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LINE-1 methylation in cleft lip tissues: influence of infant MTHFR c.677C>T genotype

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Running title: LINE-1 methylation in cleft lip and MTHFR genotype

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Abstract

Objective: To investigate the influence of MTHFR c.677C>T genotype on LINE-1 methylation in lateral and medial tissues from cleft lip (CL).

Methods: 45 consecutive non-syndromic cleft lip with or without cleft palate (nsCL/P) cases were included in the study. Genomic DNA was extracted from tissues at both sides of cleft lip, and LINE-1 methylation was detected by bisulfite conversion and pyrosequencing. MTHFR c.677C>T genotyping was carried out using the TaqMan genotyping assay.

Results: LINE-1 methylation level was significantly higher on medial side of cleft lip compared to lateral side (p=0.001). This difference was not significantly influenced by the case’s sex or cleft type. However, MTHFR c.677C>T genotyping revealed that the difference in LINE-1 methylation across cleft lip was restricted to carriers of C allele of MTHFR c.677C>T and was not apparent in TT homozygous cases (p=0.027).

Conclusion: This integrated analysis supports the previous finding of differences in DNA methylation across the two sides of cleft lip, and further suggests a possible role of MTHFR c.677C>T genotype in establishing this difference.

Introduction.

The medial and lateral tissues of upper lip structure originate from distinct and at slightly different periods early life, and are thereby susceptible to aberrant maternal environmental exposure(s). Some empirical support is provided by our recent study showing methylation reprogramming within these tissues (Khan et al., 2018) in infants with non-syndromic cleft lip and palate (nsCL/P). This has increased considerable interest to identify factors that determine the pattern of methylation in tissues from medial and lateral aspects of the upper lip.

Folate provides methyl groups for DNA methylation (Fox, & Stover, 2008), and is shown to be critical for the formation of craniofacial structures (Jiang, Bush, & Lidral, 2006; Rosenquist, 2013). The folate level and provided methyl group is influenced by the key enzyme encoded by Methyltetrahydrofolate reductase (MTHFR) gene (van der Put et al.,
MTHFR c.677C>T (p.Ala222Val; rs1801133) variant is reported to be associated with increased risk of nsCL/P (Rai, 2018), and has potential to change global DNA methylation (Shelnutt et al., 2004; Friso et al., 2002; Chen et al., 2010), particularly under low folate concentration (Friso et al., 2002).

Studies investigating the role of epigenetic variation in the etiology of non-syndromic orofacial clefts has recently begun to emerge in humans (Sharp et al., 2017; Alvezi et al., 2017; Khan et al., 2018). There are, however, no reports of the effect of the MTHFR c.677C>T variant on LINE-1 methylation and nsCL/P etiology. This motivated us to investigate, for the first time, whether MTHFR c.677C>T genotype influences LINE-1 DNA methylation levels in medial and lateral tissues from cleft lip.

Materials and Methods.

The study was approved by local institutional review board (prot. no. 08-2011). The LINE-1 methylation was detected as previously described (Khan et al., 2018). MTHFR c.677C>T genotyping was performed using TaqMan® SNP genotyping assay following manufacturers protocol (Applied BioSystem, Foster City, CA). Investigation of the relationship between MTHFR c.677C>T genotype and LINE-1 methylation was based on 45 infants with nsCL/P recruited between September 2016 and March 2018. These included 23 cases from our pilot study (Khan et al., 2018a), and 22 subsequent cases. Cases were aged 6.8 (95% CI: 6.26-7.37) months and included 26 males, ten with cleft lip (nsCL) and sixteen with cleft lip and palate (nsCLP), and 19 females (nine with nsCL and ten with nsCLP). Of these 45 cases, 23 had left, 6 had right, 7 had bilateral cleft, and for 2 data were not reported on laterality.

Normality was checked using the Kolmogorov-Smirnov test (K-S test), and differences in LINE-1 methylation within and across strata was performed using paired and unpaired Student’s t-test as appropriate.

Results

Our integrated analysis showed a significant difference in methylation level across cleft lip sides (p-value = 0.001), with the medial side having approximately 3% higher methylation (Table 1).

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On stratification, our analysis of both sexes and cleft type showed a similar difference in LINE-1 methylation across sides of cleft lip (Table 1). The minor allele frequency of MTHFR c.677C>T variant among nsCL/P infants was 43.3%, and was in Hardy-Weinberg equilibrium (p-value=0.302).

As shown in table 2, LINE-1 methylation level in wild-type CC infants was markedly (4.3%) higher on the medial side compared to the lateral side, which non-significantly reduced to 3.1% in CT infants. By contrast, TT infants showed nearly equal level of methylation in the two tissues, characterized by a higher level on the lateral side of cleft lip (74.7%), compared to CC (71.3%) or CT (71.7%) genotypes. Considering CC genotype as a reference, the absence of a difference in methylation between cleft sides in cases with TT genotype turned out significant (p-value=0.027), whereas the difference in cases with CT genotype was very similar to that of the reference group (Table 2).

**Discussion**

The integrated methylation analysis presented in this study increases confidence of our finding of differences in LINE-1 methylation between the lateral and medial sides of a cleft lip, with the medial side showing higher methylation. This difference was not influenced by the infant’s sex or cleft type.

The observed differences in methylation could be manifested as a sustained response, which is in line to our previous hypothesis (Khan et al., 2018) of these tissues experiencing different maternal environment to which they are highly responsive, and thus providing an insight into epigenetic effects of early life environmental exposures (Richmond, & Joubert, 2017).

Our observation of null effect with sex and cleft subtype suggests that the observation of higher prevalence at birth of nsCL/P in males and the presence of an accompanying cleft palate is likely unrelated to LINE-1 methylation disturbances in lip primordial tissues.

Stratification according to MTHFR c.677C>T genotype showed methylation differences only in CC and CT genotype. While an inverse association in methylation level in TT cases was observed, which could be suggested as an outcome of impaired folate metabolism in the developing embryo with TT genotype such that DNA methylation is altered at the lip prominences, ultimately impairing their correct fusion. This observation of differential
methylation at the lip prominences with functional variant in MTHFR gene is in line to previous supporting evidence linking folate metabolism and epigenetic modifications in the pathogenesis of nsCL/P (Richmond et al., 2018), and supports the hypothesis that both genetic and environmental factors can influence DNA methylation level in lip tissues (Sun, 2014; McKay et al., 2012).

Our study has some potential limitations in way to suggest a causal inference of the observed pattern of methylation differences, due to ethical limitations in collecting lip tissues from normal infants. Other potential limitations are the generalizability of our finding to populations exposed to different environmental conditions, and non-inclusion of other genes and/or variants that have potential to influence methylation. Despite these limitations, the integrated analysis presented in this study increases the confidence of our finding of methylation differences across medial and lateral tissues, and suggests possible role of MTHFR c.677C>T variant in establishing these differences. Thereby, highlighting the hypothesis that both genetic and environmental factors can influence DNA methylation level in lip tissues. Our findings warrant further investigations with larger and different cohorts of cases, and taking account of folate status of mothers during early pregnancy.

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Author contributions
MFJK, JL, and MR designed the study, MFJK, MR, LA and MCM managed recruitment of PENTACLEFt lip tissue samples, MFJK, and VA carried out the methylation analyses, VA and AR performed the genotyping. The data were analyzed by MFJK and MR. Results were
revised by JL, PM, and RPMST. MFJK, JL, and MR drafted the manuscript and all authors revised the manuscript and the final copy.

Conflicts of interest
None to declare.

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References


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Table 1. LINE-1 DNA methylation level (%) of medial and lateral cleft lip tissues and effect of sex or cleft type.

<table>
<thead>
<tr>
<th>Groups (N)</th>
<th>Medial side Mean ± SD</th>
<th>Lateral side Mean ± SD</th>
<th>Mean difference (95% CI)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total (45)</strong></td>
<td>75.13 ± 4.06</td>
<td>72.18 ± 5.39</td>
<td>2.95 (1.31 to 4.59)</td>
<td>p=0.001</td>
</tr>
<tr>
<td><strong>Males (26)</strong></td>
<td>75.70 ± 3.24</td>
<td>72.96 ± 4.97</td>
<td>2.74 (1.17 to 4.30)</td>
<td>p=0.840</td>
</tr>
<tr>
<td><strong>Females (19)</strong></td>
<td>74.13 ± 4.77</td>
<td>71.32 ± 5.93</td>
<td>2.80 (-0.67 to 6.27)</td>
<td>p=0.560</td>
</tr>
<tr>
<td><strong>CL (20)</strong></td>
<td>74.24 ± 2.38</td>
<td>71.31 ± 6.15</td>
<td>2.93 (-0.09 to 5.96)</td>
<td>p=0.560</td>
</tr>
<tr>
<td><strong>CLP (25)</strong></td>
<td>75.62 ± 4.80</td>
<td>72.97 ± 4.77</td>
<td>2.64 (0.67 to 4.61)</td>
<td></td>
</tr>
</tbody>
</table>

* Nominal p-value of comparisons of mean difference between nsCL/P cases categorized by sex or cleft subtype.  
Abbreviations: N, number of cases; SD, standard deviation; CL, 95% confidence interval (CI); CL, cleft lip; CLP, cleft lip and palate.
Table 2. LINE-1 DNA methylation level (%) in medial and lateral cleft lip tissues of total nsCL/P cases, stratified by MTHFR c.677C>T genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Medial side</th>
<th>Lateral side</th>
<th>Mean difference between medial and lateral sides</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N) Mean ± SD</td>
<td>Mean ± SD</td>
<td>(95% CI)</td>
<td></td>
</tr>
<tr>
<td>CC (17)</td>
<td>75.60 ± 3.97</td>
<td>71.29 ± 6.02</td>
<td>4.32 (1.66 to 6.98)</td>
<td>ref.</td>
</tr>
<tr>
<td>CT (17)</td>
<td>74.84 ± 4.79</td>
<td>71.70 ± 5.29</td>
<td>3.14 (0.24 to 3.41)</td>
<td>p=0.529</td>
</tr>
<tr>
<td>TT (11)</td>
<td>74.45 ± 2.66</td>
<td>74.66 ± 4.13</td>
<td>-0.02 (-3.41 to 2.97)</td>
<td>p=0.027</td>
</tr>
</tbody>
</table>

* Nominal p-value of comparisons of mean difference between medial and lateral sides considering CC genotype as reference.

Abbreviations: N, number of cases; SD, standard deviation; CL, 95% confidence interval (CI); ref., reference.