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Research Article

Role of *SATB2 5'* Untranslated Region Promoter Methylation in Formation of Non-syndromic Cleft Palate Only

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Abstract

Objectives: Accumulating evidence has implicated DNA methylation in the development of non-syndromic cleft palate only (NSCPO); however, little is known about the underlying epigenetic mechanism. This study was to elucidate the role of SATB2 5'untranslated region (UTR) promoter methylation in formation of NSCPO.

Methods: DNA methylation profiling was performed on discarded human palatal tissue after repair of NSCPO (case) or maxillofacial and palate trauma (control), using an Illumina 850K-EPIC BeadChip methylation array. The SATB2 5'UTR promoter methylation level was confirmed by pyrosequencing.

Results: Five CpG sites (cg14273610, cg22334352, cg25103650, cg22845542 and cg06199336) in the SATB2 5'UTR promoter was hypermethylated in cases compared with controls (P<0.05). Pyrosequencing revealed a mean methylation rate of 31.81% vs. 16.45% (p=0.0019) at the cg14273610 CpG site, 22.12% vs. 9.28% (p=0.0102) at the cg22334352 CpG site, 24.41% vs. 8.74% (p=0.0003) at the cg25103650 CpG site, 51.66% vs. 23.97%(p=0.0165) at the cg22845542 CpG site and 31.05% vs. 16.43% (p=0.0091) at the cg06199336 CpG site for cases and controls, respectively. The pyrosequencing results were consistent with those from the Illumina 850K-EPIC methylation BeadChip array.

Conclusion: Our results suggested that the SATB2 may be responsible for NSCPO formation and could be a potential biomarker for NSCPO.

Keywords: 5'UTR, methylation, non-syndromic cleft palate only, SATB2

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Non-syndromic cleft palate only (NSCPO) is a craniofacial birth defect of complex etiology, which arises from a failure of palatal shelf growth, elevation, adhesion, and palatal shelf midline fusion, accounting for approximately 70% of all orofacial clefts.[1] The etiopathogenesis of NSCPO remains largely unknown, but is considered to involve environmental exposure, genetic risk factors, and their interactions.[2-3] Environmental exposure factors mainly include maternal health/disease status, lifestyle, and medication.[4] Genetic risk factors involve a series of biological mechanisms such as cell migration (*ROCK1, ROCK2 and FLNB*), epithelial-mesenchymal transition (*CDH1, CRISPLD2, JAG2 and FOXE1*), cell proliferation (*MSX1, TBX22 and COL2A1*) and so on.[5-13] The maternal passive smoking and alcoholism were identified as gene and environment interactions (*PDGFRA, GSTT1 and MLLT3*).[14-16] NSCPO can have serious adverse effects on a child's health, quality of life and psychosocial well-being.[17] The treatment involves a series of complicated procedures, including cleft palate repair, speech therapy and psychological rehabilitation, which

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impose an economic and emotional burden on both affected families and society as a whole.[18] Although several studies of multiple NSCPO families have shown that NSCPO has a multifactorial etiology with a genetic component in the etiopathogenesis,[19,20] little is known about the underlying epigenetic etiology. Therefore, ascertaining genetic risk factors, especially epigenetic factors, for NSCPO would be an important milestone for improving prevention and treatment.

Craniofacial embryonic development involves a series of precisely regulated processes that require the expression of many key genes in a spatiotemporal manner. Cytosine-phosphate-guanine (CpG) island methylation at the 5' untranslated regions (UTRs) of genes has been deemed as a vital epigenetic regulator.[21] Epigenetic changes, particularly DNA methylation of CpG islands in the 5'UTR promoter, inhibit gene expression in mammalian genomic DNA.[22] Many studies have illustrated that malfunction of methylation may be involved in the development of craniofacial birth defects, including NSCPO.[23,24] Furthermore, accumulating evidence indicated that the epithelial-to-mesenchymal transition (EMT) is a crucial process for palatal fusion during palatogenesis, which involves epithelial cell proliferation, differentiation, apoptosis, and migration.[25,26] Previous studies have suggested that alterations in the *SATB2* may play a critical role in developmental anomalies of the palate in humans.[27,28] Thus, we hypothesized that aberrant DNA methylation in the 5'UTR promoter of the *SATB2* during spatiotemporal and site-specific stages of embryonic palate development might inhibit *SATB2* expression and eventually lead to NSCPO. However, little is known about the epigenetic regulation of *SATB2* in NSCPO formation.

To investigate the potential role of the *SATB2* 5'UTR promoter associated with NSCPO in this study, DNA methylation profiling was performed using Illumina 850K-EPIC methylation BeadChip on human palatal tissue samples discarded after surgical repair of NSCPO (case) or maxillofacial and palate trauma (control). The methylation level of the *SATB2* 5'UTR promoter was confirmed by pyrosequencing. These findings could demonstrate the role of DNA methylation in the *SATB2* 5'UTR promoter involved in the failure of palatal fusion that eventually results in NSCPO.

Subjects and Methods

Sample Preparation and Ethics Statements

Discarded palatal shelf tissues were collected from three NSCPO patients (cases) and three maxillofacial and palatal trauma patients (controls) during surgery at the Department of Plastic and Burn Surgery, Second Affiliated Hospital of Shantou University Medical College (Guangdong, China) between March 2019 and February 2022. The use of tissues for this study was approved by the Ethics Committee of the Second Affiliated Hospital of Shantou University Medical College (approval no. 2021-49), and written informed consent was obtained from all patients.

Illumina 850K Methylation Sequencing

The samples that passed the quality inspection were subjected to bisulfite conversion, DNA amplification, fragmentation, precipitation, and resuspension. The resuspended DNA samples were analyzed on an Illumina 850K-EPIC methylation BeadChip (Illumina, San Diego, CA, USA). The obtained data were directly imported into Genome Studio software for analysis to obtain the raw methylation data of each sample.

Illumina 850K Methylation Data Analysis

The original data were read, standardized, and pre-processed using the Minfi package.[29] The probe-type bias was filtered using the BMIQ method.[30] CpG probes with fewer than three beads, non-CG, cross-hybridizing, and located on sex chromosomes were excluded from further analysis. Principal component analysis (PCA) was conducted to identify outlier samples using standardized methylated data.[31] The correlation between sample methylation levels was validated using Pearson's correlation coefficient.[32] Significant differentially methylated sites in the 5'UTR promoter between cases and controls were identified according to $p<0.05$ and delta beta >0.1 in the normalized dataset.[33] Unsupervised hierarchical clustering of differential methylation sites of genes identified in the Illumina 850K methylation BeadChip was performed to evaluate the direct correlation and biological function of methylation.

Functional Enrichment Analysis

GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis were performed for each type of differential expressed methylation gene. Fisher's exact test was used to calculate the GO enrichment significance of each term in biological process, cellular component, and molecular function. Hypergeometric distribution test was used to calculate the significance of gene enrichment in each pathway item. Genes for subsequent research were selected according to the results of GO and analysis combined with biological significance.

Pyrosequencing to Determine the *SATB2* Methylation Level

The methylation level of the *SATB2* 5'UTR promoter was verified using pyrosequencing. Genomic DNA was prepared from the same sample used for 850K sequencing using a

QIAamp DNA mini kit (Qiagen) and was bisulfite-modified using an EpiTect Bisulfite kit (Qiagen). Amplification reactions comprised 45 cycles in 20 µL volumes, and were carried out at 95°C, 58°C, and 72°C sequentially, each lasting for half a minute, with a final extension step at 72°C for 10 min. Primer sequences used for polymerase chain reaction were listed in Table 1. The amplification products were pyrosequenced using a PyroMark Gold Q96 kit (Qiagen) and CpG methylation values were analyzed using Pyro Q-CpG software (Qiagen).

Statistical Analysis

PCA was conducted to identify outlier samples with standardized methylated data. Pearson's correlation coefficient was used to estimate the correlation between samples. Unsupervised hierarchical clustering of differentially methylated sites was performed to evaluate their direct correlation and biological function. $P<0.05$ and delta beta >0.1 were used to evaluate the differential methylation levels. $P<0.05$ and false discovery rate <0.01 were considered to indicate significance.

Results

Participant Characteristics

The patients' information was presented in Table S1. Three patients with NSCPO (mean age 1.025 years) and three controls (mean age 32.67 years) were enrolled. Three tissue specimens from each group were used for methylation array sequencing and validation of methylation levels in the *SATB2* 5'UTR promoter by pyrosequencing.

Quality Control and Standardization of the Illumina 850K Methylation Array Data

The original data were read, standardized, and filtered according to beta values. The beta-value density curve showed a bimodal distribution (Fig. 1a). The first peak represented the hypomethylated or unmethylated probes with a beta value close to 0, whereas the second peak represented hypermethylated or fully methylated probes with a beta value close to 1. In addition, box-and-whisker plots of the beta-value distribution and concentration trends of the six samples were approximately symmetrically distributed (Fig. 1b). PCA revealed that the two sets of samples displayed the same clustering (Fig. 1c). The Pearson correlation coefficient showed that the two groups of samples were strongly correlated, ranging from 0.97 to 1 (Fig. 1d).

Identification of Differential Methylation Sites

Based on 850K BeadChip sequencing, a total of 20,455 differentially methylated CpG sites were identified, including 15,983 differentially hypermethylated CpG sites and 4472 differentially hypomethylated CpG sites between cases and controls ($p<0.05$, $|\Delta beta| > 0.1$; Table S2). Figure 1e displayed a bar plot showing the proportions of high, low, and total CpG methylation sites in the different methylated regions of the gene. The distribution of important CpG sites was shown using a volcano plot in Figure 1f. To evaluate the methylation levels at different CpG sites, unsupervised hierarchical clustering analysis was performed to quantify changes in CpG site methylation levels between cases and controls. The unsupervised hierarchical clustering heat map of the 5'UTR was shown in Figure 3a.

Table 1. The primers of SATB2 for cg14273610, cg22334352, cg25103650, cg22845542 and cg06199336 in analysis of the CpG island loci

Probe ID	Group	DNA sequence (5→3)
cg14273610	Forward primer	AGTTTTATAGTAGAGGGGTAGAAGAAG
	Reverse primer	ACAAAAATTACCAAAACCAAAACTTAAC
	Sequencing primer	GTTAAATGGGTTGTTGG
cg22334352	Forward primer	AGTTTTATAGTAGAGGGGTAGAAGAAGTAG
	Reverse primer	ACAAAAATTACCAAAACCAAAACTTAAC
	Sequencing primer	AAGTTGAGGTTGATTGTTTTATT
cg25103650	Forward primer	AGTTTTATAGTAGAGGGGTAGAAGAAGTAG
	Reverse primer	ACAAAAATTACCAAAACCAAAACTTAAC
	Sequencing primer	AGTTGAGGTTGATTGTTTTATT
cg22845542	Forward primer	TTTAGTAATTGGGTTTGTTGGTTAT
	Reverse primer	ACAACAATTCACCTTTAAAAACTCTTC
	Sequencing primer	CACTTACACACCCCA
cg06199336	Forward primer	GGGTGTGTAAGTGTGAGTGTA
	Reverse primer	AACAACAATTCACCTTTAAAAACTCTTC
	Sequencing primer	GTTATTTGTTTTAGTAGTTTTTGT

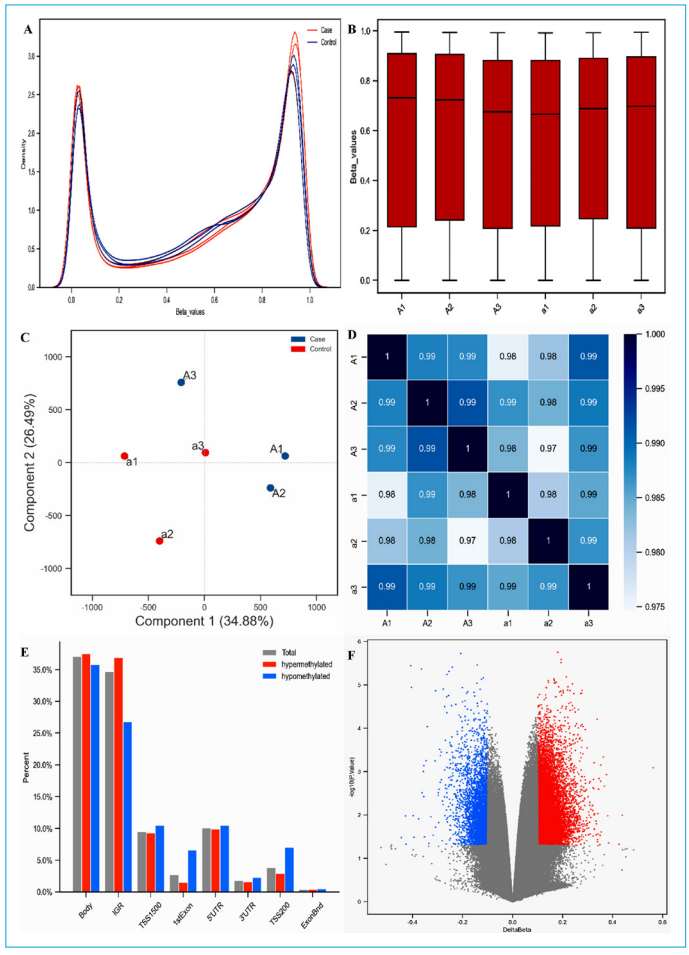

Figure 1. Quality control of the methylation array data. **(a)** Delta beta distributions of the data from the case and control groups. **(b)** Boxplots of the delta beta from the six samples. **(c)** PCA analysis of the two sets of samples. **(d)** Pearson correlation coefficients. **(e)** Bar plot showing the proportion of high, low and total methylation of CpG sites in different methylated regions of the SATB2. **(f)** Volcano plot of the methylated data indicating the differentially-methylated regions between NSCPO cases and controls. PCA: principal component analysis.

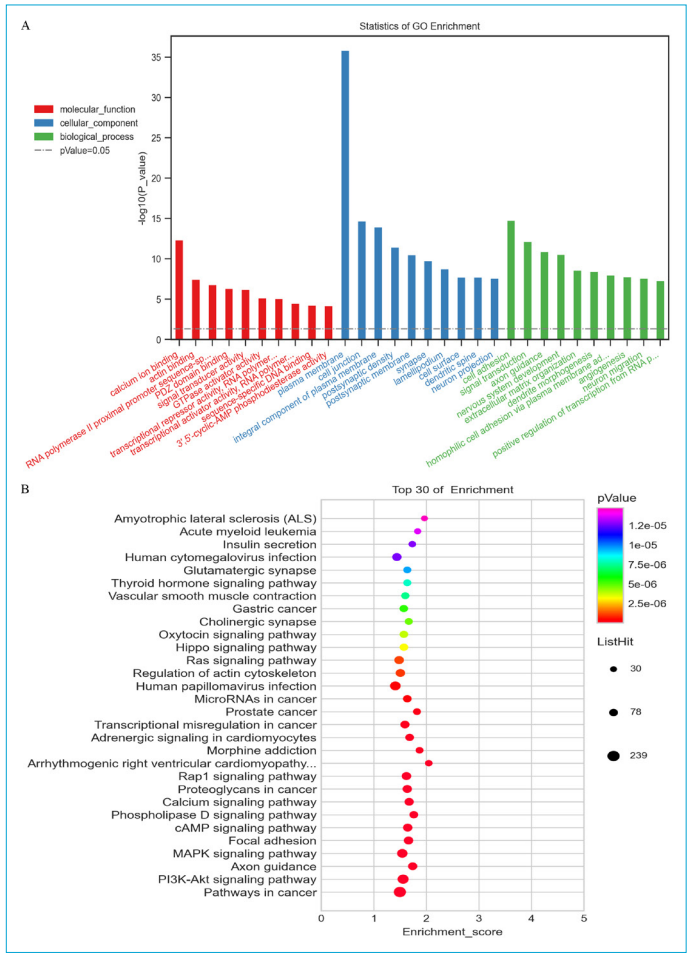

Figure 2. The GO and KEGG enrichment analyses of the differentially methylated genes. **(a)** The top 10 of GO enrichment. **(b)** The top 30 of KEGG enrichment.

Promoter 5'UTR *SATB2* Methylation Level

According to the distribution of differentially methylated sites, we identified the DNA hypermethylation level in the *SATB2* 5'UTR promoter among the Five differentially methylated sites in the case vs. control samples (Table S2). Five CpG regions of *SATB2* showed substantial predominance in the 5'UTR promoter (cg14273610, cg22334352, cg25103650, cg22845542 and cg06199336).

Functional Enrichment Analysis

According to the combination analysis results that were annotated and classified ($p<0.05$ and FDR <0.05), the top 10 GO terms of differential significantly methylated genes were shown in Figure 2a and the top 30 KEGG terms were shown in Figure 2b. Moreover, we further mined *SATB2* related to GO and KEGG items. We discovered that the biological processes for *SATB2* were 'neuron migration', 'osteoblast development', 'palate development' and 'negative regulation of transcription from RNA polymerase II promoter'. The cellular components were 'transcription factor complex' and the molecular functions were 'RNA polymerase II proximal promoter sequence-specific DNA binding', 'transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding' and 'sequence-specific DNA binding' (Table 2). However, the KEGG items related to *SATB2* were not enriched.

Pyrosequencing Validation

Pyrosequencing of the three CpG sites in the *SATB2* 5'UTR promoter (cg14273610, cg22334352, cg25103650, cg22845542 and cg06199336) showed that methylation levels were higher in patients with NSCPO than in those patients with palatal trauma. The mean percentages of meth-

Table 2. The SATB2 related to GO enrichment analysis

Category	Term ID	Term description	p	FDR
Biological process	GO:0001764	neuron migration	2.57E-08	8.80E-06
	GO:0002076	osteoblast development	0.000596	0.021592
	GO:0060021	palate development	0.001300	0.034241
	GO:0000122	negative regulation of transcription from RNA polymerase II promoter	0.002036	0.046545
Cellular component	GO:0005667	transcription factor complex	2.48E-05	0.000492
Molecular function	GO:0000978	RNA polymerase II proximal promoter sequence-specific DNA binding	1.67E-07	5.17E-05
	GO:0001077	transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding	3.16E-05	0.003669
	GO:0043565	sequence-specific DNA binding	5.50E-05	0.005676

ylation were 31.81% vs.16.45% at the cg14273610 CpG site (p=0.0019, Fig. 3b), 22.12% vs. 9.28% at the cg22334352 site (p=0.0102, Fig. 3c), 24.41% vs. 8.74% at the cg25103650 site

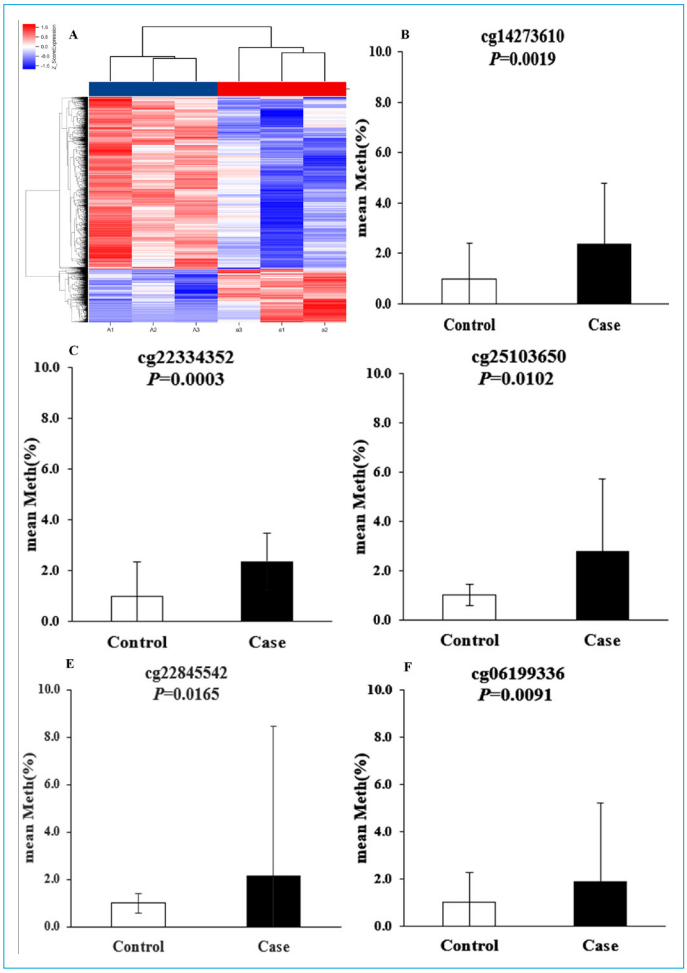

Figure 3. Unsupervised hierarchical clustering heat-map of the differentially-methylated CpG sites between NSCPO cases and controls, and validation of the results of the Illumina 850K DNA methylation bead array analysis by pyrosequencing. **(a)** Heat-map of the differentially-methylated CpG sites in the 5'UTR promoter, **(b)** cg14273610, **(c)** cg22334352, **(d)** cg25103650, **(e)** cg22845542, **(f)** cg06199336.

(p=0.0003, Fig. 3d), 51.66% vs. 23.97% at the cg22845542 CpG site (p=0.0165, Fig. 3e) and 31.05% vs. 16.43% at the cg06199336 CpG site (p=0.0091, Fig. 3f) in cases vs. controls, respectively. These pyrosequencing results were consistent with the Illumina 850K-EPIC methylation BeadChip results.

Discussion

In the present study, we demonstrated that the *SATB2* 5'UTR promoter hypermethylation was directly related to the occurrence of NSCPO. First, we compared the *SATB2* methylation pattern in palatal tissues in patients with NSCPO or with maxillofacial and palate trauma. Subsequently, we analyzed the methylation levels of CpG sites in the *SATB2* 5'UTR promoter and corroborated the methylation via pyrosequencing. The relationship between NSCPO and the methylation level of CpG sites in the *SATB2* 5'UTR promoter was demonstrated based on the following observations: (1) the methylated CpG sites in the *SATB2* 5'UTR promoter were located within CpG islands and were hypermethylated; (2) pyrosequencing of the three CpG sites in the *SATB2* 5'UTR promoter (cg14273610, cg22334352, cg25103650, cg22845542 and cg06199336) showed that methylation was higher in patients with NSCPO than in controls with palatal trauma, which was consistent with the Illumina Human Methylation 850K BeadChip sequencing results; (3) GO enrichment analysis of *SATB2* for biological processes including 'neuron migration', 'osteoblast development', 'palate development' and 'negative regulation of transcription from RNA polymerase II promoter' were all related to palatogenesis. These results indicated the participation of epigenetic mechanisms in regulating palatogenesis, which may confirm the regulatory role of CpG sites in the *SATB2* 5'UTR promoter during NSCPO formation.

DNA methylation is a vital epigenetic process involved in gene silencing, genomic imprinting, X-chromosome inac-

tivation, genomic instability, somatic reprogramming, and embryonic development. Gene expression during embryonic development is partly mediated by the reprogramming of DNA methylation. Although DNA methylation in different tissues has been well-studied in recent years, little is known about its regulation mechanism demonstrated that the persistence of specific epigenetic markers in somatic cells is associated with reprogramming-related epigenetic differences in abnormal methylation patterns.[34] This implies that NSCPO may result from the abnormal methylation of key genes. The promoter is a conserved DNA sequence, most of which is located at the 5'end upstream of the transcription start site for structural genes. RNA polymerase must recognize, bind, and initiate transcription. Luo et al.,[35] and Suzuki et al.,[36] demonstrated that DNA methylation in promoter regions is a stable repressive regulator of promoter activity.

Development of the palate is due to the growth and fusion of the palatal shelves, which involves cell migration, proliferation, differentiation, and apoptosis. The cleft palate results from the failure of palatal shelf growth, elevation, adhesion, and palatal shelf fusion of neural crest–derived mesenchymal cells at the midline.[37] In palatogenesis, epithelia from both palatal medial edges adhere to form a two-cell-thick midline epithelial seam.[38,39] EMT is a crucial step for palatal fusion during palatogenesis, which involves high levels of epithelial cell proliferation, differentiation, apoptosis, and migration.[25,26] *SATB2* is a nuclear matrix-associated protein that plays an important role in palate development, craniofacial formation, and cortical neuron differentiation during EMT by influencing chromatin structural remodeling.[40-43] Hypermethylation of the *SATB2* 5'UTR promoter may inhibits *SATB2* expression near the midline epithelial seam and could inhibit palatal shelf fusion by decreasing medial epithelial cell proliferation and migration, and thus reducing EMT. Therefore, our results suggested that the *SATB2* hypermethylation in the 5'UTR promoter is involved in EMT by reducing EMT of the basal epithelial layer, which may result in NSCPO.

Although our study suggested that the DNA hypermethylation in the *SATB2* 5'UTR promoter could be related to NSCPO, there are some limitations to be clarified in this study. For example, sex ratio and differences in ages between cases and controls may be related to NSCPO, but we did not have sufficient data to compare NSCPO subtype differences. The relatively small sample sizes (model: n=3 vs. normal: n=3) was due to the difficulty to obtain palatal tissues from normal controls. Therefore, the *SATB2* 5'UTR promoter and its verification at gene levels need further evaluation.

Conclusion

Taken together, our study indicated that the DNA hypermethylation in the *SATB2* 5'UTR promoter could be related to NSCPO, which may inhibit palatal shelf fusion by decreasing medial epithelial cell proliferation and migration. This could reduce EMT and eventually result in NSCPO. Therefore, our results suggested that the *SATB2* could be a potential biomarker and a promising intervention target for NSCPO. However, the detail molecular and biological mechanisms by which *SATB2* mediates EMT in NSCPO require further investigation.

Disclosures

Ethics Committee Approval: The use of tissues for this study was approved by the Ethics Committee of the Second Affiliated Hospital of Shantou University Medical College (approval no. 2021-49), and written informed consent was obtained from all patients.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

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Table S1. Characteristics of the three NSCPO cases and three controls

Pools	Samples	Sex	Age (year)
Case	Non-syndromic cleft palate only (NSCPO)		
Pool1	1	Male	1.0
	2	Female	0.9
	3	Female	1.2
Control	Maxillofacial and palate trauma		
Pool2	1	Male	54
	2	Male	28
	3	Female	16

Supplement Table 2 can be downloaded from the following link: https://www.ejmo.org/table/table1.csv