

RESEARCH PAPER

MiR-199-3p–Dnmt3a–STAT3 signalling pathway in ovalbumin-induced allergic rhinitis

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Abstract

Recent research has pointed out the involvement of epigenetic modifications in allergic rhinitis (AR), especially DNA methylation. However, the detailed mechanism has remained largely uncovered. We used ovalbumin (OVA) to induce AR in mouse, and behaviour scores were used to confirm its successful establishment. Histamine and other inflammatory factors were detected to further verify success of the model. Real-time PCR was employed to identify the overexpression of miR-199-3p and subsequent down-regulation of DNA methyltransferase 3a (Dnmt3a). Western blotting was utilized to detect Dnmt3a and signal transducer and activator of transcription 3 (STAT3) at the protein level. Bisulfite sequencing PCR was applied to reveal the methylation status of the *Stat3* promoter region. A dual-reporter assay was used to confirm the direct targeting of miR-199-3p on the Dnmt3a mRNA and an antagomir specific to miR-199-3p was injected to rescue the symptoms of AR. The AR model was successfully established in mouse and confirmed by both behaviour and molecular markers. We also found lowered expression of Dnmt3a and consecutive hypomethylation of *Stat3* promoter and elevated expression of STAT3, which then led to overexpression of IgE and other inflammatory factors. MicroRNAs that worked on the Dnmt3a 3'-untranslated region were predicted and then verified by dual-reporter assay. Finally injection of a miR-199-3p antagomir successfully attenuated the symptoms of AR. We propose that the miR-199-3p–Dnmt3a–STAT3 signalling pathway is involved in OVA-induced AR.

KEYWORDS

allergic rhinitis (AR), Dnmt3a, miR-199-3p, ovalbumin (OVA), STAT3

1 | INTRODUCTION

Allergic rhinitis (AR), also termed pollinosis or hay fever, is caused by overreaction of the immune system to environmental allergens in the air, typically including pollen, pet hair, dust and mould. It causes an inflammation of the nasal membranes, and the symptoms include sneezing, airflow obstruction, nasal itching and rhinorrhoea. These immune reactions against environmental allergens are mediated by IgE (Bousquet et al., 2008), which results in release of inflammatory factors such as histamine from the mast cells (Pawankar, Mori, Ozu, & Kimura, 2011; Wheatley & Togias, 2015). It has been revealed that signal transducer and activator of transcription 3 (STAT3) is required for release of IgE and this process is mediated by interleukin (IL)-21 in human naïve B cells (Avery et al., 2008). Other recent studies have also indicated that

STAT3 is involved in AR, both in the mouse model (Wang et al., 2019) and in patients (Chen et al., 2016). However, how STAT3 is regulated in AR is poorly understood.

An earlier study showed that maternal AR symptoms during early pregnancy, but not during late pregnancy, were strongly associated with higher prevalence of AR in the offspring, which suggested that epigenetic modifications might be involved in the onset of AR (Shinohara, Wakiguchi, Saito, & Matsumoto, 2007). Using seasonal allergic rhinitis (SAR) as a disease model, Nestor et al. found that the DNA methylome, rather than the transcriptome, of CD4⁺ T cells was distinct between SAR patients and healthy controls both *in vitro* and *in vivo* (Nestor et al., 2014). Two recent publications identified elevated DNA methylation level in the promoter regions of interferon (IFN)- γ and IL-4 in CD4⁺ T cells of pediatric AR (Li, Mu, Wang,

Liu, & Jiang, 2018; Li, Rui, Ma, Jiang, & Chen, 2019), which was further revealed to be regulated via the extracellular signal-regulated kinase–DNA methyltransferase pathway as illustrated in the murine model (Li, Zhou, Rui, Zhou, & Mo, 2019). By comparing DNA methylation profiles in peripheral blood mononuclear cells before and after grass pollen exposure, 42 sites showing a significantly higher level of DNA methylation were discovered, among which three genes were further validated by pyrosequencing, namely *TPSG1*, *SLFN12* and *MUC4* (North et al., 2018). Hypomethylation of the *IL13* gene was observed, in company with increase expression of IL-13 and serum IgE overloading, which in turn led to higher susceptibility to AR from house dust mite sensitization (Li et al., 2016). Thus, DNA methylation is proposed to be involved in the pathogenesis of AR.

MicroRNAs (miRNAs) are a group of non-coding RNAs of around 22 nt in length. They regulate gene expression post-transcriptionally by targeting the 3'-untranslated region (UTR) of their target mRNAs, resulting in degradation or translational repression (Bartel, 2004). MiRNAs play important roles in various developmental progressions such as stem cell self-renewal and pluripotency (Gangaraju & Lin, 2009), organogenesis (Asli, Pitulescu, & Kessel, 2008), the cell cycle (Chen, Farwell, & Zhang, 2010), cardiovascular disorders (Wronska, Kurkowska-Jastrzebska, & Santulli, 2015) and tumorigenesis (Exposito-Villen, A, & Franco, 2018; Quan et al., 2018). Some miRNAs have also been found to be involved in distinct aspects of AR: miR-375 works on the Janus kinase 2–STAT3 pathway to inhibit apoptosis of nasal mucosa cells and improves AR symptoms (Wang, Chen, Wang, Xu, & Li, 2018); miR-16 suppresses secretion of inflammatory cytokines and mucus production by inhibition of the nuclear factor κ B signalling pathway (Gao & Yu, 2018); and miR-487b ameliorates AR symptoms by inhibiting the IL-33/sulfotransferase 2 signalling pathway (Liu, Liao, & Liu, 2018). Other miRNAs are under investigation, including miR-199-3p in AR and its involvement in epigenetic modification.

In this study we revealed that miR-199-3p directly targeted DNA methyltransferase (Dnmt) 3a, resulting in hypomethylation of the promoter of *Stat3* and overexpression of STAT3, which finally caused the AR symptoms.

2 | MATERIALS AND METHODS

2.1 | Animals

BALB/c mice were obtained from the Shanghai Experimental Animal Centre (Shanghai, China), and housed four mice per cage in a 12 h light/12 h dark cycle at 20–22°C with 50 ± 10% humidity. Standard food and water were available *ad libitum*. Eight mice were used in each group unless stated otherwise.

2.2 | Ethical approval

All experimental procedures were approved by Qilu Hospital of Shandong University, NHC key laboratory of Otolaryngology (no. QHSU2017584a). Animal studies were carried out according to the

New Findings

- **What is the central question of this study?**

What is the mechanism of DNA methylation in allergic rhinitis?

- **What is the main finding and its importance?**

A miR-199-3p–Dnmt3a–STAT3 signalling pathway is involved in ovalbumin-induced allergic rhinitis, and miR-199-3p antagomir can relieve the symptoms in the mouse model.

guidelines for animal studies in Qilu Hospital of Shandong University and the NIH's *Guide for the Care and Use of Laboratory Animals* (8th edition).

2.3 | Chemicals and reagents

Chicken egg ovalbumin (OVA, grade V, A-5503) and aluminium hydroxide (alum, 23918-6) were purchased from Sigma-Aldrich (St Louis, MO, USA). Eighty milligrams of OVA was dissolved in 40 ml of ice-cold sterile saline, and vortexed at 2000 rpm for 5 min to obtain a 2 mg ml^{−1} solution. Stock solution was aliquoted and frozen at −20°C for further experiments.

*mirVana*TM PARISTTM Kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA) to isolate protein and miRNA-containing RNA from the same experimental sample. TaqMan Reverse Transcription kits for regular mRNA (N8080234) and miRNA (4366596) were both from Thermo Fisher Scientific. SYBR Green Master Mix was from Bio-Rad Laboratories (Hercules, CA, USA).

2.4 | OVA induced AR in murine model

Six-week-old BALB/c mice were sensitized on day 0, 7, 14 and 21 by intraperitoneal (i.p.) injection of 75 µg OVA emulsified in 2 mg alum in 200 µl PBS, after which eight mice were randomly assigned into a negative control group and a treatment group. Mice in the treatment group were further sensitized with daily nasal instillation with 500 µg OVA in 20 µl phosphate-buffered saline (PBS) into bilateral nasal cavities, while those in the negative control group were only dripped with 20 µl PBS (Figure 1a). Mice were killed 24 h after the last treatment on day 30 with CO₂.

2.5 | Measurement of behaviour scores

Behaviour scores were measured to evaluate establishment of the AR model in mouse as previously described (Shin, Kim, Kim, & Park, 2014). Total number of sneezing and rubbing motions was recorded 15 min after each intranasal instillation, and was compared with that in the negative control group.

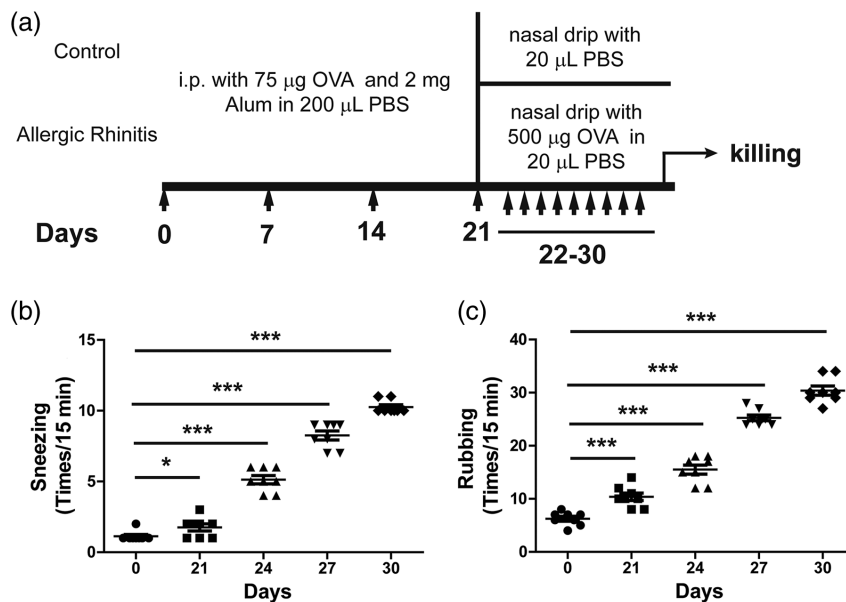


FIGURE 1 Establishment of murine OVA-induced AR model. (a) BALB/c mice were sensitized with OVA and aluminium hydroxide on days 0, 7, 14 and 21. The treatment group was sensitized with intranasal instillation every day with OVA on days 22–30. Negative control group was treated with PBS. $n = 8$ for each group. Total number of sneezing (b) and rubbing (c) motions was significantly increased in AR group in a time-dependent manner. * $P < 0.05$, *** $P < 0.001$

2.6 | Detection of histamine and OVA-specific cytokines

Blood specimens were withdrawn by cardiac puncture under anaesthesia 24 h after the last treatment on day 30. Blood samples were left undisturbed at room temperature for 30 min to clot. After brief centrifugation at 2000 g for 10 min, the clot was removed and the supernatant was designated as serum. Serum was aliquoted and stored in -80°C .

The expression level of histamine and OVA-specific inflammatory factors in serum (IgE, IL-1 β , IL-10, IFN- γ and tumour necrosis factor (TNF)- α) was detected using a commercially available enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol. Histamine and IgE concentrations were measured using ELISA kits from Pharmingen (San Diego, CA, USA). IL-1 β and TNF- α ELISA kits were purchased from BD Biosciences (San Jose, CA, USA). ELISA kits for IL-10 and IFN- γ were from R&D Systems (Minneapolis, MN, USA). All samples were measured in duplicate.

2.7 | Total RNA isolation, cDNA synthesis and real-time PCR

RNA isolation, cDNA preparation and real-time qPCR of Dnmts were performed as described in a previous study (Chen, Pan, Xiao, Farwell, & Zhang, 2011). Total RNA was extracted from mouse serum with a mirVanaTM PARISTM Kit (Thermo Fisher Scientific) according to the manufacturer's instruction. RNA concentration was quantified with a NanoDrop ND-2000 Microvolume UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was determined by absorbance ratios of 260/280 and 260/230.

One microgram of total RNA was used for reverse transcription (RT) using the TaqMan Reverse Transcription Kit; 180 μ L of DNase/RNase free water was added to 20 μ L RT products to make the working template for real-time qPCR; 10 μ L of qPCR mixture consisted of 5 μ L of SYBR Green Master Mix, 0.8 μ L of forward/reverse primer mix and

4.2 μ L of working template. qPCR of each sample was conducted with three technical replicates.

2.8 | Western blotting

Antibodies used for western blotting were anti-Dnmt1 (Abcam, Cambridge, MA, USA; ab19905), anti-Dnmt3a (Cell Signaling Technology, Inc, no. 2160), anti-Dnmt3b (Abcam, ab16049), anti-STAT3 (Cell Signaling Technology, Inc., Danvers, MA, USA, no. 9139) and Gapdh (Abcam, ab8245). Total protein samples were prepared by cell lysis with EBC buffer (Labscoop, Little Rock, AR, USA) at 4°C for 1 h, followed by five cycles of brief sonication. Quantitation of protein samples was performed using the Bradford protein assay (Bio-Rad Laboratories). Twenty micrograms of total protein was loaded onto a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was blocked with 5% non-fat milk in tris-buffered saline and Tween-20 (TBST) for 30 min then incubated with primary antibodies overnight at 4°C , followed by three washes with TBST buffer and 1 h incubation with secondary antibodies at room temperature. After another three washes with TBST buffer, the member was covered by Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Exposure and further analysis of protein expression were performed via Image-Pro Plus software 6.0 (Media Cybernetics, Rockville, MD, USA).

2.9 | Bisulfite sequencing PCR

The CpG island on the *Stat3* promoter region was predicted using online software MethPrimer and primers targeting the *Stat3* CpG island for bisulfite sequencing PCR (BSP) were designed using the same software (Li & Dahiya, 2002). Genomic DNA of the nasal tissue was bisulfite converted using the EpiMark Bisulfite Conversion Kit (New England Biolabs, Ipswich, MA, USA, no. E3318S) and then amplified via EpiMark Hot Start Taq DNA Polymerase (New England Biolabs M0490S) following the manufacturer's instructions.

2.10 | Dual-luciferase reporter assay in TMNE cells

Human polymeric immunoglobulin receptor gene (*hplgR*)-transfected mouse nasopharyngeal epithelial cells (TNMEs) transformed with *N,N'*-dinitrosoperazine were used to test whether miR-199-3p directly targets Dnmt3a.

The potential target of miR-199-3p on Dnmt3a was predicted using TargetScan (www.targetscan.org), PicTar (pictar.mdc-berlin.de) and miRanda (www.microRNA.org). The 3'-UTR of firefly luciferase in pMIR plasmid was replaced by wild-type or mutated 3'-UTR of Dnmt3a. MiR-199-3p or the scramble miRNA was transfected together with the luciferase plasmid into the TMNE cells. *Renilla* luciferase activity in pRL-CMV plasmid was used as the internal control. Forty-eight hours after co-transfection, luciferase activities were analysed via the dual-luciferase reporter assay system (Promega, Madison, WI, USA) (Li et al., 2015).

2.11 | Statistical analysis

Statistical analyses were conducted with SPSS 11.0 (SPSS Inc., Chicago, IL, USA) and Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). All data are presented as the mean $\pm$ standard deviation (SD) unless otherwise indicated. Student's *t* test or one-way ANOVA followed by a Bonferroni *post hoc* test was used for statistical comparisons. The distribution normality of the samples was determined by Kolmogorov-Smirnov test before applying Student's *t* test.

3 | RESULTS

3.1 | Establishment of OVA-induced AR model in mouse

The procedure for establishing the model of OVA induction is illustrated in Figure 1a. Briefly, initial sensitization was carried out in 6-week-old BALB/c mice by i.p. injection of 75 μ g OVA emulsified in 2 mg alum along with 200 μ l PBS at day 0, 7, 14 and 21. From day 22 to day 30, daily intranasal instillation of 500 μ g OVA in 20 μ l PBS was performed in the treatment group, while 20 μ l PBS was utilized in the negative control group. Behaviour scores (number of sneezing and number of rubbing motions) were recorded on day 21, 24, 27 and 30, 15 min after OVA treatment as previously described (Shin et al., 2014), and mice were killed 24 h after treatment to withdraw blood by cardiac puncture under anaesthesia. As shown in Figure 1b,c, both sneezing times and rubbing times in 15 min were significantly increased in the AR group in a time-dependent manner. Since the behaviour scores are the gold standard of AR models, we concluded that the AR model via OVA induction had been successfully established in mouse.

3.2 | Up-regulation of histamine and inflammatory factors in OVA-sensitized mice

Previous studies showed an elevated level of histamine, IgE, TNF- α and IL-1 β in the serum of OVA-sensitized mice (Zhang, Li, Jia, & He, 2015). Other studies showed up-regulation of IL-10 and IFN- γ in

serum in patients with AR compared with a normal population (Bayrak Degirmenci et al., 2018; Uguz, Sanlioglu, Yuzbey, Coskun, & Yegin, 2005). In order to further investigate whether OVA induction could increase the expression level of histamine and other OVA-specific inflammatory factors *in vivo*, blood specimens were collected on day 21, 24, 27 and 30 followed by ELISA assays, as illustrated in Figure 2. Augmentation of histamine (Figure 2a) and all OVA-specific cytokines (IgE, IL-1 β , IL-10, IFN- γ and TNF- α , Figure 2b-f) was observed in mouse serum of the AR group with statistical significance and in a time-dependent manner. These results confirm the successful establishment of the AR model by detecting the molecular markers.

3.3 | Down-regulation of Dnmt3a in AR mouse model

In order to find out whether DNA methyltransferases (Dnmts) are involved in the OVA-induced AR model, we detected the expression level of all three Dnmts (Dnmt1, Dnmt3a and Dnmt3b) in nasal tissues of the AR group compared with the control group. Dnmt1 and Dnmt3b did not show much differentiation between the two groups (Figure 3a-e). However, Dnmt3a demonstrated a decline in expression with high significance after OVA induction, both on the mRNA level (Figure 3b) and on the protein level (Figure 3d,e). The results imply that Dnmt3a is the pivotal enzyme that is involved in the progress of AR by modification of the methylation status of other genes.

3.4 | CpG island of Stat3 promoter was hypomethylated in AR model

Since overexpression of STAT3 is usually the cause of diseases with higher IgE, we detected the expression level of STAT3 in the AR mouse *versus* control mouse. Western blotting result showed that STAT3 expression was indeed elevated in nasal tissue, as shown in Figure 4b,c. In order to find out whether the augmented expression of STAT3 was due to hypomethylation in the promoter region resulting from a decline in expression of Dnmt3a, we identified the CpG island within this region using MethPrimer online software. It turned out that a CpG island, which included 16 CpGs, was present from -342 to -145 bp upstream of Stat3 transcription start sites (TSS) (Figure 4a). Bisulfite sequencing PCR (BSP) was employed to measure the methylation status of the CpG island in the Stat3 promoter region in the nasal tissues of the AR group *versus* the control group. The result showed notable hypomethylation in the CpG island, which was consistent with overexpression of STAT3 in the AR mouse (Figure 4b,c).

3.5 | MiR-199-3p was up-regulated in AR model

For the purpose of discovering the miRNA(s) that drive the decreased expression of Dnmt3a in the treatment group, real-time PCR was utilized to detect the expression level of all miRNAs that were predicted to target the 3'-UTR region of Dnmt3a using TargetScan, PicTar and miRanda. The real-time PCR result showed that only miR-199-3p expression was significantly elevated in the nasal tissues of AR mice (Figure 5), which indicated that this miRNA may cause the down-regulation of Dnmt3a after OVA induction.

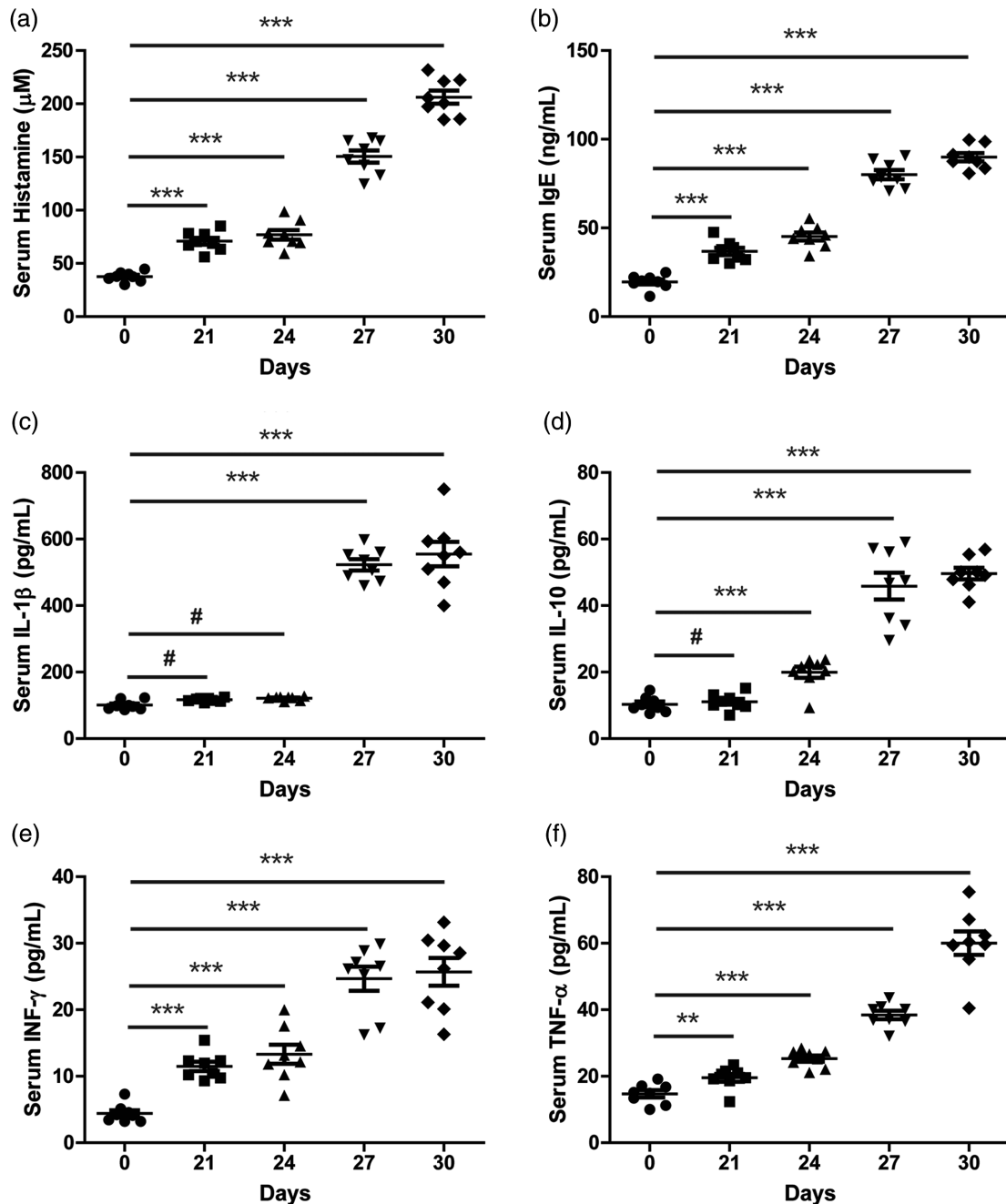


FIGURE 2 The expression level of the inflammatory factors was up-regulated in OVA-sensitized mice. The levels of histamine (a), OVA-specific IgE (b), IL-1 β (c), IL-10 (d), INF- γ (e) and TNF- α (f) were measured in mouse serum from AR group by ELISA. # $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$

3.6 | MiR-199-3p directly targeted Dnmt3a in TMNE cells

To prove that augmented expression of miR-199-3p indeed lowered the expression of Dnmt3a by targeting the 3'-UTR region of its mRNA, a dual-reporter assay was employed. Either the wild-type or mutated 3'-UTR sequence of Dnmt3a was used to replace that of firefly luciferase in the pMIR plasmid, which was then co-transfected with miR-199-3p or scramble miRNA into the TMNE cells. The result showed that only when wild-type 3'-UTR and miR-199-3p were co-transfected was the luciferase activity dramatically decreased

(Figure 6). Along with other results of this study, we hypothesized that miR-199-3p targeted the mRNA of Dnmt3a and lowered its protein level, which caused hypomethylation of the *Stat3* promoter region and enhanced its expression, and finally resulted in overexpression of IgE and other inflammatory factors.

3.7 | Down-regulation of miR-199-3p could attenuate the OVA-induced AR

In order to verify our hypothesis of a miR-199-3p-Dnmt3a-STAT3 signalling pathway, the antagomir specific to miR-199-3p was i.p.

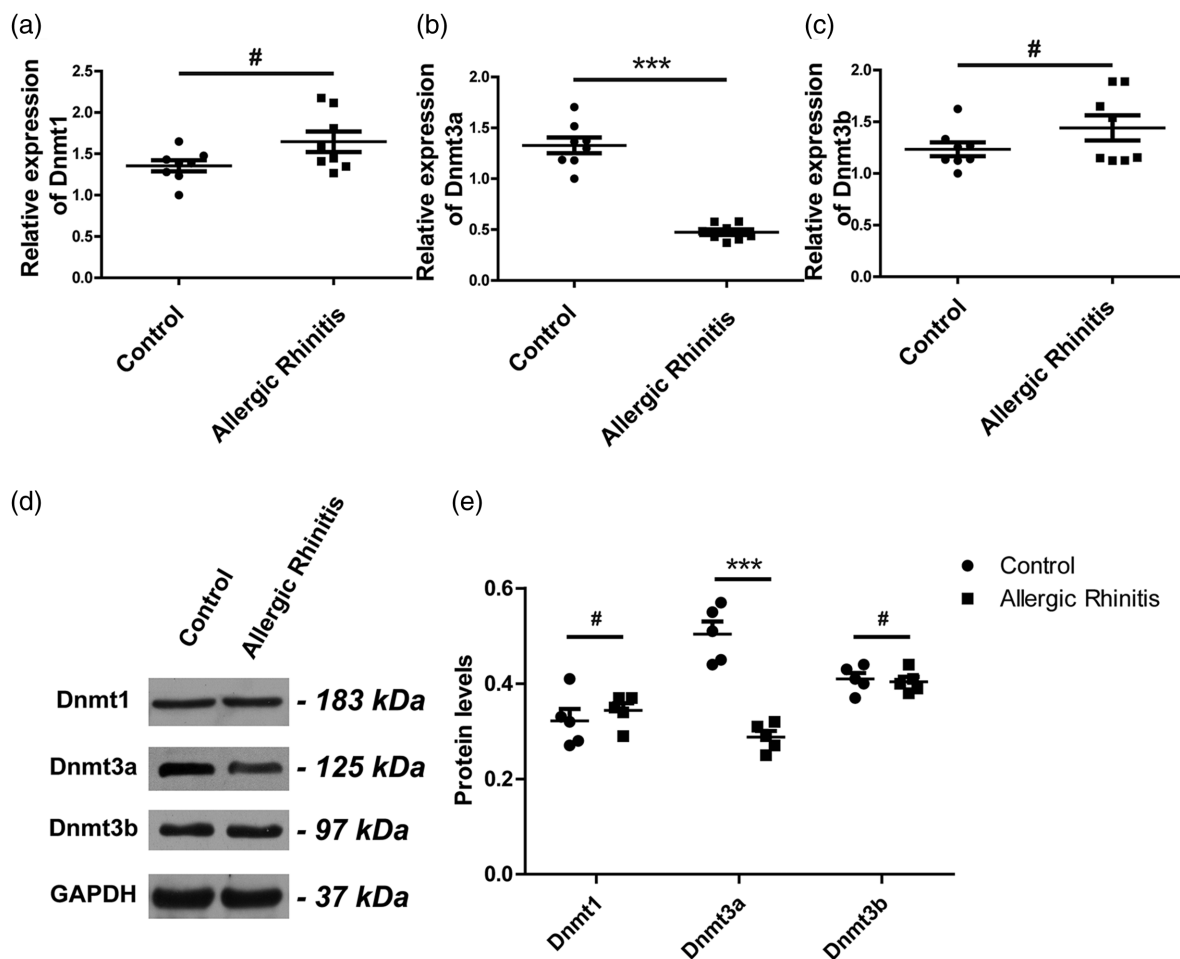
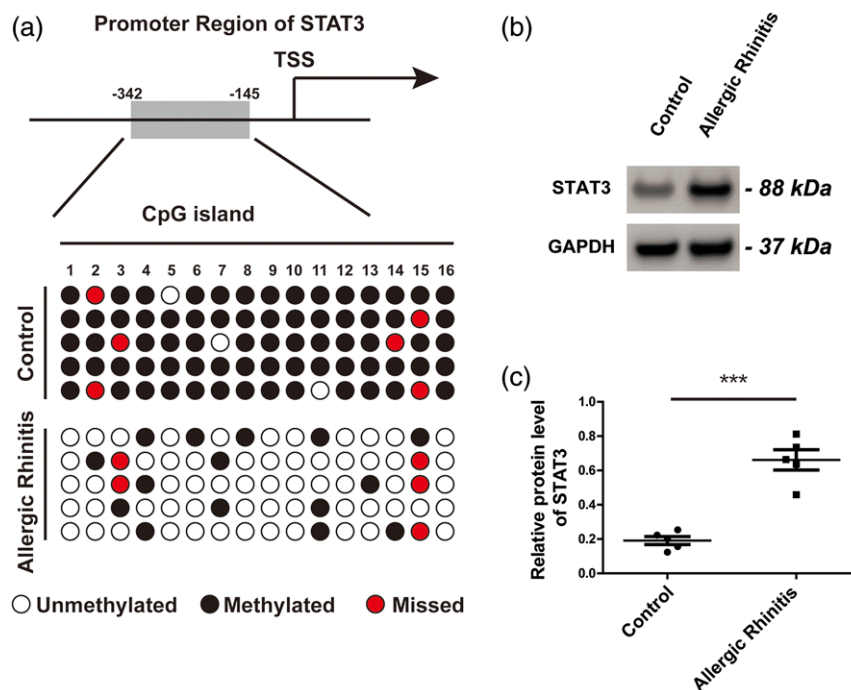


FIGURE 3 Dnmt3a was down-regulated in OVA-induced AR model. (a–c) The mRNA levels of Dnmt1, Dnmt3a and Dnmt3b in nasal tissue were measured in mouse serum from AR group compared to control group by real-time PCR. (d) The protein levels of Dnmt1, Dnmt3a and Dnmt3b in nasal tissue were determined by western blotting. (e) Statistical analysis of the western blot

FIGURE 4 CpG island of *Stat3* promoter was hypomethylated in AR model. (a) CpG island (from –342 to –145 bp) of *Stat3* promoter region was predicted via online software MethPrimer. BSP analysis of the promoter CpG island methylation status in the nasal tissue from control group and AR model group is shown at the bottom. (b,c) The protein levels of STAT3 in nasal tissue were determined by western blotting



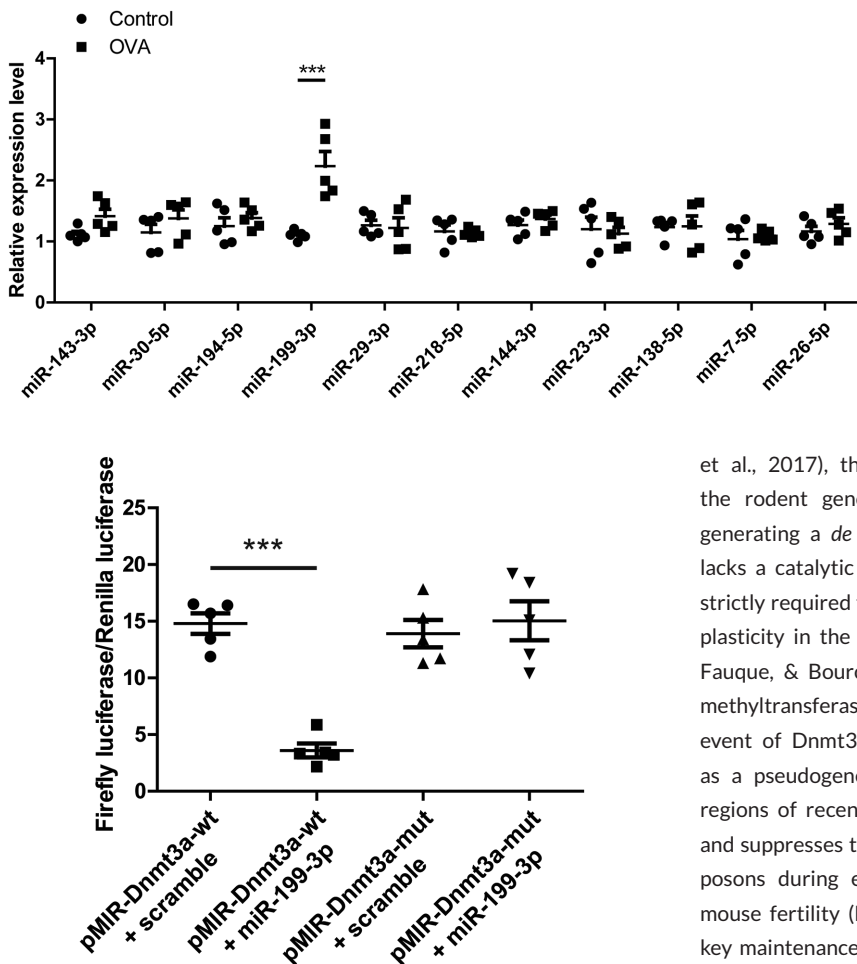


FIGURE 5 MiR-199-3p was up-regulated in AR model. Candidate miRNAs which may directly target the mRNA of Dnmt3a were determined by real-time PCR in the nasal tissue from AR group and compared to the control group. MiR-199-3p was significantly up-regulated in the AR group. *** $P < 0.001$

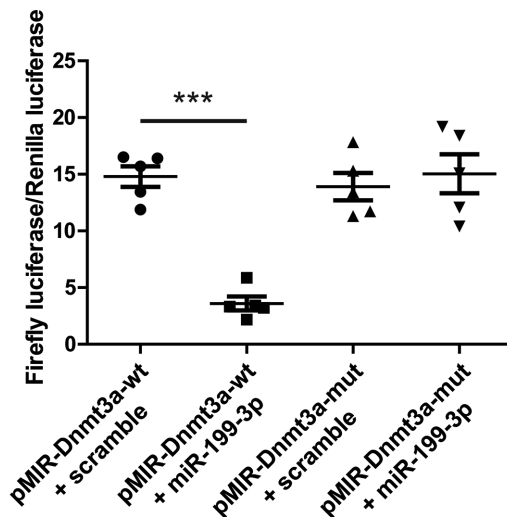


FIGURE 6 MiR-199-3p directly targets Dnmt3a in TMNE cells. Dual-reporter assay was used to determine whether miR-199-3p directly targeted the mRNA of Dnmt3a. The 3'-UTR of firefly luciferase in pMIR plasmid was replaced by wild-type or mutated 3'-UTR of Dnmt3a and miR-199-3p or the scramble miRNA was transfected together with the luciferase plasmid into the TMNE cells. Renilla luciferase activity in pRL-CMV plasmid was used as the internal control. The ratio of firefly luciferase/Renilla luciferase was used to determine the luciferase activity in different groups as indicated. Data are presented as mean $\pm$ SD, $n = 5$ independent experiments. *** $P < 0.001$

injected during OVA-induction as the rescue group (Figure 7a). With the addition of antago-mir-199-3p, we found out that both behaviour scores (sneezing times/15 min and rubbing times/15 min) were significantly decreased (Figure 7b,c), which implied the attenuation of OVA-induced AR.

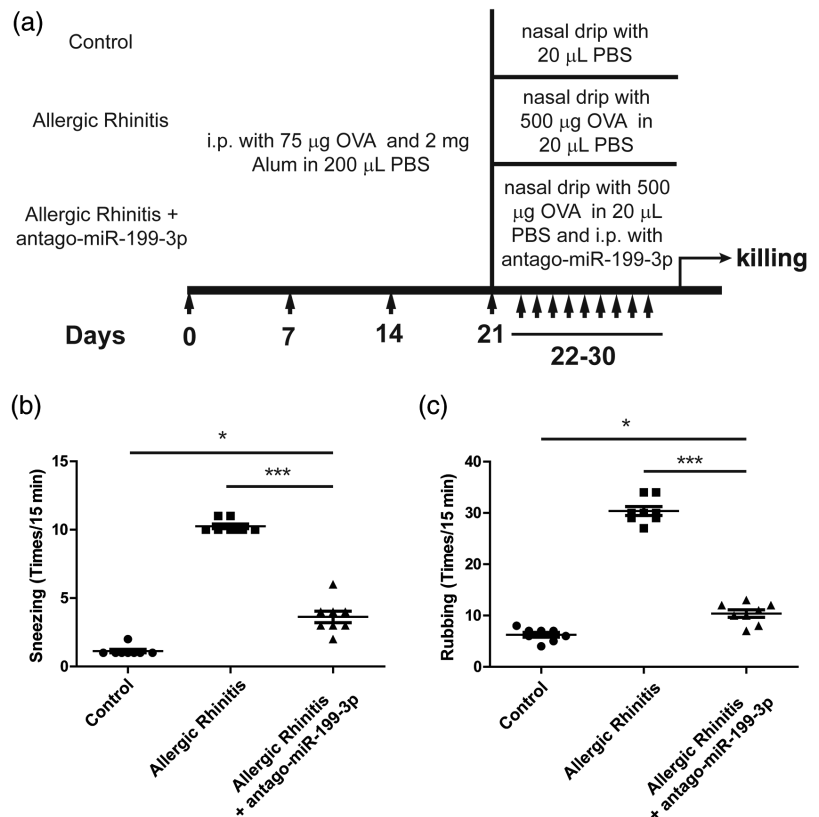
4 | DISCUSSION

There are mainly three types of DNA methyltransferases in mammals, Dnmt1 (Svedruzic, 2008), Dnmt3a (Yang, Rau, & Goodell, 2015) and Dnmt3b (Duan et al., 2015). The Dnmt3 family consists of Dnmt3a, Dnmt3b, Dnmt3l and Dnmt3c (Barau et al., 2016; Jain

et al., 2017), the last of which was recently discovered only in the rodent genome. Dnmt3a and Dnmt3b are well known for generating a *de novo* methylation pattern (Chedin, 2011). Dnmt3l lacks a catalytic domain, but can assist Dnmt3a and Dnmt3b. It is strictly required for the methylation of the germline, but shows more plasticity in the postimplantation embryo (Guenatri, Duffie, Iranzo, Fauque, & Bourc'his, 2013). Dnmt3c is a newly identified *de novo* methyltransferase, which seems to have arisen from a duplication event of Dnmt3b in the rodent genome, and was first annotated as a pseudogene. It is responsible for methylating the promoter regions of recently evolved retrotransposons in the male germline and suppresses the expression of those 'young and dangerous' transposons during early spermatogenesis, and thus is necessary for mouse fertility (Barau et al., 2016; Jain et al., 2017). Dnmt1 is the key maintenance methyltransferase, which has much higher enzyme activity in methylating CpGs on hemimethylated DNAs than on unmethylated ones (Hermann, Goyal, & Jeltsch, 2004). Dnmt1 is the most abundant DNA methyltransferase, and is even more actively transcribed in the S phase, when it is needed for methylating the newly synthesized daughter DNA strands (Robertson, Keyomarsi, Gonzales, Velicescu, & Jones, 2000).

As early as 2007, researchers had found that maternal AR symptoms during early pregnancy were positively associated with a higher prevalence of AR in their offspring. However, this association did not occur with late pregnancy or with paternal AR symptoms, which clearly demonstrated this disease was not solely due to genetic information transferred from the parents. Instead some epigenetic modifications must be involved in the onset of AR (Shinohara et al., 2007). Because of their heterogeneity, most allergic diseases are not suitable for genetic/epigenetic analyses. However, seasonal allergic rhinitis (SAR) is an ideal model due to its well-characterized phenotype and aetiology. By comparing CD4⁺ T cells from SAR patients and from healthy controls, Nestor et al. found that DNA methylation patterns, but not the transcriptomes, were distinguishable (Nestor et al., 2014). Recently more and more case studies have revealed aberrant methylation status in the promoter regions of AR-related genes, such as *IFNG* and *IL4* in CD4⁺ T cells of paediatric AR (Li et al., 2018, 2019), *TPSG1*, *SLFN12* and *MUC4* in the peripheral blood mononuclear cells (North et al., 2018), and *IL13* in house dust mite-sensitized AR patients (Li et al., 2016).

FIGURE 7 Down-regulation of miR-199-3p could attenuate the OVA-induced AR. (a) BALB/c mice were sensitized with OVA and aluminium hydroxide as described in Figure 1. The third group was injected with antago-miR-199-3p i.p. with the treatment of OVA every day on days 22–30. $n = 8$ for each group. Total number of sneezing (b) and rubbing (c) motions was significantly reduced in the antago-miR-199-3p group. *** $P < 0.001$



In this study we wanted to find out which DNA methyltransferase is responsible for the aberrant methylome in AR: Dnmt1, Dnmt3a or Dnmt3b? We first established a murine AR model by OVA induction (Figure 1a), which was confirmed both by behaviour scores and by molecular markers. Both indexes, sneezing times and rubbing times, showed significant increase in a time-dependent manner during OVA induction (Figure 1b,c). Furthermore, augmentation of histamine as well as OVA-specific cytokines (IgE, IL-1 β , IL-10, IFN- γ and TNF- α) was observed in mouse serum also in a time-dependent manner (Figure 2). After establishment of the AR murine model, we detected expression of Dnmts compared between treatment and control groups using real-time PCR and western blotting, and both assays suggested down-regulation of Dnmt3a after OVA induction, but not of the other two Dnmts (Figure 3).

In order to check the genes that are affected by down-regulation of Dnmt3a, we then detected the methylation status of *Stat3*, over-expression of which is usually the cause of diseases with higher IgE, using a BSP assay. It was not surprising to find that the promoter of *Stat3* was hypomethylated in the AR group, resulting in overexpression of STAT3, which was confirmed by western blotting (Figure 4).

Using TargetScan, PicTar and miRanda, 11 miRNAs were predicted to potentially target the 3'-UTR region of Dnmt3a. Expression of those 11 miRNAs was measured by real-time PCR, and only miR-199-3p was found to be significantly elevated in the nasal tissues of AR mice (Figure 5). A dual-reporter assay further verified that Dnmt3a was directly regulated by miR-199-3p in TMNE cells (Figure 6). Finally a miR-199-3p antagomir was also i.p. injected during OVA-induction *in vivo*, which indeed rescued the AR symptoms and indicated the

correctness of the miR-199-3p–Dnmt3a–STAT3 signalling pathway (Figure 7).

A miR-199-3p–Dnmt3a–STAT3 signalling pathway was proposed for the first time in this study and proved to be involved in the onset of AR disease. Since Dnmt3a methylates plenty of genes and regulates their expression, a follow-up study would be an investigation of other genes and pathways that are involved in this process. Whole genome bisulfite sequencing and RNA-seq could be applied to identify alterations of the methylome and transcriptome, respectively, after down-regulation of Dnmt3a. Further research into the detailed functions of miR-199-3p and Dnmt3a in AR are warranted.

5 | CONCLUSION

A murine AR model was first established with OVA induction, and then a miR-199-3p–Dnmt3a–STAT3 signalling pathway was verified in this AR model. A miR-199-3p antagomir successfully rescued the AR symptoms in the model. This new mechanism will help in better understanding the aetiology of AR and in its clinical treatment.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

X.C., Y.G. and Q.W. performed the experiments; X.C. and Y.G. analysed the data; X.C. and X.L. wrote the paper; X.L. supervised the study. All

authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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