原著論文

The chicken homolog of KIAA0319L functions as a receptor of avian adeno-associated virus (A3V)

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Abstract: Mammalian *KIAA0319L* was discovered as a gene associated with dyslexia and subsequently identified as an essential receptor for adeno-associated virus (AAV) infection in mammals. KIAA0319L expression is found ubiquitously, but its function is only reported in the nervous system and spermatogenesis. In mammals, various receptors are known to be involved in AAV infection, but many serotypes of AAV infection commonly require KIAA0319L. Although avian AAV (A3V) is being studied as a viral vector, little analysis has been done on its receptor. Therefore, in this study, functional analysis in A3V infection was performed by cloning chicken KIAA0319L cDNA and its transient expression in animal cells. In chicken cells, chicken KIAA0319L increased A3V infection but did not increase A3V binding to the cell surface. Furthermore, when cells in which the CRISPR-Cas9 system disrupted the chicken *KIAA0319L* gene were prepared, A3V infection was significantly reduced and recovered by the transient expression of chicken KIAA0319L. From these results, it was strongly suggested that chicken KIAA0319L functions as an essential receptor for A3V infection, and its main action is the intracellular uptake of A3V bound to the cell surface.
Key words: KIAA0319L, adeno-associated virus (AAV), avian AAV (A3V), CRISPR-Cas9, chicken

1. Introduction

KIAA0319L, a membrane protein associated with dyslexia, was identified as a homolog of KIAA0319[1)]. These genes are found in human loci associated with dyslexia[2)]. In the knockdown experiment in rats, nerve movement abnormalities were observed; therefore, it is a guide molecule for nerve movement during development[3)]. However, in a double-knockout experiment with the *KIAA0319* gene in mice, no effect was observed on nerve migration, and abnormalities were observed in the auditory system[4)]. In addition, a single-knockout experiment of the *KIAA0319L* gene in mice suggested its involvement in acrosome formation in sperm[5)]. Although the *KIAA0319L* gene is expressed in many tissues and cells (https://www.proteinatlas.org/ENSG00000142687-KIAA0319L/tissue[6)]), its function in them has not been elucidated at all.

Adeno-associated virus (AAV) is a small virus that belongs to the genus *Dependoparvovirus*. As

it is not pathogenic to humans, it is being developed as a viral vector for gene therapy[7]. Although the infection requires cell membrane receptors, glycan adhesion factor is involved in adhesion to the cell surface, and proteinaceous co-receptors are involved in cell invasion[8]. Various receptors have been identified as co-receptors for AAV, but human KIAA0319L has been identified as an essential receptor regardless of the AAV serotype[9–14]. Mammalian KIAA0319L is considered an essential receptor for infection because its expression is observed in many cell lines. The infection by AAV is almost eliminated by the knockout experiment by genome editing in mammalian cells[9].

Four strains of avian AAV (A3V) have been reported from different origins[15–19], but little research has been done on the receptors involved in the infection. Therefore, in this study, the role of chicken KIAA0319L homolog in A3V infection was examined in terms of infection efficiency and cell surface binding.

2. Materials and Methods

2.1. Construction of plasmid vectors

The cDNA of chicken homologs of KIAA0319L was amplified by polymerase chain reaction (PCR) from the cDNA of chick embryonic fibroblasts (CEFs) and chicken adult testis. The cDNA of the human KIAA0319L was amplified by PCR from the cDNA of AAVpro® 293T cells (Takara Bio). The primers used for the amplified KIAA0319L cDNA were chKIAA0319L: 5′-GAGAAGAGGTTGGAAGCCAAGCTCAGCATT-3′ and 5′-TAGGATCTCCTCCCTCTGGCTCTTGGCTTT-3′ and hKIAA0319L: 5′-GAGAAGAGGCTGGGAGTCAAGCCAAATCCT-3′ and 5′-CAGGATCTCCTCCCGCGGGCTCCTGGCCTT-3′. The amplified fragments were introduced into the plasmid vector pTriEx-4 (Novagen), and then the nucleotide sequences were confirmed.

The plasmid vectors pAAV-CMV, pRC2-mi342, and pHelper for producing recombinant AAV2 were purchased from Takara Bio. The plasmid vector pAAV-CMV-luc2 was constructed with the pAAV-CMV, and the luciferase reporter gene *luc2* was prepared from the plasmid vector pGL4.10[luc2] (Promega).

The plasmids vectors pA3V-EGFP, pCA3V-RC, and pSR449B for producing recombinant A3V were provided by Dr. Chiorini[16]. The plasmid vector pA3Vd4-luc2 was prepared from pA3V-EGFP by excising a part of the cytomegalovirus (CMV) promoter sequence and replacing the *EGFP* gene with the *luc2* gene. The plasmid vector pCA3V-RC(DA-1) was prepared by replacing the VR-865-capsid gene of the plasmid vector pCA3V-RC with the synthesized DA-1-capsid gene[17].

The Cas-expressing plasmid pA3Vd4-hSaCas9 was prepared from pA3V-EGFP by excising a part of the CMV promoter sequence and then replacing the *EGFP* gene with the Cas-expressing gene units from the plasmid vector pX602-AAV-TBG::NLS-SaCas9-NLS-HA-OLLAS-bGHpA;U6::BsaI-sgRNA (Addgene).

2.2. Cell culture and transfection

All cells were maintained in a medium supplemented with 10% fetal bovine serum (Hyclone) and 100 U/mL penicillin and 100 µg/mL streptomycin (Nacalai Tesque) in a humidified incubator with 5% CO_2 at 37°C. AAVpro® 293T cells and CEFs (primary cultured) were cultured in complete Dulbecco's modified Eagle's medium (Sigma). LMH cells[20] (from the JCRB Cell Bank) were cultured in complete Waymouth's medium (Thermo Fisher

Scientific). All knockout clones were grown in the same medium as parent cell lines.

2.3. AAV production and purification

Triple-plasmid transfection using polyethylene-imine (PEI) was carried out to produce the recombinant AAV for this study. The following plasmids were co-transfected into AAVpro® 293T cells for AAV2: pAAV-CMV-luc2, pRC2-mi342 encoding Rep and Cap protein, and pHelper. The following plasmids were co-transfected into AAVpro® 293T cells for A3Vd4(VR-865) and A3Vd4(DA-1): pA3Vd4-luc2, pCA3V-RC or pCA3V-RC(DA-1) encoding Rep and Cap protein, and pSR449B. When the cells reached 80% confluence, they were transfected in 100 mm plates, each with 5 μg plasmid DNA at a PEI-max/DNA ratio of 2:1 (w/w). Three days after transfection, recombinant AAVs were extracted using the AAVpro® Extraction Solution (Takara Bio) according to the manufacturer's instruction.

2.4. AAV genome titration

DNase I-resistant viral genomes (vg) were quantified using TB Green® Premix *Ex Taq*™ II (Tli RNaseH Plus; Takara Bio) and QuantStudio® 3 Real-Time PCR System (Thermo Fisher Scientific) using a primer set detecting the CMV promoter. The primers against the CMV promoter were 5′-CATGGTGATGCGGTTTTG G-3′ and 5′-CAATGGGGTGGAGACTTGGA-3′.

2.5. AAV transduction and luciferase assay

All cells were seeded in a 12-well plate on the day before 80% confluence and transfected with the Avalanche®-Everyday Transfection Reagent (EZ Biosystems) and 1 μg plasmid DNA/well using a reagent/DNA ratio of 2:1 (w/w). The medium was changed after 4 h of the transfection, and AAV transduction was performed at 10,000 vg/cell. Two days after transduction, the cell culture medium was removed, and cells were lysed with 250 μL/well Passive Lysis Buffer (Promega) and then frozen at −80°C. After thawing, luciferase expression was measured in relative luciferase units (RLUs) per second on a Sirius Luminometer (Berthords) using the PicaGene Luminescence Kit (25 μL; TOYO B-Net). RLU values were standardized per cell by protein quantification using the Protein Assay (Bio-Rad).

2.6. Virus binding assay

Transfection was performed in the same manner as the luciferase assay. The medium was changed after 4 h of the transfection, and the cells were cultured for 2 days. AAVs were allowed to bind to cells (10,000 vg/cell) at 4°C for 1 h. After binding, cells were washed once with PBS(−), and bound AAVs were directly extracted using the AAVpro® Extraction Solution.

2.7. Generation of knockout cell lines

The CRISPR-Cas9 genome editing system was used to generate knockout cell lines. The target sequences were designed using the web tool CHOPCHOP (https://chopchop.cbu.uib.no/). Oligonucleotides corresponding to the guide RNA (gRNA) sequences were synthesized (Thermo Fisher Scientific). gRNA oligonucleo-tides were directly cloned into the Cas-expressing plasmid pA3Vd4-hSaCas9. LMH cells were co-transfected with EGFP-expressing plasmids pEGFP-C1 (Clontech), including target sequences at the *Dra*I site using the Avalanche®-Everyday Transfection Reagent. After 2 days, cells were selected by adding G418 disulfate aqueous solution (Nacalai Tesque) at a final concentration of 500 μg/mL, and neomycin-resistant and EGFP-expressing colonies were isolated.

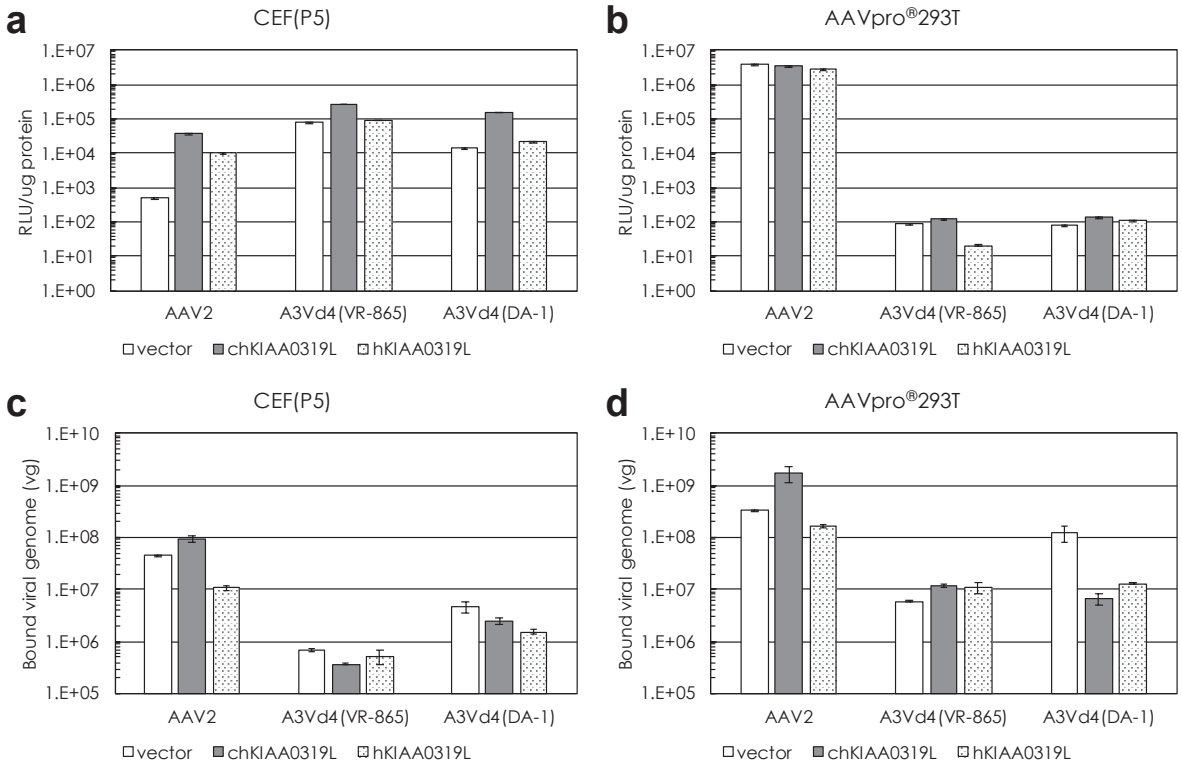

Fig. 1 Transduction of recombinant AAVs in chicken and human cells. (**a** and **b**) After transfection with plasmid vectors pTriEx-4 (negative control), pTE-chKIAA0319L, and pTE-hKIAA0319L, cells were infected with recombinant AAVs [AAV2, A3Vd4(VR-865) and A3Vd4(DA-1)]. Luciferase activities (relative luciferase units, RLUs) were measured to assess AAV transduction and standardized based on protein amounts. (**c** and **d**) Two days after transfection with plasmid vectors pTriEx-4 (negative control), pTE-chKIAA0319L, and pTE-hKIAA0319L, cells were incubated for 1 h with recombinant AAVs [AAV2, A3Vd4(VR-865), and A3Vd4(DA-1)] at 4°C. After removing unbound AAVs, bound AAVs (viral genomes, vg) were quantified by real-time PCR. The data are from one representative experiment of three independent trials and standard deviations of technical replicates. P5 indicates that the number of passages is 5.

3. Results and Discussion

3.1. Cloning of KIAA0319L cDNAs

When *KIAA0319L* gene expression in chicken adult tissues and CEFs was quantified by real-time PCR (RT-PCR), the highest expression was observed in adult testis (data not shown). Therefore, chicken KIAA0319L was amplified by PCR using cDNA derived from CEFs and adult testis. When the amplified DNA fragments were introduced into a plasmid vector and the nucleotide sequences were confirmed. When each of the four clones was analyzed, one clone derived from CEFs contained only one synonymous substitution compared to the nucleotide sequence registered in the database (GenBank accession no. XM_417781). Therefore, this clone was used thereafter. When the nucleotide sequence of the human KIAA0319L DNA fragment amplified from the cDNA derived from AAVpro® 293T cells was confirmed in the same manner, 3 out of 8 clones completely matched the nucleotide sequence registered in the database (GenBank accession no. NM_024874). When these were transiently expressed in AAVpro® 293T cells and the His-tag added to the C-terminal was detected by immunoblotting, a specific band of about 150 kDa was detected in each case (data not shown). Human AAVR has been detected as a glycoprotein of about 150 kDa, and recombinant human KIAA0319L protein has also been identified as a glycoprotein of about 150 kDa, which is in good agreement[10]. The molecular weight of chicken KIAA0319L predicted from the nucleotide sequence was

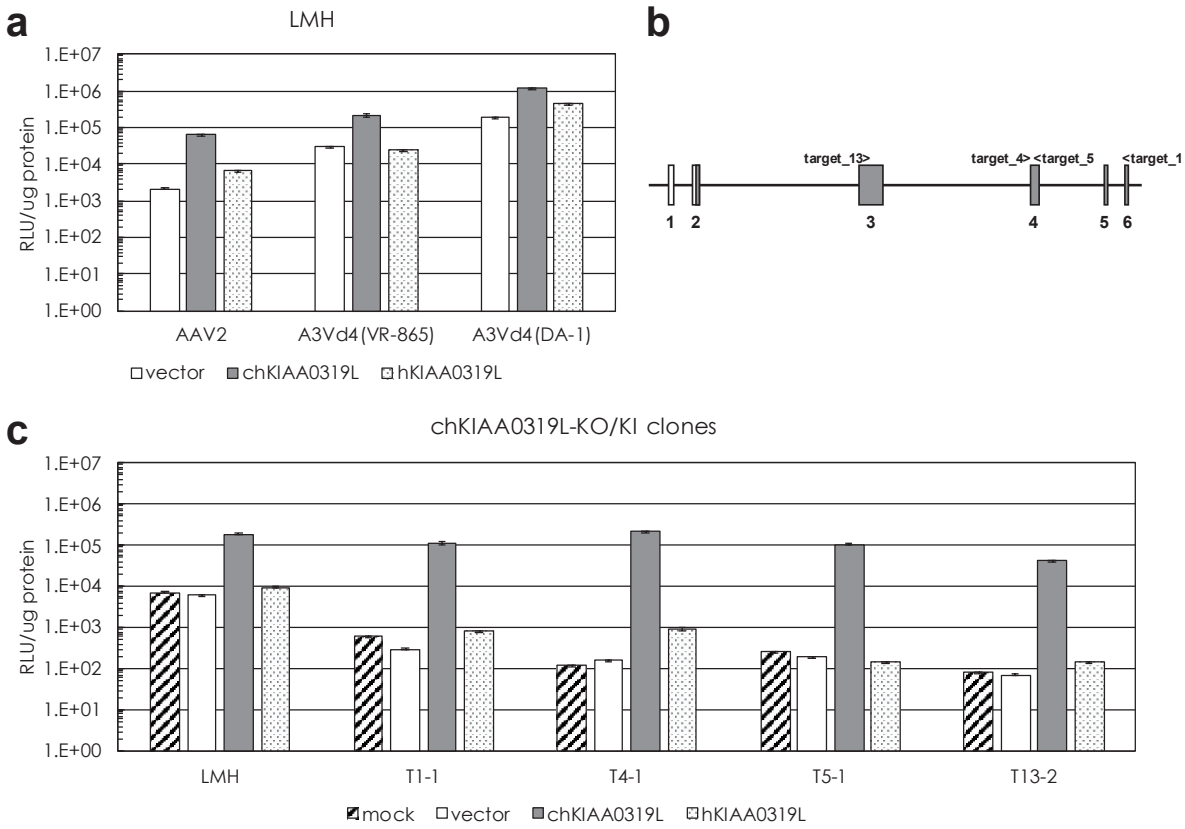

Fig. 2 Transduction of recombinant AAVs in the chicken cell line LMH and disruption of the chicken *KIAA0319L* gene. (**a**) After transfection with plasmid vectors pTriEx-4 (negative control), pTE-chKIAA0319L, and pTE-hKIAA0319L, the cells were infected with recombinant AAVs [AAV2, A3Vd4(VR-865) and A3Vd4(DA-1)]. Luciferase activities (relative luciferase units, RLUs) were measured to assess AAV transduction and standardized based on protein amounts. (**b**) A part of the structure of the chicken *KIAA0319L* gene is shown. The closed box represents the coding region of the exons, and the open box represents the non-coding region of the exons. The four target sequences and their directions are shown above. (**c**) After transfection with plasmid vectors pTriEx-4 (negative control), pTE-chKIAA0319L, and pTE-hKIAA0319L, wild-type LMH cells and chKIAA0319L-knock-out/knock-in clones (T1-1, T4-1, T5-1, and T13-2) were infected with recombinant A3Vd4(VR-865). Luciferase activities (RLUs) were measured to assess AAV transduction and standardized based on protein amounts. The data are from one representative experiment of three independent trials and standard deviations of technical replicates.

slightly larger than that of humans, but a clear distinction could not be made.

3.2. Transduction of recombinant AAVs

Chicken and human KIAA0319L cDNAs were transiently expressed in chicken cells (CEFs) and human cells (AAVpro® 293T cells), and the effect on recombinant AAV infection was investigated (Fig. 1). It was confirmed by immunoblotting that these recombinant proteins were also expressed in CEFs. Two strains A3Vd4(VR-865) and A3Vd4(DA-1) were used as recombinant A3V. Although four A3V strains have been reported, one of them, DA-1, was used for comparison because the three strains other than VR-865 are extremely closely related in comparison by the molecular phylogenetic tree. In these preparations, plasmid DNA in which only the capsid protein gene was substituted was used. As a result, only the capsid protein was different. Recombinant AAV2, which infects mammalian cells, was used as a negative control. All recombinant AAVs also contain the CMV promoter and luciferase gene, and infection efficiency is detectable as luciferase activity. In chicken cells, AAV2 was hardly infected, but KIAA0319L expression significantly increased the infection efficiency (Fig. 1a). In addition, A3Vd4(VR-865) and A3Vd4(DA-1)

significantly infected chicken cells compared to AAV2, and KIAA0319L expression increased the infection efficiency (Fig. 1a). In each case, chicken KIAA0319L was more effective than human KIAA0319L. In human cells, AAV2 showed very high infection efficiency, but KIAA0319L expression did not change its efficiency (Fig. 1b). A3Vd4(VR-865) and A3Vd4(DA-1) hardly infect human cells, and KIAA0319L expression did not significantly change the infection efficiency (Fig. 1b).

Furthermore, the effect of KIAA0319L on the binding of recombinant AAV to the cell surface was investigated (Fig. 1c and d). In chicken cells, AAV2 had a higher binding amount than A3Vd4(VR-865) and A3Vd4(DA-1) and was slightly increased by chicken KIAA0319L expression (Fig. 1c). The binding of A3Vd4(VR-865) and A3Vd4(DA-1) was low, and the amount of binding decreased by KIAA0319L expression (Fig. 1c). The amount of bound AAV2 was high in human cells and increased by chicken KIAA0319L expression (Fig. 1d). A3Vd4(VR-865) and A3Vd4(DA-1) had more binding than those in chicken cells, and KIAA0319L expression caused A3Vd4(VR-865) to bind slightly more, and A3Vd4(DA-1) markedly reduced the amount of binding (Fig. 1d).

These results suggest that, in chicken cells, chicken KIAA0319L increased the infection efficiency of A3V but not because of increased binding to the cell surface. In mammals, the involvement of glycan adhesion factor is considered more important for the adhesion of AAV to the cell surface[11]. KIAA0319L is thought to rather increase the uptake of bound AAV into cells in mammals[11]. It is also possible that the intracellular uptake is promoted so that the apparent binding to the cell surface is reduced. In human cells, chicken KIAA0319L had little effect on A3V infection efficiency, presumably because chicken KIAA0319L did not coordinate well with the endocytosis mechanism of human cells. The cytoplasmic domain of the KIAA0319L molecule plays an important role in endocytosis[8]. The cytoplasmic domain of human KIAA0319L contains an internalization motif similar to cation-independent mannose-6-phosphate receptor （CI-MPR)[9,21]. Human KIAA0319L, which lacks the cytoplasmic domain, has been shown to inhibit reverse transport to the Golgi apparatus, resulting in an extremely low AAV infection efficiency[9]. It is speculated that the difference of amino acids sequence in this domain between humans and chickens influences the intracellular uptake of AAV.

3.3. Disruption of the chicken KIAA0319L *gene*

To further analyze the role of chicken KIAA0319L in A3V infection, cells were generated in which chicken KIAA0319L was genetically disrupted (Fig. 2). The LMH cells used were cell line derived from chicken liver cancer. KIAA0319L expression was slightly higher than that of CEFs (data not shown). When an infection experiment similar to that for CEFs was performed using LMH cells, similar results for CEFs were observed (Fig. 2a). However, a reversal was observed in the infection efficiency of A3Vd4(VR-865) and A3Vd4(DA-1). In CEFs, the infection efficiency of A3Vd4(VR-865) was high; in LMH cells, that of A3Vd4(DA-1) was high. They only differed in capsid proteins, and the differences in infection efficiency may reflect tissue tropism, as seen in mammalian AAV serotypes[22].

The chicken *KIAA0319L* gene registered in the database consists of 20 exons (NCBI Gene ID:

419634). Four target sequences were selected based on this nucleotide sequence (Fig. 2b), and the gene was disrupted by the CRISPR-Cas9 system. A knock-in vector with an *EGFP* gene and a neomycin resistance gene was used to visualize gene disruption, and several EGFP-positive and neomycin-resistant cell colonies were isolated. The target sequences of these cells were confirmed. A3Vd4(VR-865) infection experiments were performed on cloned cells in which both alleles were deleted or destroyed by knock-in (Fig. 2c). All clones showed less than 10% infection efficiency of wild-type LMH. Transient expression of chicken KIAA0319L in these cells showed a marked recovery of infection efficiency, equivalent to wild-type LMH cells (Fig. 2c).

3.4. Conclusions

Chicken KIAA0319L, like mammalian KIAA0319L, functions as an essential receptor for AAV infection. This was confirmed in at least two A3V strains (VR-865 and DA-1). Moreover, its main role is to take up AAV bound to the cell surface into the cell, which requires other molecules involved in endocytosis. It seems that KIAA0319L and endocytosis-related molecules are incompatible in mammalian and avian classes and cannot simply be exchanged. Analyzing the mechanism of A3V infection is expected to greatly contribute to the use of A3V as a viral vector in birds.

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