Original Article

MiR-133a suppresses the migration and invasion of esophageal cancer cells by targeting the EMT regulator SOX4

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Abstract: MicroRNAs (miRNAs) are small, non-coding RNAs which can function as oncogenes or tumor suppressor genes in human cancers. In the present study, we demonstrated that the expression of miR-133a was dramatically decreased in examined esophageal squamous cell carcinoma (ESCC) cell lines and clinical ESCC tissue samples. Additionally, miR-133a expression was inversely correlated with tumor progression in ESCCs. We have found that over-expression of miR-133a significantly suppressed the proliferation, migration and invasion of ESCC cells in vitro. miR-133a over-expression also significantly suppressed the aggressive phenotype of ESCC in vivo, suggesting that miR-133a may function as a novel tumor suppressor. Further studies indicated that the EMT-related transcription factor Sox4 was a direct target gene of miR-133a, evidenced by the direct binding of miR-133a with the 3'UTR of Sox4. Notably, the EMT marker E-cadherin or vimentin, a downstream of Sox4, was also down-regulated or up-regulated upon miR-133a treatment. We have also shown that over-expressing or silencing Sox4 was able to elevate or inhibit the migration and invasion of ESCC cells, similar to the effect of miR-133a on the ESCC cells. Moreover, knockdown of Sox4 reversed the enhanced migration and invasion mediated by anti-miR-133a. These results demonstrate that miR-133a acts as a tumor suppressor in ESCC through targeting Sox4 and the EMT process. miR-133a may serve as a potential target in the treatment of human esophageal cancer.

Keywords: MiR-133a, esophageal squamous cell carcinoma, EMT, Sox4

Introduction

Esophageal cancer is the eighth most common cancer and the sixth common cause of cancer-related death in the world [1]. Esophageal squamous cell carcinoma (ESCC) is the most predominant type of esophageal cancer in China [2, 3]. Despite the rapid advancement in diagnosis and therapy for esophageal cancer, the average 5 year overall survival is still poor, due to that most cases of ESCC could not be diagnosed until the disease is at the advanced stages [4, 5]. Therefore, elucidating the potential mechanism of tumor development would help us with the early diagnosis, as well as understanding of the progression and the therapy of ESCC.

MicroRNAs (miRNAs) are a class of small, non-coding RNAs, approximately 19-25 nucleotides. It negatively regulates gene expression at post-transcription level by interacting with the 3’ untranslated regions (3’-UTRs) of target miRNAs [6, 7]. MiRNAs are phylogenetically conserved and play crucial roles in a number of biological processes including proliferation, differentiation, apoptosis, metabolism, immunity and development progress [8]. Emerging evidence indicates miRNAs are aberrant expressed in various tumors, and it can modulate tumor ini-
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MicroRNA-133a (miR-133a) is a multicopy gene with two copies distributed in chromosome 18 and chromosome 20, respectively. miR-133a has long been recognized as a muscle specific miRNA which may regulate myoblast differentiation and participate in many myogenic diseases [13-15]. In the tumorigenesis, miR-133a is expressed at a low level in several cancer cell lines and solid tumors including ovarian cancer [16], colorectal cancer [17], bladder cancer [18, 19], breast cancer [20], prostate cancer [21] and esophageal cancer [22]. The biological targets of miR-133a have been partially identified, and miR-133a induces G1 arrest, apoptosis, and senescence by regulation of key factors in cell cycle and apoptosis such as Fascin1, IGF1R, SP1, BCL-xL, and EGFR [16-24]. However, the specific role of miR-133a in ESCC progression is yet to be fully determined.

SOX4 is a member of the Sox (SRY-related HMG-box) family of transcription factors with critical role in embryonic development and in cell-fate determination during organogenesis of the heart, pancreas and brain, and lymphocyte differentiation [25-30]. SOX4 was reported to be upregulated in multiple cancer types, and increased SOX4 activity contributes to cellular transformation, cell survival and metastasis [31-35]. Most recently, Sox4 has been reported to induce epithelial-mesenchymal transition (EMT) via the polycomb epigenetic regulator EZH2 [36, 37]. Sox4 functions as a high-order master that governs traditional EMT transcription factors, such as snail, zeb and twist family members [37]. Furthermore, Sox4 was required in TGF induced EMT [38]. Sox4 could regulate Wnt signal pathway by directly binding to β-catenin and TCF family members [39].

In the present study, we investigated the role of miR-133a in ESCC progression and metastasis. We found that miR-133a is down-regulated in ESCC cell lines and clinical tissue samples, suggesting that miR-133a might act as a tumor suppressor miRNA. Furthermore, we identified that the EMT regulator SOX4 is one of direct target genes of miR-133a. MiR-133a is able to inhibit EMT and metastasis of ESCC cells through suppressing the function of SOX4. These results demonstrate that miR-133a is potentially a therapeutic target for esophageal cancer.

Materials and methods

Samples

Fresh samples from ESCC and corresponding adjacent tissue were obtained from 45 patients at Second Hospital Affiliated to Hebei Medical University between January 2008 and November 2010. The samples immediately snap frozen in liquid nitrogen, and stored at -80°C until RNA extraction. The tumors were classified according to World Health Organization classification. The study was approved by hospital ethical committee, and every patient had written informed consent. Clinicopathological information of the patients about age, sex, stage and lymph node metastasis was obtained from patient records, which were summarized in Table 1.

Cell culture and transfection

The human ESCC cell lines KYSE-150, KYSE-510, EC-9706 and immortalized human esophageal epithelial cell SHEE were kindly provided by Dr. Zhang Xun (Tianjin Chest Hospital). TE13 was purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) medium containing 10% fetal bovine serum (FBS, GIBCO), 100 IU/ml penicillin and 100 mg/ml streptomycin maintained at 37°C in humidification.
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Fried air containing 5% CO₂. For transfection, cells were cultured to 80% confluence and transfected with recombinant eukaryotic vector and empty vector using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer’s recommendation.

**Quantitative real-time PCR**

Quantitative RT-PCR was performed to validate the miRNA expression level. qRT-PCR was carried out using SYBR®Premix Ex TaqTM (Takara, Japan). PCR were carried out in triplicate and analyzed using the ABI Prism 7900HT fast real-time PCR system (Applied Biosystems, Life technologies, USA). The relative quantification values for each gene were calculated by the 2^ΔΔCt method using U6 or GAPDH as an internal reference. All primers were shown in Table S1.

**Plasmid constructions**

Genomic sequence of human miR-133a, including 200 bp flanking sequence, was amplified from human genome, then inserted into the BamHI/EcoRI site of the pcDNA3.1 vector (Invitrogen), named as pcDNA3.1-miR-133a. The full-length 3’ untranslated region (3’UTR) of Sox4 was amplified from human genomic DNA, and was cloned into the downstream of the firefly luciferase coding region of pMIR-GLOTM Luciferase vector (Promega, USA). The recombinant vector was named as pMIR-Sox4. Mutations of miR-133a binding sites were introduced by site-directed mutagenesis and the resulted vector was named pMIR-Sox4 -Mut. Primers used for the constructions were listed in Table S1. All the constructions were confirmed by sequencing.

**Cell migration assay**

Boyden chamber assay was used to examine cell invasion capability. TE13 and KYSE-150 cells were transfected with miR-133a mimic, mimic control, miR-133a inhibitor or inhibitor control. Sixteen hours later, transfected cells were trypsinized and resuspended, 1.0×10⁴ cells in 200 μl RPMI-1640 medium were placed into the upper chambers (8-mm pore size; Millipore). The lower chambers were filled with 600 μl complete medium with 10% FBS. After incubation for 12 h at 37°C, non-invading cells were removed from the top of the chamber with a cotton swab. The invasion cells on the lower surface of the inserts were fixed and stained with 0.1% crystal violet, and five random fields for each insert were counted at 200× magnifications.

**Proliferation assay**

MTT assay was used to analyze cell proliferation. TE13 and KYSE-150 cells were transfected with either miR-133a mimic (miR-133a) or mimic control (NC). After 24 h transfection, cells were seeded into 96-well plate at 5.0×10³ cells/ml and continue cultured for 24, 48, 72, 96 h, and 120 h, respectively. At each time point, 10 μl MTT reagent (5 mg/ml, Sigma) was added to each well, successive incubated for 4 h at 37°C. The supernatant was removed and 200 μl DMSO (Invitrogen) was added to dissolve the formazan crystals for 30 min. Spectrometric absorbance at a wavelength of 570 nm was measured on microplate reader (Spectra Max M5, MD, USA). Each sample was tested in triplicate and all experiments were performed three times.

**Cell cycle analysis**

Flow cytometry assay was used to analyze cell cycle. TE13 and KYSE-150 cells were plated onto the 6-well plate, respectively. After 24 h, cells were transfected with miR-133a mimic (miR-133a) or mimic control (NC) using lipofectamine 2000. Six hours after the transfection, we exchanged the medium with RPMI-1640 containing 10% FBS. At 48 hours post-transfection, cells were harvested, fixed over-night at 4°C and stained with a propidium iodide solution for 30 min, and then were analyzed using a Flow Cytometry (Beckman Coulter, USA) and WinCycle.
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In vivo assay

For the in vivo metastasis assays, the stable cell line TE13-luc-miR-133a and control cell line TE13-luc-pcDNA3.1 were collected and suspended in 0.2 ml PBS for each mouse (four in each group, 6-8 weeks age), and the cells were injected into left side of the posterior flank of nude mouse. Thirty minutes after cell injection, luciferase substrate was added at a dose of 150 mg/kg and live images of the mouse were obtained using an IVIS200 (Xenogene, USA). These data were classified as Day 0. Luciferase activity was measured every 7 days using the same protocol. Tumor growth was measured periodically. The fluorescence intensity was calculated using Plus Image-Pro software (Media Cybernetics, Bethesda, MD, USA).

Dual-luciferase reporter assay

Cells were seeded into 24-well plates and cotransfected with 200 ng of pMIR-Sox4 or pMIR-Sox4-Mut vector and 100 ng of miR-133a mimic or mimic control, and the pRL-TK plasmid (Promega, Madison, WI) which was used as internal normalization. After 48 h, cells were lysed using the lysis buffer (Promega). Luciferase reporter gene assay was implemented using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. All experiments were performed at least three times.

Western blotting

Cells were transfected with either miR-133a, pCMV-Tag-2b-Sox4 or small interfere RNA target Sox4 (si-SOX4). Total cell extracts prepared from cells using RIPA buffer (Beyotime, China), were resolved on 10% gradient SDS-polyacrylamide gel and transferred NC membranes. Membranes were blocked for 1 hour in 5% skim milk in TBST and incubated with primary antibody overnight at 4°C, followed by the incubation with appropriate HRP-conjugated secondary antibody at optimized concentration. The primary antibodies used in this study were as follows: anti-Sox4 antibody (1:1000, Santa Cruz), anti-E-cadherin antibody (1:1000, Santa Cruz), anti-vimentin antibody (1:1000, Santa Cruz) and anti-β-actin antibody (1:5000, CST). The densitometry of Western blot results was measured using ImageJ software.

Immunofluorescence

TE13 Cells were seeded onto glass coverslips in 24-well plates, washed with PBS, fixed with 4% formaldehyde solution for 20 min at room temperature, and permeabilized with 0.2% Triton X-100 for 15 min. Subsequently, cells were blocked with 2% BSA in PBS for 30 min at room temperature. Cells were incubated with primary antibodies (E-cadherin 1:300, and vimentin 1:400) for 1 h, followed by incubation with TRITC-conjugated secondary antibodies.
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Figure 2. MiR-133a inhibited the aggressiveness of ESCC in vitro. A. The wound healing was used to detect the migration ability of TE13 and KYSE150 cells, respectively. B, C. Boyden chamber assay was employed to examine the invasion ability of TE13 and KYSE150 cells, respectively. The results were from three independent experiments. The migratory cell number in each group was normalized to the control. Cells were transfected with miR-133a mimic or mimics control, and miR-133a inhibitor or inhibitor control. D. MTT assay was employed to examine the proliferation ability of TE13 and KYSE150 cells, respectively. Cells were transfected with miR-133a mimic or mimics control. E. Flow cytometry analysis was performed to examine the effect of miR-133a on the cell cycle in TE13 and KYSE150 cells, respectively. The right panel showed the percentage of cells in G1, G2 and S phase after transfection of miR-133a mimics or mimics control. (*P<0.05, t-test).

Figure 3. Downregulation of miR-133a contributes to ESCC progression in vivo. The miR-133a overexpressed TE13-luc-miR-133a cells (miR-133a) and negative control cells TE13-luc-pcDNA3.1 (NC) were injected into the mice (5/group). A. Representative images of tumors are shown in the right on the indicated days. and tumor growth curve in mice are shown in the left. Data are mean ± SD. B. The luciferase intensity of each tumor was measured (right) on Day 28 and mean luciferase intensity of each group was shown in the left. The pseudo-color scale bars represent the intensity of light emission with different colors. Data are reported as mean ± SD (*P<0.05, t-test).

( Zhongshan Golden Bridge Biotechnology, China) for 1 h, and then stained with Hoechst. Finally, coverslips were observed under a fluorescence microscope.

Statistical analysis

The data were presented as mean ± standard deviation (SD). MiR-133a expression in 45
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**Results**

miR-133a is down-regulated in ESCC cells and tissue specimens

Firstly, we analyzed the expression level of miR-133a in human esophageal epithelial cell SHEE and four human esophageal carcinoma cell lines including KYSE150, KYSE510, EC9706 and TE13 by qRT-PCR. The results showed that miR-133a was down-regulated significantly in
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all the four esophageal carcinoma cell lines compared with the SHEE cell line (Figure 1A). Further, we detected miR-133a expression in both ESCC tissues and corresponding adjacent tissues by qRT-PCR. We found that miR-133a expression was decreased in ESCC tissues compared with adjacent tissue (n=45, Figure 1B, p<0.05), which indicated that miR-133a was acted as a tumor suppressor. After analyzing the clinical information of patients, we found that the aberrant expression level of miR-133a was associated with the pathological stage and lymph node metastasis of patients (Table 1, p<0.05). Taken together, these results suggest a possible link between reduced expression of miR-133a and the progression of human ESCC.

Downregulation of miR-133a promotes the aggressiveness of ESCC cells in vitro

Next, we tested the functional significance of miR-133a in ESCC cells. Wound healing assay showed that the ectopic expression of miR-133a in TE13 and KYSE150 cells significantly inhibited cell migration, compared to the control group (Figure 2A). Additionally, we performed the Boyden chamber assay to investigate the effect of miR-133a on cell invasion. As shown in Figure 2B and 2C, after transfected...
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with miR-133a mimics, the invasion ability of KYSE150 and TE13 cells decreased, compared to the control group. However, the cells showed an increased invasion upon the treatment of miR-133a inhibitor (Figure 2B, 2C). Taken together, the data strongly suggest that miR-133a is able to suppress the migration and invasion of ESCC cells in vitro.

Additionally, we used MTT assay to investigate the effect of miR-133a on cell proliferation. As shown in Figure 2D, when transfected with miR-133a mimics, the proliferation ability of TE13 and KYSE150 cells was down-regulated compared to the control group. Flow cytometric analysis of cell cycle was performed to investigate the mechanism by which ectopic expression of miR-133a inhibits cell proliferation. As showed in Figure 2E, the ectopic expression of miR-133a in TE13 decreased the percentage of cells in G2/S phase (54.7%) compared with control group (68.1%). Similarly, ectopic expression of miR-133a in KYSE150 cells resulted in a decreased percentage of cells in G2/S phase from 41.4 to 27.0 (P<0.05). These results suggest that miR-133a could inhibit cell proliferation through regulate cell cycle progression.

**Downregulation of miR-133a contributes to ESCC progression in nude mouse xenograft model**

To confirm the tumor suppressor role of miR-133a, we established a stable cell line with overexpression of miR-133a, termed TE13-luc-miR-133a (miR-133a), and prepared another stable cell line TE13-luc-pcDNA3.1 as negative control (NC). miR-133a and NC cells were injected into nude mice, and the fluorescence intensity was measured every 7 days until Day 28. As shown in Figure 3A, the luciferase intensity of tumor in miR-133a group increased slowly after Day 7 compared to the negative control group. The result showed that the growth ability in miR-133a overexpressed group was down-regulated compared to the control group. Figure 3B showed the the luciferase intensity of tumor in all two groups at Day 28, the cells proliferation ability in miR-133a group was significantly reduced relative to the control group (P<0.05). The results indicate that miR-133a significantly inhibits the tumorigenicity of TE13 cells in the nude mouse xenograft model.

**MiR-133a directly inhibits the expression of Sox4 through its 3'UTR**

To gain further insight into the molecular mechanism by which miR-133a suppresses migration and invasion of esophageal carcinoma cells, we searched for genes targeted by miR-133a using biological target prediction softwares TargetScan and PicTar. The gene encoding sex-determining region Y-box 4 (SOX4) harbored a potential miR-133a binding site within its 3'UTR (Figure 4A). To detect whether SOX4 is regulated by miR-133a, the wild type or mutant 3'UTR sequence of Sox4 was cloned into pMIR reporter vector, respectively, as shown in Figure 4B. The luciferase activity of pMIR-SOX4-3'UTR-wt construct was significantly decreased upon the over-expression of miR-133a in TE13 cells, whereas its mutant counterpart was not (Figure 4C). In addition, the protein level of SOX4 in TE13 and KYSE150 cells was dramatically reduced by miR-133a (Figure 4D-F). Taken together, these data indicated that SOX4 is a direct target of miR-133a at least in esophageal cancer.

It is reported that Sox4 acted upstream of the EMT inducers during EMT, through suppressing E-cadherin or inducing vimentin expression in human cancer [37]. To further confirm that Sox4 acts as a target of miR-133a, we examine the effect of miR-133a on those two downstream effectors of Sox4 by Western blot. As shown in Figure 4D, 4E, the EMT maker E-cadherin or vimentin was dramatically up-regulated or down-regulated upon the over-expression of miR-133a in TE13 and KYSE150 cells. Taken together, these data indicate that miR-133a directly inhibits Sox4 expression via targeting its 3'UTR and induces EMT of ESCC cells.
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Sox4 inversely correlate with miR-133a expression in ESCC tissue specimens

Then, we examined whether miR-133a downregulation mediated Sox4 activation in ESCCs was clinically relevant. As shown in Figure 5A, correlation studies in 45 ESCCs specimens showed that miR-133a levels was inversely correlated with the expression of Sox4. In addition, Sox4 expression was markedly up-regulated compared to adjacent tissues in 45 pairs of matched esophageal cancer specimens (Figure 5B). We also found that the expression of EMT markers, E-cadherin and vimentin were also changed related to SOX4 expression by immuno-ﬂuorescence and Western blot methods (Figure 5C, 5D). As can be seen, the epithelial marker E-cadherin was decreased in response to SOX4 overexpression, and the mesenchymal marker vimentin was elevated in response to SOX4 overexpression, which suggested that Sox4 could affect the EMT process in TE13 cells.

Sox4 contributes to miR-133a suppressed migration and invasion of ESCC cells

Having demonstrated Sox4 as a direct target of miR-133a, we next examined the importance of Sox4 in miR-133a mediated migration and invasion of ESCC cells. Ectopic expression of Sox4 in TE13 cells signiﬁcantly enhanced cell migration and invasion ability (Figure 6A, 6B), however, silencing Sox4 by siRNAs in TE13 cells decreased migration and invasion ability of the cells (Figure 6C, 6D), revealing its positive roles in the contribution of ESCC cells migration and invasion. Meanwhile, the transfecting or silencing efﬁciency of Sox4 in the cells was detected by Western blot (Figure 6B, 6D lower panel). Then, we assessed whether the functional effect of miR-133a on ESCC cells was dependent on Sox4. As shown in Figure 6E and 6F, ectopic expression of Sox4 abrogated miR-133a effects on cell migration and invasion. In parallel, the protein level of Sox4 was conﬁrmed by Western blot (Figure 6F, lower panel). Collectively, these results suggested that Sox4 functions as a target of miR-133a, responsible for miR-133a mediated regulation of the migration and invasion of ESCC cells.

Discussion

In this study, we found that miR-133a was markedly decreased in ESCC cell lines and tissue specimens. Thus, we supposed that miR-133a may be a novel tumor suppressor miRNA and its dyregulation may involve in initial tumorigenesis and the advanced progress of human cancer. These results are similar to those for other malignant cancers, such as colorectal cancer [17], breast cancer [20] and prostate cancer [21]. For the mechanism involving miR-133a downregulation, Chen WS, et al. reported that the hyper-methylation in the promoter region was responsible for the reduced expression of miR-133a [40]. Accordingly, we speculated that this DNA modiﬁcation might cause the alteration of miR-133a expression in human ESCC.

Next, we investigated the function of miR-133a in ESCC cells. Our data showed that the introduction of miR-133a dramatically repressed proliferation, migration, invasion and cell cycle of ESCC cells in vitro. Therefore, our data suggested that the decreased expression of miR-133a may contribute to the growth and metastasis of cancer and consequently facilitate the advanced development of ESCC. Furthermore, we used nude mouse xenograft model to conﬁrm the role of miR-133a in vivo, we found that miR-133a signiﬁcantly inhibited xenograft tumorigenesis and growth. These results indicated that downregulation of miR-133a contributes to ESCC progression.

A miRNA can coordinate a large number of target genes [41]. Several miR-133a targets have been identifed in different cells and organs. Such as, EGFR was found to be a target of miR-133a, which mediates an effect on cell proliferation, migration and invasion ability in prostate cancer cells [21]. Dong et al indicated that miR-133a could induce a G0/G1-phase arrest by directly target RFFL and activating p53/p21 pathway [42]. miR-133a directly target Fascin1 (FSCN1) inhibited colorectal cancer cell invasion ability [17]. Our present study found that Sox4 was a functional target of miR-133a by luciferase reporter gene assays and western blot analysis method.

Increasing evidence suggests that Sox4 is over expressed in several human cancers and is closely correlated with tumor invasion and metastasis [31-35]. It is also one of members of EMT-transcriptional inducers [36-38], EMT is a key developmental program that is often activated during cancer progression and may promote resistance to therapy [43]. Zhang et al
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[44] showed that overexpression of SOX4 in human mammary epithelial cells led to the acquisition of mesenchymal traits, and enhanced cell migration and invasion. Furthermore, SOX4 positively regulated the expression of known EMT inducers and activated the TGF-β pathway to contribute to EMT [38]. To date, EMT is an attractive target for therapeutic interventions, provides a new basis of the progression of carcinoma towards dedifferentiated and more malignant states [45, 46]. In our study, we found that Sox4 had a frequently high expression in ESCC tumor specimens compared with adjacent tissues. Sox4 expression inversely correlated with miR-133a expression in ESCC tissue specimens. And Sox4 was responsible for miR-133a modulated migration and invasion of ESCC cells. Notably, we found that E-cadherin or vimentin, the downstream effector of Sox4, was also down-regulated or up-regulated by miR-133a, indicating that miR-133a may exert functions in migration and invasion of ESCC cells by modulating EMT progress.

In summary, we investigated the role of miR-133a in ESCC progression and metastasis. Our study indicates that miR-133a may be a novel tumor suppressor miRNA. miR-133a inhibits the migration and invasion of ESCC cells through targeting the EMT regulator Sox4. Our data provide new insight into the mechanism responsible for the development of human ESCC. Therefore, targeting miR-133a could be a promising therapeutic strategy in ESCC.

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Disclosure of conflict of interest
None.

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References
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### Table S1. Primers used in the paper were listed

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