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Position-dependent correlation between TBX22 exon 5 methylation and palatal shelf fusion in the development of cleft palate

KE LI[1], XUAN SHU[1], HUI GONG[2], LIUHANGHANG CHENG[1], ZEJUN DONG[1] and SHENYOU SHU[1]

[1]The Cleft Lip and Palate Treatment Center, Second Affiliated Hospital of Shantou University Medical College, 69, Dongxia North Road, Jinping District, Shantou, 515041, China
[2]The Department of Gynaecology, Second Affiliated Hospital of Shantou University Medical College, 69, Dongxia North Road, Jinping District, Shantou, 515041, China

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Abstract: DNA methylation is essential for spatiotemporally-regulated gene expression in embryonic development. TBX22 (Chr X: 107667964-107688978) functioning as a transcriptional repressor affects DNA binding, sumoylation, and transcriptional repression associated with X-linked cleft palate. This study aimed to explore the relationship and potential mechanism between TBX22 exon 5 methylation and palatal shelf fusion induced by all-trans retinoic acid (ATRA). We performed DNA methylation profiling, using MethylRAD-seq, after high throughput sequencing of mouse embryos from control (n=9) and ATRA-treated (to induce cleft palate, n=9) C57BL/6J mice at embryonic gestation days(E) 13.5, 14.5 and 16.5. TBX22 exon 5 was hyper-methylated at the CpG site at E13.5 (P=0.025, log2FC=1.5) and E14.5 (P=0.011, log2FC:1.5) in ATRA-treated, whereas methylation TBX22 exon 5 at the CpG site was not significantly different at E16.5 (P=0.808, log2FC=-0.2) between control and ATRA-treated. MSP results showed a similar trend consistent with the MethylRAD-seq results. qPCR showed the change in TBX22 exon 5 expression level negatively correlated with its TBX22 exon 5 methylation level. These results indicate that changes in TBX22 exon 5 methylation might play an important regulatory role during palatal shelf fusion, and may enlighten the development of novel epigenetic biomarkers in the treatment of CP in the future.

Key words: DNA methylation, cleft palate, TBX22, CpG site.

INTRODUCTION

DNA methylation is an epigenetic event that plays an essential role in the regulation of temporal and spatial gene expression during embryonic development (Shiota 2004). It is the process by which a methyl group is added to a DNA molecule and usually occurs at the C5 position of cytosine within CpG islands of genomic DNA (Barrès et al. 2009, Law and Jacobsen 2010), where a gene-exon region (gene body) uses methylation to repress exon expression or affect gene transcription between the first coding exon and last exon (Chuang et al. 2012, Gelfman et al. 2013). Promoter methylation is associated with gene silencing (Zaidi et al. 2010), and intergenic methylation may affect gene expression through enhancer regulation

Correspondence to: Shenyou Shu
E-mail: syshu@stu.edu.cn
ORCid: https://orcid.org/0000-0001-9283-4114

(Stadler et al. 2011). During embryogenesis, DNA methylation of certain genomic sequences or even an entire chromosome definitively inactivates gene transcription for dose compensation, such as the X chromosome (Li et al. 1993, Beard et al. 1995), or in differentiated cells (Kafri et al. 1992). Failure to establish the normal methylation patterns can result in cleft palate formation (Bliek et al. 2008, Kuriyama et al. 2008, Loenarz et al. 2010).

Cleft palate (CP) is a congenital malformation in which both genetic and environmental factors are involved, and is due to the failure of palate shelves to appose at the midline and fuse (Vieira 2008, Stuppia et al. 2011, Rahimov et al. 2012). Palatal fusion involves not only temporal and spatial-regulated disruption of embryonic palatal medial edge epithelia (MEE), but also dynamic cellular and molecular processes that result in adhesion and intercalation of the embryonic MEE to form an inter-shelf epithelial seam, and subsequent remodeling and fusion to form the intact roof of the oral cavity (Rice 2005, Thiery and Sleeman 2006, Nawshad 2008, Bush and Jiang 2012, Lan et al. 2015). In the mouse, the palatal shelf grows first vertically in the oral cavity, then elevates into a horizontal position above the tongue at E13.5. The opposing palatal shelves adhere along the MEE to form the midline epithelial seam (MES) at E14.5, then complete palatal shelf fusion at E16.5 (Gritli-Linde 2007). Increasing evidence indicates that TBX22 is a susceptible gene for CP (Braybrook et al. 2001, Bush et al. 2002, Jiang et al. 2012). TBX22 (Chr X: 107667964-107688978) functions as a transcriptional repressor affect DNA binding, sumoylation, and transcriptional repression associated with X-linked cleft palate (Braybrook et al. 2001b). Several studies show that mutations in TBX22 exon 5 makes a significant contribution to prevalence of cleft palate (Braybrook et al. 2002, Marçano et al. 2004, Suphapeetiporn et al. 2007). Sequence analysis shows a C→T transition in TBX22 exon 5 at nucleotide 785C→T which causes a nonconservative amino acid substitution from threonine to methionine (Braybrook et al. 2001b). DNA methylation can significantly increase the rate of spontaneous C→T mutations at CpG dinucleotides (Ehrlich and Wang 1981, Hwang and Green 2004, Mugal and Ellengren 2011). However, it is not clear whether TBX22 exon 5 methylation is involved embryonic palatal shelf fusion induced by maternal exposure to all-*trans* retinoic acid (ATRA).

To explore the mechanisms of TBX22 exon 5 methylation during embryonic palatal shelf fusion, we integrated methylation data for TBX22 exon 5 from previous MethyRAD-seq data, used MSP and qPCR to validate TBX22 methylation at a CpG site in exon 5, and correlated DNA methylation with TBX22 exon 5 mRNA expression level to elucidate the involvement of DNA methylation during embryonic palatal shelf fusion.

MATERIALS AND METHODS

ANIMALS AND TREATMENT

C57BL/6J mice, 20–28 g in body weight and 8 to 10 weeks of age, were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). In this study, female mice were mated with male mice of similar weight and age overnight (n=18, 3 groups, 3 ATRA-treated samples *vs*. 3 control samples). For this study, an ATRA (Sigma-Aldrich, St. Louis., MO, USA)-induced mouse cleft palate model was used, and embryonic palatal shelf tissues were resected at E13.5, E14.5, and E16.5, as reported previously (Qin et al. 2014, Shu et al. 2018). This animal study protocol was approved by the Laboratory Animal Ethical Committee of Medical College of Shantou University (SUMC2015-106; Shantou, China), and experiments were carried out in accordance with the animal care guidelines of the US National Institutes of Health.

DETERMINATION OF METHYLATION LEVEL IN THE CpG SITE OF TBX22 EXON 5

To determine enhancer DNA methylation level in a CpG site, genomic DNA at E13.5, E14.5, and E16.5 was extracted from embryonic mouse palatal shelves, using the conventional cetyltrimethylammonium bromide method, and a genomic library was prepared using MethylRAD (Cohen-Karni et al. 2011, Wang et al. 2015). Clean reads were then subjected to paired-end sequencing on a HiSeq X Ten sequencer (Illumina Inc, San Diego, CA, USA), according to the manufacturer's protocol, by the Shanghai Oebiotech Co. Ltd (Shanghai, China). According to the reference sequence, individual enzyme reads were mapped to the reference sequence of the C^mCGG sites using the SOAP program (version 2.21) (Li et al. 2009). The differential in DNA methylation at different methylation sites was assessed between ATRA-treated and control in three biological replicates.

METHYLATION-SPECIFIC PCR (MSP) VALATION

The level of the TBX22 exon 5 methylation was validated using MSP. In brief, genomic DNA was extracted from mouse palatal shelf tissues using a rapid DNA extraction kit (Sino Gene Scientific, China). After quantification, 1 μg of each DNA sample was subjected to bisulfite modification using a DNA methylation modification kit (Zymo Laboratories, Inc., South San Francisco, CA, USA) and PCR amplification according to the manufacturer's protocol. MSP primers were designed to amplify TBX22 exon 5 using the online software MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi), and synthesized by Sino Gene Biotech (Beijing, China) (Table I). The PCR products were then separated in a 2% agarose gel by electrophoresis, stained with ethidium bromide, and visualized under an ultraviolet illuminator (JY04S-3C, Beijing). The distinct visible band of the amplicon with methylation-specific primers was considered the DNA methylation band, and the density of each band was analyzed using image analysis software (Gel-Pro 4.5) for quantitation.

qPCR VALIDATION

To validate the MethylRAD-seq data, qPCR was conducted in 18 individual samples (triplicates). The qPCR primers used in this study are listed in Table I. The mRNA expression level was analyzed as described in a previous study, and the $2^{-\Delta\Delta}$Ct method was used to calculate the level of gene expression relative to the expression of β-actin, as an internal control (Livak and Schmittgen 2001).

STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). The difference in methylation level, between control and ATRA-treated, was assessed using the R package edge R, as described in a previous study (Robinson et al. 2010). The PCR data were analyzed using Student's *t*-test to compare the means between ATRA-treated and control samples. A p-value <0.05 and $\log_2 FC > 1$ were considered statistically significant.

RESULTS

MORPHOLOGY AND HISTOLOGY OF EMBRYONIC PALATE SHELF TISSUE

In palate shelf histological sections of control embryos, it can be observed that the palatal shelf was separated at E13.5, contacted the midline at E14.5, and completely fused at E16.5, whereas in palate histological sections from ATRA-treated embryos, the palatal shelf remains separated without fusion at E13.5, E14.5, and E16.5 (Fig. 1).

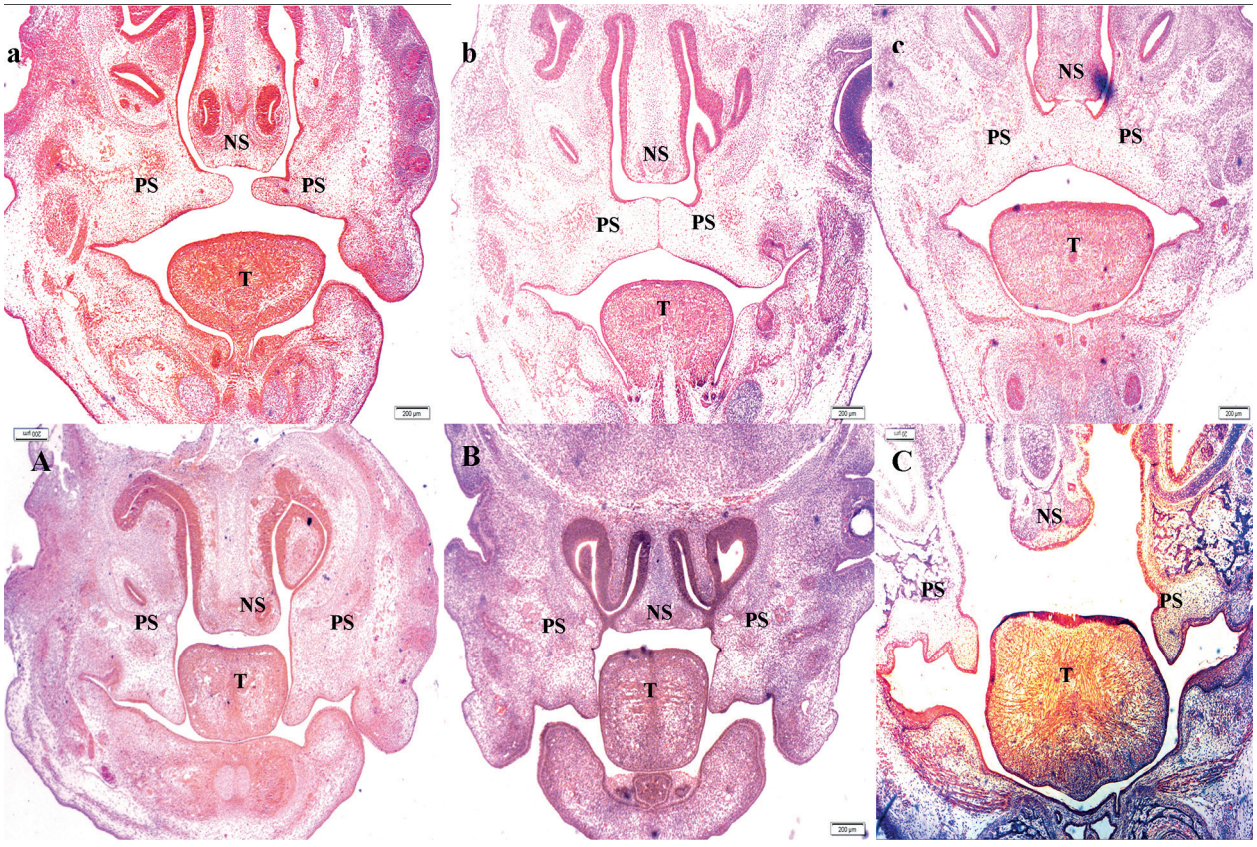

Figure 1 - Morphology and histology of palate shelf tissues at E13.5(a, A), E14.5(b, B), and E16.5(c, C) between control *vs.* ATRA-treated mice. Pregnant mice were administered ATRA (70 mg/kg) by gavage at E10.5 to establish a cleft palate (CP) model in C57BL/6J mice (ATRA-treated). Control groups were given the equivalent volume of corn oil. Pregnant mice were dissected at E13.5, E14.5, E16.5 (n=24, 3 groups, 4 ATRA-treated samples *vs*. 4 control samples) to obtain the embryonic palates, and then paraffin coronal sections of palatal shelves and hematoxylin and eosin staining was performed to study the changes of morphology and histology (n=6, 3 groups, 1 ATRA-treated samples *vs*. 1 control samples). (A–C) Unfused, separated palatal shelf from an embryo of an ATRA-treated mouse. **a**. The palatal shelf separated. **b**. The palatal shelf contacted the midline. **c**. The palatal shelf completely fused. PS, palatal shelf; T, tongue; NS, nasal septum.

TABLE I
Primers of TBX22 used for MSP and qPCR.

Primer	Primer sequence	Size (bp)
MF	5′- TTTTTAGATTTTATGTTTATTCGG-3′	100
UF	5′- tAATTTTTAGATTTTATGTTTATTt-3′	
UR	5′-TACATAACCCTTATCATCCATCTCATT-3′	
Sense	5′-CTTCTCACACTTTTGCGCCTT-3′	90
Antisense	5′-GTCACTGGAGATGAGCCACT-3′	

MF: forward primer sequence for the methylation reaction; UF: forward primer sequence for the un-methylation reaction; UR: reverse primer sequence for un-methylation reactions.

COMPREHESIVE ANALYSIS OF MethylRAD-seq DATA FOR DETERMINATION OF TBX22 EXON 5 METHYLATION

According to the MethylRAD-seq data, hierarchical cluster analysis indicated that the methylation level of differentially methylated site among ATRA-treated palates is higher than those of control at E13.5, E14.5, and E16.5 (Fig. 2). TBX22 exon 5 is among the sequences of differentially-methylated DNA, based on the annotation and sequence alignment in MethylRAD. TBX22 (Chr X: 107667964-107688978) exon 5 (http://asia.ensembl.org/Mus_musculus/Transcript/Exons?db=core;g=ENSMUSG00000031241;r=X:107667964-107688978;t=ENSMUST00000118986)(Fig. 3). The enzyme read sequence was GATTCTATGTCCAC**C^{m}CGG**ACTCCCCTTGCTCT, clearly showing that TBX22 exon 5 methylation occurs at the C5 position of cytosine within the CpG site in the genome. According to MethylRAD-seq, ATRA treatment resulted in hypermethylation of DNA within TBX22 exon 5 at the CpG site at E13.5 (P=0.025, $\log_2$FC=1.5), and E14.5 (P=0.011, $\log_2$FC:1.5), but there was no significant difference in methylation, between ATRA-treated and control, at E16.5 (P=0.808, $\log_2$FC=-0.2) (Files 1, 2 and 3). The files 1, 2 and 3 can be found at the following link: https://pan.baidu.com/s/1R2M252-jHj6BemXYSduHCw. Password: 7qzl.

CONFIRMATION OF TBX22 EXON 5 METHYLATION AT THE CpG SITE BY MSP

Methylation of TBX22 exon 5 at the CpG site was examined by MSP. The MSP results showed a similar trend consistent with the MethylRAD-seq results. The relative density of methylated MSP to that of unmethylated MSP in ATRA-treated was increased at E13.5 and E14.5, whereas TBX22 exon 5 methylation at the CpG site was not significantly different, from the controls, at E16.5, indicating that DNA at TBX22 exon 5 was hypermethylated at E13.5 and E14.5 in CP embryos, but not significantly different at E16.5 in mice during palatal fusion induced by maternal exposure to ATRA (Fig. 4a).

DECREASED TBX22 EXON 5 EXPRESSION CORRELATES WITH ELEVATED METHYLATION

At E13.5, the relative expression levels of TBX22 exon 5 in ATRA-treated was down-regulated compared with controls (P=0.018), as well as at E14.5 (P=0.008). TBX22 exon 5 was not significantly different between ATRA-treated and controls at E16.5 (P=0.286) (Fig. 4b). When comparing mRNA expression and methylation, reciprocal relationships were found in TBX22 exon 5 in ATRA-treated embryos, which indicated hyper-methylation at a CpG site within the TBX22 exon 5, concomitant with decreased exon 5 expression when compared with control at E13.5 and E14.5.

DISCUSSION

Altered gene expression can be due to mutations and/or epigenetic regulation, such as DNA methylation, of genes. A previous study showed that several aberrant DNA methylation genes are involved in cleft lip and palate formation, such as CDH1and TGF-β3 (Liu et al. 2016, Alvizi et al. 2017). There are three major aspects of molecular control of palatal fusion, i.e. global alterations, site-level local alterations (e.g. exons), and the impact of these alterations on gene expression (Kuriyama et al. 2008, Lan et al. 2015). In this study, we compared the genome-wide DNA methylation profiles of embryonic mouse palatal tissues between ATRA-treated and controls at E13.5, E14.5, and E16.5, and then assessed methylation and the implication of aberrantly methylated TBX22 exon 5 in cleft palate formation. We then confirmed our data using MSP and qPCR and further showed, by qPCR.

TBX22, encodes an X-linked T-box transcription factor, and is a major genetic determinant for familial cleft palate (Kantaputra

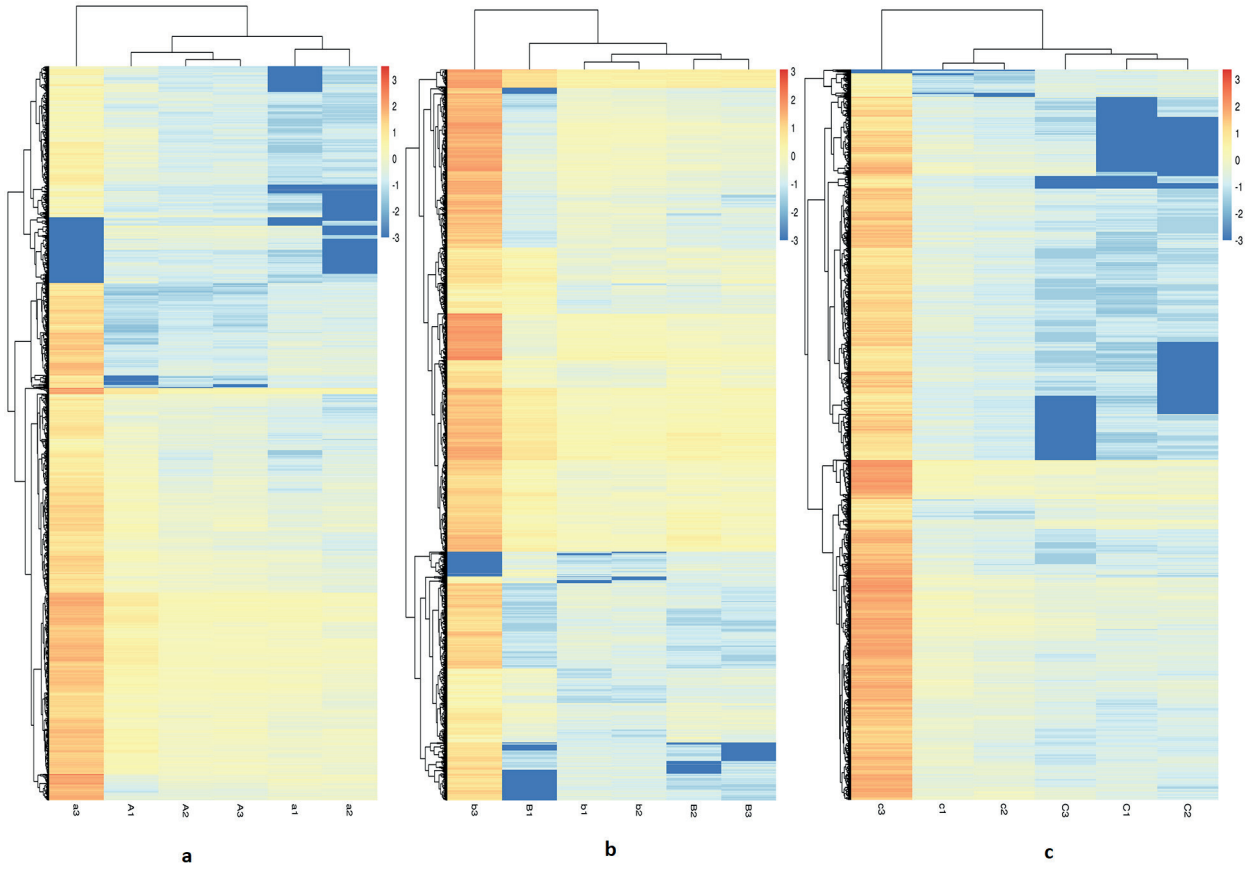

Figure 2 - Hierarchical cluster analysis heat-map of differential C^{m}CGG methylation sites between ATRA-treated and control in the stage of differential development. Using MethylRAD technology combined with Paired-End sequencing on a HiSeq X Ten platform, a label containing C^{m}CGG sites was proposed as the reference sequence. The individual enzyme reads were mapped to the reference sequence of the C^{m}CGG sites using the SOAP program (version 2.21, parameter: -M4-v2-r0). For assessing the methylation level of differential methylation sites between ATRA-treated and control for the three biological replicates, cluster analysis was performed to further reveal the changes in C^{m}CGG sites methylation level (n=18, 3 groups, 3 ATRA-treated samples *vs*. 3 control samples). Brown indicates hypermethylation sites, and blue indicates hypomethylation sites respectively (P<0.05, $\log_2$FC >1). (**a**):at E 13.5, (**b**): E14.5, (**c**): at E16.5.

et al. 2011). Some studies have shown that TBX22 expression disappears before palatal shelf fusion and is thought instead to be required for mesenchymal proliferation and palatal shelf elevation (Marçano et al. 2004, Suphapeetiporn et al. 2007). DNA methylation can significantly increase the rate of spontaneous C→T mutations at CpG dinucleotides (Ehrlich and Wang 1981, Hwang and Green 2004, Mugal and Ellegren 2011). Therefore, DNA methylation is more strongly correlated with C→T mutations at CpG dinucleotides in TBX22 exon 5. However, the underlying mechanism of TBX22 involved in CP is still unknown.

In the current study, we achieved four research objectives of elucidating the role of TBX22 exon 5 epigenetics in palatogenesis following ATRA-induced cleft palate formation: 1) identification of histology associated with cleft palate (Fig. 1); 2) identification of a TBX22 exon 5 at a CpG site involved in cleft palate; 3) identification of changes in TBX22 exon 5 expression related to cleft palate *vs*. DNA methylation level; and 4) characterization of the DNA methylation patterns in TBX22 exon 5 at the CpG site. However, our current study is preliminary and much more research is needed to disclose the relationship of TBX22 exon 5

Figure 3 - Methylation site of TBX22 exon 5 is at a CpG site (http://asia.ensembl.org/Mus_musculus/Transcript/Exons?db=core;g=ENSMUSG00000031241;r=X:107667964-107688978;t=ENSMUST00000118986).

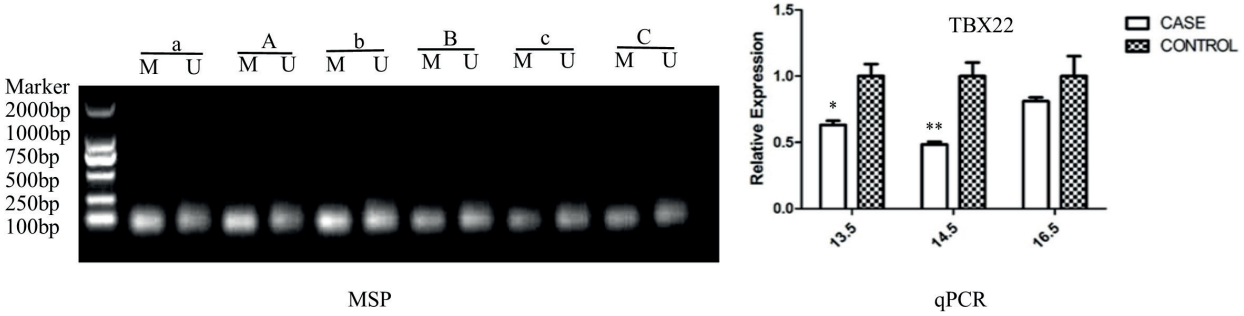

Figure 4 - MSP and qPCR were performed at E13.5, E14.5, and E16.5 in ATRA-treaded (case) *vs*. control. MSP: Genomic DNA was extracted from mouse palatal shelf tissues using a rapid DNA extraction kit. After quantification, each DNA sample was subjected to bisulfite modification using a DNA methylation modification kit and PCR amplification. The PCR products were then separated in a 2% agarose gel by electrophoresis, stained with ethidium bromide, and visualized under an ultraviolet illuminator. The distinct visible band of the amplicon with methylation-specific primers was considered the DNA methylation band, and the density of each band was analyzed using image analysis software (Gel-Pro 4.5) for quantitation. qPCR: The same RNA samples were used for reverse transcription. cDNA was synthesized using the Thermo First cDNA Synthesis Kit. The qPCR data are shown as the mean ± S.E.M, and the $2^{-\Delta\Delta}$Ct method was used to calculate the level of gene expression relative to the expression of β-actin, as an internal control. All reactions were carried out in triplicate for technical and biological repetitions (n=18, 3 groups, 3 ATRA-treated samples *vs*. 3 control samples). (**a**): TBX22 exon 5 methylation level validation by MSP. The lengths of markers from top to bottom are 2000, 1000, 750, 500, 250, and 100 bp. "U" and "M" indicate unmethylated and methylated sites, respectively. Lane a-c: control; Lane A-C: ATRA-treated. (**b**): TBX22 exon 5 expression validation by qPCR. TBX22 exon 5 in ATRA-treated was obviously down-regulation compared with control at E 13.5 (P=0.018) and at E 13.5 (P=0.008), TBX22 exon 5 was no significant difference of expression level at E16.5 (P=0.286). MSP, methylation-specific polymerase chain reaction; qPCR, quantitative real-time PCR.

alterations and cleft palate formation. Our sample size was relatively small, and palatal shelves were directly obtained from embryonic mouse tissues that could be mixed with other tissues. We also cannot exclude the effect of ATRA and its endogenous small molecule metabolites on methyltransferases, which further affects the bias of our experimental results. Our data revealed a novel site (TBX22 exon 5 CpG site) of DNA methylation that is associated with cleft palate formation.

CONCLUSIONS

Taken together, our results demonstrate that hypermethylation TBX22 exon 5 at CpG site could inhibit TBX22 exon 5 expression induced by ATRA. The change in methylation of TBX22 at the exon 5 CpG site could be associated with the manifestation of CP. DNA methylation at the TBX22 exon 5 CpG site may provide a foundation for future studies and may serve as a future epigenetic target for development of novel therapeutics for CP.

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AUTHOR CONTRIBUTIONS

KL: Conception and design of research, performed experiments, analyzed data, interpreted results of experiments, edited and revised manuscript, approved final version of manuscript. XS: Performed experiments, analyzed data, interpreted results of experiments, approved final version of manuscript. ZJD: Analyzed data. SYS: Edited and revised manuscript. HG: Conception and design of research, interpreted results of experiments, edited and revised manuscript, approved final version of manuscript. LHHC: Edited and revised manuscript, interpreted results of experiments, approved final version of manuscript.

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