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CRISPR/Cas9-INDUCED MUTATIONS OF *chNHE1* GENE IN CHICKEN PRIMORDIAL GERM CELLS

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ABSTRACT

In recent years, CRISPR/Cas9 technology has been extensively applied in gene editing research across plants as well as animal subjects, making it an effective tool in aiding the investigation of interactions between pathogens and hosts. Avian leukosis virus subgroup J (ALV-J) is the first known virus-related tumor disease causing avian leukosis, which can lead to major economic losses due to decreased productivity and increased mortality in infected chicken flocks. This poses a significant threat to the poultry industry worldwide, including Vietnam, as ALV-J is considered one of the most difficult pathogens to control. ALV-J infects the host cells by the meditation of the chicken NA^+/H^+ exchange type 1 (*chNHE1*) receptor. In this study, a CRISPR/Cas9 construct was developed and successfully delivered into the primordial germ cells of H'Mong-Vietnamese chickens. The insertion and expression of the transgenes in chicken cells were demonstrated by GFP expression and PCR with specific primers. The CRISPR/Cas9 induced mutations in the target gene *chNHE1* were identified and characterized via ICE analysis (Synthego). The knockout score was indicated at 23 points from one of three tested cell batches. Various induced mutations were observed at the target locations, indicating the presence of different mutant cell lines in the tested cell batch. This is the first success in utilizing the CRISPR/Cas9 system to induce targeted mutations of the *chNHE1* gene in the primordial germ cells of a local chicken variety in Vietnam. Our results provide a potential approach for further research to enhance the poultry health and productivity of local livestock.

Keywords: NHE1, CRISPR/Cas9, chicken, Avian leukosis

INTRODUCTION

Currently, proteins derived from chicken and eggs are considered one of the most important protein sources for human consumption due to their affordability and lack of limitations, based on religious beliefs. Therefore, poultry farming is substantial to every nation's livestock systems. However, many existing chicken breeds face

challenges such as susceptibility to disease, slower growth rates, and lower productivity, which limit their efficiency in meeting the growing global demand for protein (Negash et al., 2021). Consequently, there is a pressing need for research to focus on the enhancement and development of new chicken breeds. Such advancements are important to improve the efficiency and productivity of poultry farming, ensuring a stable and sustainable protein supply for the growing global population (Fiorilla *et al.*, 2024). Moreover, chicken is a valuable subject in research as it serves as a model for studying vertebrate development due to its accessibility and the ability to manipulate it experimentally. Namely, in the research of Sheng et al. (2021), genome editing in chickens was employed as a powerful tool for investigating the genetic and developmental processes underlying gastrulation and early embryonic development. By allowing precise modifications of genes, researchers can explore the roles of specific pathways and mechanisms underlying amniotic membrane development in a model organism that closely resembles vertebrate, specifically human development. Therefore, research related to gene editing in chickens not only contributes significantly to basic research needs but also holds great potential for practical applications.

In recent years, CRISPR-Cas9 technology has been widely acknowledged for its major applications in biotechnology, particularly for editing the genome of various organisms to study the interactions between pathogens, as well as for other purposes. CRISPR-Cas9 genome editing technology involves the precise insertion, deletion, or replacement of nucleotides at specific positions to alter genetic information. This method is highly effective for studying gene function both *in vitro* and *in vivo*, with applications in both fundamental and applied research (Atimango *et al.*, 2024). CRISPR/Cas9 technology has been successfully applied for chicken genome editing in a number of research projects, such as generating virus-resistant gene-edited chickens *(Idoko-Akoh et al.*, 2023) and generating chickens with eggs that can be sex-selective early on (Luo *et al.*, 2023). While there are still many obstacles to applying this technique to chickens, it has the potential as a sustainable solution to enhancing the efficiency of poultry productions, which contributes to addressing global food security challenges (Khwatenge & Nahashon, 2021).

Diseases caused by viruses have seriously damaged the poultry industry globally, including in Vietnam, leaving a negative impact on the economy. Among these, the damage of avian leukosis viruses (ALV) is particularly severe, with the ALV-J subgroup evolving into numerous strains that affect both broiler and egg-laying chickens by causing various malignant tumors in chickens, including myelocytoma, hemangioma, and other types of tumors (Payne & Nair, 2012; Li *et al.*, 2021). ALV-J utilizes the chicken Na^+/H^+ exchanger type 1 (*chNHE1*) as its cellular receptor. Through a combination of biochemical purification and functional cloning approaches, such as immunoprecipitation, western blot analysis, etc, the viral envelope protein (EnvJ) was indentified to specifically bind to *chNHE1*, facilitating the entry of the virus into the host cells. This interaction is crucial for the virus to infect the cells and initiate the infection process (Chai & Bates, 2006). Comparison of the amino acid sequence in ALV-J-susceptible species (such as chickens, wildfowl, and turkeys) and virus-resistant

birds has revealed that Trp38 is an important structural regulator of the *chNHE1* receptor that mediates ALV-J infection into the host cell. Therefore, deletion of Trp38 will increase resistance to ALV-J in chickens (Lee *et al.*, 2017).

In Vietnam, the CRISPR/Cas9 system has been developed and utilized for genome editing of various plant species in recent reports, however, success in chicken genome editing is still very limited. In this study, a CRISPR/Cas9 construct was designed to induce targeted mutations of *chNHE1* gene in the chicken genome in order to lower the expression of *chNHE*1 gene. The activity of the constructed vector was demonstrated by induced mutations on the *chNHE1* gene in Vietnamese H'mong chicken, an endemic species that exclusively habitats in Vietnam, primordial germ cells.

MATERIALS AND METHODS

Materials

H'Mong chicken breed was provided by the National Institute of Animal Sciences. Plasmid pSpCas9 was provided by Alexander Meissner Lab (Addgene plasmid #172221). DNA fragments and primers were conducted by PHUSA Biochemistry (Can Tho, Vietnam).

gRNA design and vector construction

Wu *et al.* (2021) protocol was followed in order to generate the pSpCas9-*NHE1* gene editing vector. First, the *chNHE1* gene fragment from H'mong chickens was amplified using PCR technique and sequenced. The pSpCas9 plasmid was then digested with the restriction enzymes BbsI and SapI. The digestion products were separated via agarose gel electrophoresis and purified using the GeneJET Gel Extraction Kit (Thermo Scientific). The gRNA sequence was designed based on Wu *et al.* (2021) by synthesizing the NHE1-gRNA-F/R oligo pair. Equal amounts of these oligos were mixed, denatured at 95˚C for 5 minutes, and then cooled to room temperature to form the product using T4 DNA ligase. This product was then transformed into *E. coli* G10 cells. The transformed cells were transferred on LB agar containing 100 mg/L ampicillin (Sigma). Colonies were screened by PCR using NHE1-gRNA-F and CMV-enhan-R primers, and positive colonies were confirmed through sequencing. This method ensured the successful construction of the pSpCas9-NHE1 vector for subsequent gene editing applications. An expression cassette of the GFP gene was also inserted into the pSpCas9-NHE1 vector for rapid screening of transgenic chicken cells.

Primordial germ cells (PGCs) cultivation

Primordial germ cells (PGCs) were obtained from the gonads of 5.5- to 6-day-old chicken embryos, following the method described by (Ji *et al.*, 2016). Firstly, the embryonic gonadal tissues were dissociated by gentle pipetting in 500 µl of 0.25% trypsin/ 0.02% EDTA solution (Gibco) at 37°C for 5 minutes. Fifty (50) µl of fetal bovine serum (FBS, Gibco) was added to the mixture. The gonadal cells were collected by centrifugation at 250 g for 5 minutes. After the supernatant was discarded, the precipitated cells were resuspended in pre-warmed PGCs culture medium at a density of 10^5 cells/ml and transferred into 96-well culture plates. The PGCs culture medium consisted of DMEM (Gibco Invitrogen) supplemented with 7.5 ml FBS, 2.5% chicken serum (Sigma-Aldrich), 1%

GlutaMAX-I Supplement (Invitrogen), 1% nucleosides (Millipore), 1% antibiotic/antimycotic (Abcam), 1% insulin-transferrin-selenium supplement (Gibco), 1% non-essential amino acids (Invitrogen), 0.1% β-mercaptoethanol (Sigma-Aldrich), and 0.01 ng/µl basic human fibroblast growth factor (bFGF, Sigma-Aldrich).

After 7-14 days of culture, primary PGCs formed into colonies. These colonies were collected by gentle pipetting and centrifugation at 200 g for 5 minutes. PGCs were then separated from the colonies using Accutase, centrifuged again, and reseeded into fresh PGCs culture medium in a 12-well plate. This culturing process was repeated every 3-4 days to maintain the PGCs.

Delivery of the CRISPR/Cas9 vector into PGCs

The gene editing vector pSpCas9-NHE1 was transferred into primordial germ cells (PGCs) using Lipofectamine LTX (Thermo Scientific) with some modifications to the manufacturer's protocol. Briefly, 1.6 µg of the pSpCas9-NHE1 plasmid was combined with Lipofectamine LTX reagent, which had been diluted in 100 µl of OPTI-MEM (Thermo Fisher Scientific). This mixture was incubated with 0.5–1 × 10^5 cells for 5 minutes, followed by a 2-hour incubation at 37°C in 400 µl of antibiotic-free culture medium. Post-incubation, cells were transferred to 6-well plates and cultured at 37°C with 5% CO_2. For selection , the cells were grown in the culture medium supplemented with 1 µg/ml puromycin (Sigma, USA) from days 2 to 4 after infection. Subsequently, the cells were transferred to the antibiotic-free medium to proliferate. Gene transfer efficiency was then assessed using laser microscopy.

Analysis of CRISPR/Cas9 induced mutations

At 15 days after infection, the chicken cells were collected for genotyping. The chicken cells were analyzed using PCR with Cas9-specific primers to verify the presence of the transgene. After that, the CRISPR/Cas9-induced mutations were identified through PCR-gel electrophoresis on 1.5% agarose gel using specific primers for the *chNHE1* gene (sequences noted in Table 1). The PCR products were purified and used for Sanger sequencing. Sequencing data were analyzed using the Synthego ICE tool (Conant *et al.*, 2022) to identify any potential mutations introduced by the CRISPR/Cas9 system.

Table 1: Sequences of used primers

Primers name	Sequence 5'-3'	Description
chNHE1-F	CATCTCCTCGCAGCGTCTCT	Used for PCR, amplifying of *chNHE1* gene
chNHE1-R	GAGTAGTCGATGCCCAGCAC	
NHE1-gRNA-F	CACCGCCCCACGGCTGCTCCCAGGT	*Guide sequence for chNHE1 gene, used in vector construction*
NHE1-gRNA-R	AACACCTGGGAGCAGCCGTGGGGC	
CMV-enhan-R	GGAAAGTCCCTATTGGCGTTA	

Cas-F	GCTGTTGTCTGGGTTCAGGT	Used for PCR, amplifying of *Cas9* gene
Cas-R	AGGTGGCGTACCATGAAAAG	

RESULTS AND DISCUSSION

Construction of the CRISPR/Cas9 vector for chNHE1 gene editing

Specific primer pair *chNHE1*-F/R were designed to amplify the exon 1 of *chNHE1* from the Vietnamese H'Mong chicken genome. Electrophoresis analysis (Figure 1) showed a single DNA band of 312 bp, consistent with the design. The PCR product was sequenced and compared with the reference sequence on NCBI (DQ256198.1), confirming that there were no differences between the isolated gene segment and the published reference gene (Figure 2).

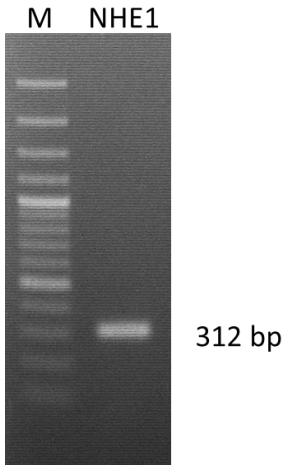

Figure 1. PCR- gel electrophoresis of the *chNHE1* gene from the H'Mong chicken. M: GeneRuler 1 kb DNA Ladder. NHE1: *chNHE1* gene fragment amplified with *ChNHE1*-F/R primer.

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                10        20        30        40        50        60        70        80        90
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NHE1 (ref)      CCCTCGGCCGAGTGGTTGTGGTGCTGTTTGTTCAGCGGGTGCACCTCCTGGGCCGTGGCCAGCGGGGCGGCGGTGATACCCCCGGGCTCT
NHE1-(cloned)   CCCTCGGCCGAGTGGTTGTGGTGCTGTTTGTTCAGCGGGTGCACCTCCTGGGCCGTGGCCAGCGGGGCGGCGGTGATACCCCCGGGCTCT

               100       110       120       130       140       150       160       170       180
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NHE1 (ref)      CCCCACGGCTGCTCCCAGGTGGGCTCGGAGACACGCGTGGCGTCGGCCTGCAAGCGCTGGCCGGGCAGCAGCGGGCCCAGCAGCAGCAGC
NHE1-(cloned)   CCCCACGGCTGCTCCCAGGTGGGCTCGGAGACACGCGTGGCGTCGGCCTGCAAGCGCTGGCCGGGCAGCAGCGGGCCCAGCAGCAGCAGC
                                gRNA
                190       200       210       220       230       240       250
                ....|....|....|....|....|....|....|....|....|....|....|....|....|.
NHE1 (ref)      AGAGCCCAGGGAAGGGCTCGGAGGGCCGCGGCCCCCATGGCGGAGGGAGCCGGGGCCGCCCCGCAGAGACG
NHE1-(cloned)   AGAGCCCAGGGAAGGGCTCGGAGGGCCGCGGCCCCCATGGCGGAGGGAGCCGGGGCCGCCCCGCAGAGACG
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Figure 2. Analysis of the *chNHE1* gene sequence of the H'Mong chicken. The sequence highlighted in the red box indicates the target location for CRISPR/Cas9 construction

Based on the sequencing results of the *chNHE1* gene, we compared it with the gRNA sequence previously used to induce mutations in the *chNHE1* gene (Wu *et al.*, 2021). The results indicate that the gRNA designed by Wu and colleagues is suitable

for use in Vietnamese chicken breeds (Figure 2). We synthesized this gRNA sequence by designing an oligo pair, NHE1-gRNA-F/R. The gRNA was then ligated to the plasmid backbone with the pSPCas9 expression cassette.

To verify the sequence of the vector, plasmids isolated from colonies 1 and 3 were sequenced using the primer CMV-enhan-R. The obtained sequences were compared with the theoretical vector sequence, confirming that both plasmid samples contained the desired sequence. Consequently, we successfully designed the pSpCas9-NHE1 vector for gene transformation in chickens (Figure 3).

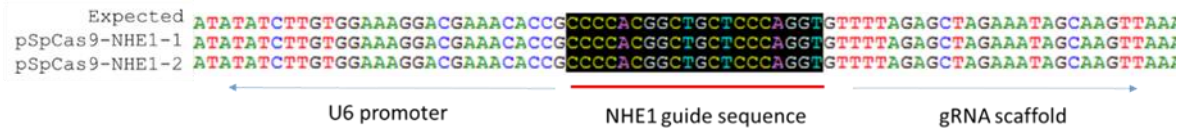

Figure 3. Validation of the pSpCas9_NHE1 construct by Sanger sequencing

Creating transgenic PGCs

After 7 days of culture, the morphology of PGCs cells was illustrated in Figure 4. The cells exhibited the typical round shape of PGC cells and a darker color. The cell density on the culture dish increased, leading to the formation of cell clusters. This result indicated that the PGCs cells could keep their property to survive and proliferate on the culture medium after infection.

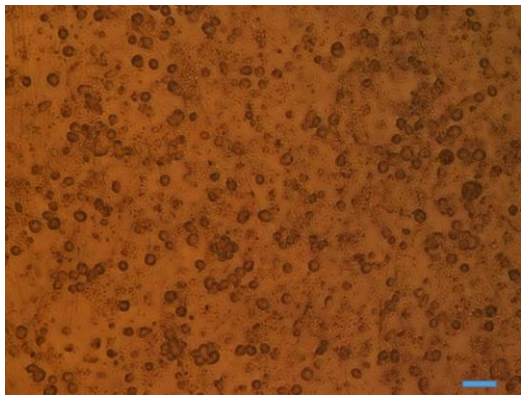

Figure 4. PGCs cells at 7 days of infection. Images were captured under a 20x microscope Nikon Eclipse Ti with a 20x objective lens (bars = 100 µm).

In addition, the expression of the GFP reporter gene in PGCs cells was accessed under a confocal microscope (Figure 5). The images exhibited clear, bright expression of green fluorescence of the GFP gene. These results confirmed that the CRISPR/Cas9 constructs were successfully delivered into the PGC cells.

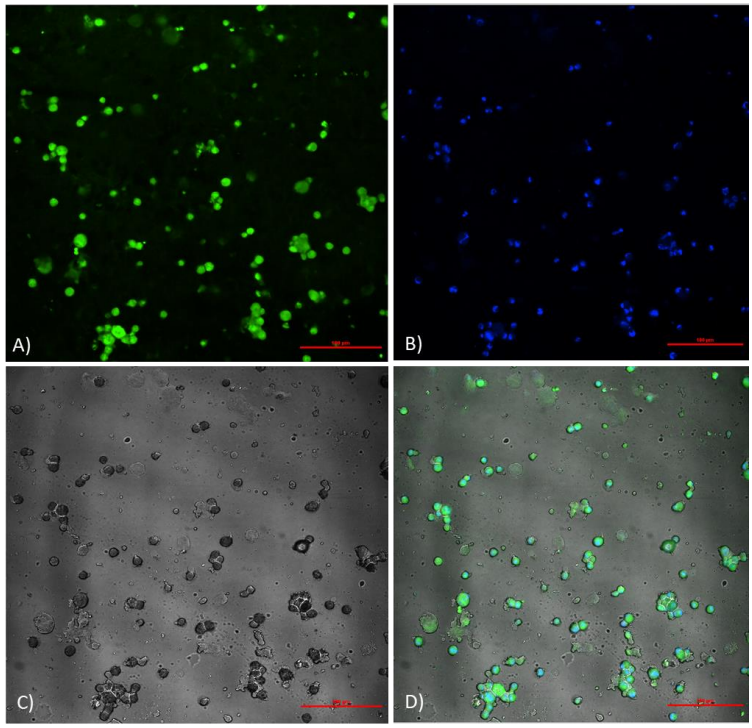

Figure 5. GFP expression in the infected PGCs after 15 days of transfection . Images were captured under confocal microscopy Nikon Eclipse 80i with a 20x. A) GFP gene expressed (green), B) stained with DAPI (blue), C) bright field, D) merged (bars = 100 µm)

Transgenic chickens offer numerous advantages, including a relatively short maturation period and easier breeding (Ivarie, 2003; Sang, 2004). Retroviral vectors, such as avian leukemia virus (Harvey *et al.*, 2002), Moloney murine leukemia virus (Kamihira *et al.*, 2005), and equine infectious anemia virus (McGrew *et al.*, 2004), have been employed to establish transgenic chickens. However, there are only a few reports demonstrated the successful application of nonviral methods (Love *et al.*, 1994; Zhu *et al.*, 2005). In most studies, transgenes were directly introduced into embryos within eggs, with a portion of the DNA being incorporated into primordial germ cells (PGCs). Generally, the frequency of obtaining G1 transgenic progeny is very low. However, recent advancements have enabled the generation of transgenic birds by transferring genes into cultured PGCs and infecting these transgenic PGCs into the anterior portion of the sinus terminales (Van De Lavoir *et al.*, 2006). In the current study, the CRISPR/Cas9 fused with a GFP expression cassette was delivered into chicken PGCs via Lipofectamine LTX. The infected PGCs keep proliferation and growth in the culture medium. In addition, the expression of the GFP gene was confirmed by confocal microscopy. This indicates that the Lipofectamine LTX system has potential for chicken cell transformation.

Characterization of CRISPR/Cas9 induced mutations in the *chNHE1* gene

The PCR-gel electrophoresis (Figure 6A) confirmed insertion of the CRISPR/Cas9 constructs in the genome of chicken cell

batches. For the investigation of the targeted mutation region, the *chNHE1* gene was amplified and analyzed by PAGE (Figure 6B). There was no difference in the DNA pattern between the control (cells without transformation) and tested cell batches, indicating the absence of large indels in the targeted gene or the very low edited cell rates. The PCR products were then purified, ligated to the cloning vector and subjected to Sanger sequencing using the Applied Biosystems 3500 system.

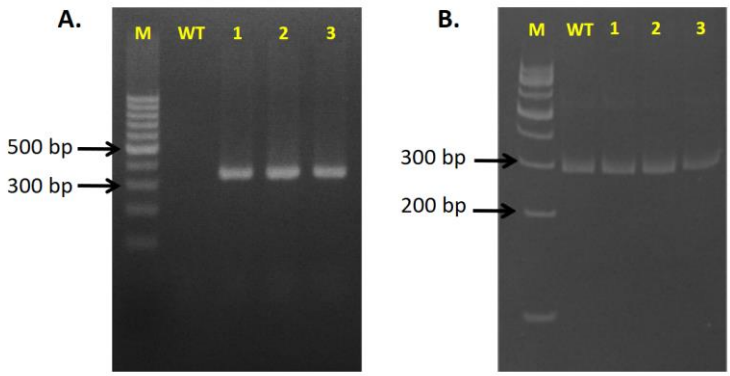

Figure 6. Electrophoresis of PCR products from different experimental batches. (A) PCR analysis of the Cas9 gene. (B) PCR analysis with chNHE1-F/chNHE1-R primer. M: GeneRuler 100 bp DNA Ladder (Thermo Scientific). WT: Wild-type. 1-3: DNA samples collected from primordial germ cells of three different experimental batches.

The Sanger sequencing data was analyzed using ICE software and showed overview data for each sample (Figure 7). This data includes the sample name, the guide target of each template strand (consistent across all samples due to the provided gRNA), the PAM sequence, ICE score, R2 value, and KO score.

The current outcome indicated that cell batch 2 contained gene-edited cells, with an indel percentage of 28% and a knockout score of 23 points. However, other cell batches (1 and 3) showed no difference compared to the wild-type cell lines, suggesting no detectable CRISPR/Cas9 induced mutations of the targeted gene in these samples. Therefore, we performed further analysis for mutant characterization of the generated *chNHE1* sequence from the cell batch 2 via the ICE tool (Synthego).

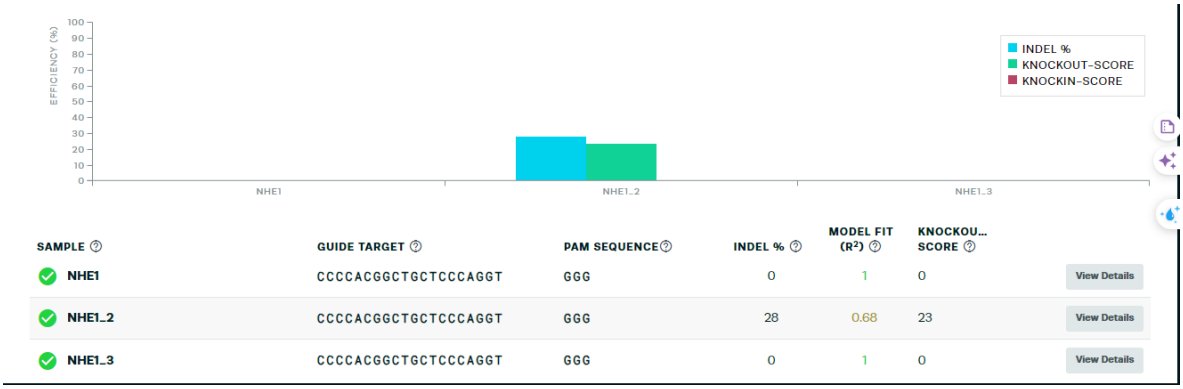

Figure 7. Sequences analysis of the *chNHE1* gene from three cell batches 1,2 and 3 using ICE tool (Synthego)

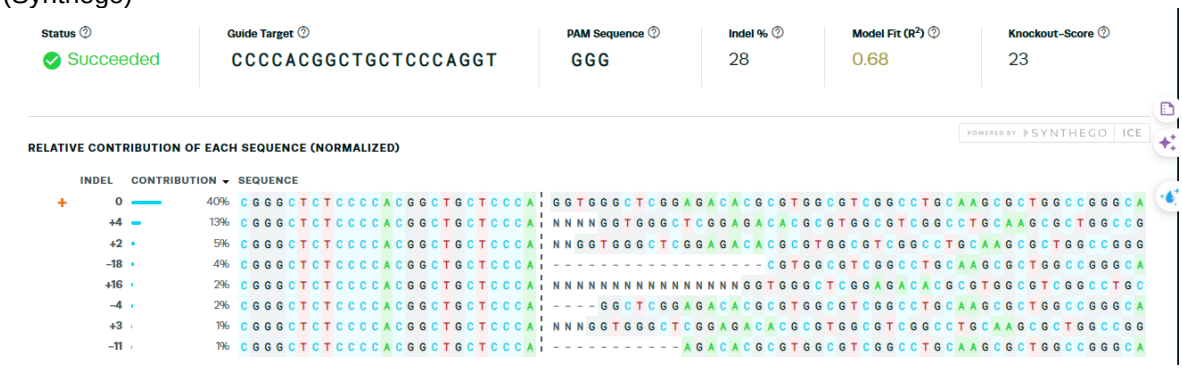

Figure 8. Mutation analysis of the *chNHE1* gene from the cell batch 2

The results from the relative contribution of each sequence analysis in the ICE tool revealed the presences of CRISPR/Cas9 induced mutations in the *chNHE1* gene from cell batch 2. The specific mutation rates were as follows: 13% for +4bp, 5% for +2bp, 4% for -18bp, 2% for +16bp, 2% for -4bp, and 1% each for +3bp and -11bp mutations (Figure 8). Different induced mutations were observed from the sequences, which indicated that batch 2 contained various mutant cell lines. Additional steps need to be performed to isolate and proliferate single cell lines for further analysis.

Park *et al.* (2014) reported that gene transformation efficiency, based on marker genes, could reach up to 36.4% to 60.0% when GFP-positive cells were screened using fluorescence signals through fluorescence-activated cell sorting (FACS) analysis. However, the efficiency of β-actin editing was significantly lower, achieving only 8.3% to 9.1%, which included one 33-nucleotide deletion and one nucleotide insertion. A similar observation was reported by Oishi *et al.* (2016), in which they demonstrated that the percentage of GFP-positive could reach up to 50%. However, it is noteworthy that despite the high percentage of GFP-positive cells, the corresponding mutation frequencies were markedly lower. These findings suggest that the rate of successful gene editing is often lower than the rate of cells that receive the transgene. Similarly, in our study, while all cell batches showed GFP expression, the proportion of cells with CRISPR/Cas9 induced mutations was very limited. Further optimization needs to be conducted to

improve editing efficacy through the CRISPR/Cas9 system in chicken cells.

CONCLUSION

A CRISPR/Cas9 system was constructed and successfully delivered into primordial germ cells of the H'Mong chicken variety. The presence and insertion of the transgenes in the chicken cells were confirmed. The activity of the constructed the CRISPR/Cas9 system was validated by targeted mutations in the *chNHE1* gene. This is the first success in utilizing CRISPR/Cas9 system to induce targeted mutations of the *chNHE1* gene in the primordial germ cells of a local chicken variety in Vietnam. This study provides a potential approach for further research to enhance the poultry health and productivity of local livestock.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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