #28) are presented in figure 4. The mean fluorescent intensities (MFI) of CD47 on cell surface of clone #4 and #11 (Figure 4, middle and right upper graphs) determined by flow cytometry were approximately 10 times lower than KKU-213 (Figure 4, left upper graph), while the MFI of CD47 on cell surface of clone #19, #23 and #28 (figure 4, lower panel graphs) were comparable to isotype control antibody stained cells, indicated the CD47 expression is lower than instrumental detectable levels.

Conclusion

CD47 is a transmembrane glycoprotein that plays an important physiological role on self-recognition and self-destruction. Various cancer cells acquire CD47 over-expression on cell surfaces. These signals serve as the protection signal against immune surveillance and destruction and these contribute to cancer promotion and progression. To date, CD47 targeting treatment is under investigation in a clinical trial (17). Functions of CD47 in CCA, however, are not well understood. To elucidate the functional importance of CD47 in CCA, CD47 deficient CCA cells are required. In the current study, CD47 deficient KKU-213 clones were established by a lentiviral transduced plasmid containing CD47 directed gRNA and Cas9 endonuclease. CD47 deficient KKU-213 clones were successfully generated by FACS and limiting dilution. CD47 was undetectable on the cell surfaces of CD47 deficient clones. Clone #19, #23 and #28 were selected for further studies of CD47 functional significances in CCA cells. Molecular characterization of CD47 mutation on CD47 deficient clones are currently under investigation.

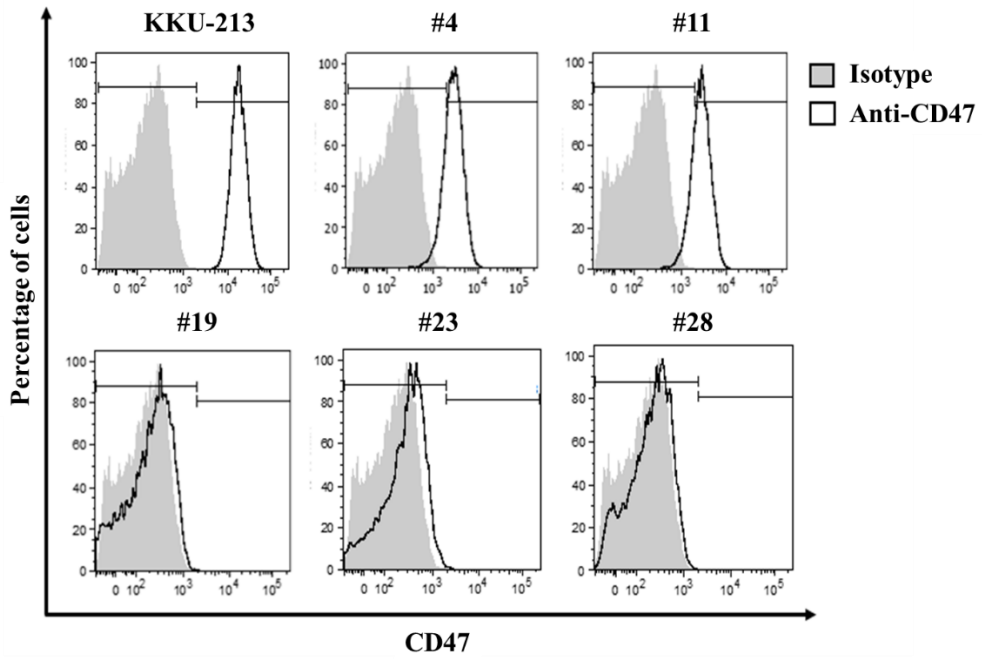

Figure 4 CD47 expression on CCA cells. CD47 expressions on the surface of KKU-213 parental (KKU-213) and CD47 CRISPR/Cas9 transduced KKU-213 clones #4, #11, #19, #23 and #28 were detected by flow cytometry using anti-human CD47 antibody conjugated with PerCP/Cy5.5. Clone #4 and #11 represented CD47 suppressed clones and clones #19, #23 and #28 represented CD47 deficient clones. Filled histograms represented isotype control while empty histogram represented anti-CD47 bounded cells.

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