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**Evaluating LINE-1 methylation in cleft lip tissues and its association with early pregnancy exposures**

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1    **Abstract**

2    **Aim:** To pilot investigation of methylation of long interspersed nucleotide element-1 (LINE-

3    1) in lip tissues from infants with non-syndromic cleft lip, and its association with maternal

4    periconceptional exposures.

5    **Methods:** The lateral and medial sides of the cleft lips of 23 affected infants were analyzed for

6    LINE-1 methylation by bisulfite conversion and pyrosequencing.

7    **Results:** The medial side showed 1.8% higher methylation compared to the lateral side;

8    $p=0.031$, particularly in male infants (2.7% difference; $p=0.011$) or when the mothers did not

9    take folic acid during periconceptional period (2.4% difference; $p=0.011$). These results were

10   not statistically significant when Bonferroni adjustment was used.

11   **Conclusion:**  The observed differences in DNA methylation, although non-significant after

12   correction for multiple comparisons, suggest that differential regulation of the two sides may

13   impact lip fusion and warrant larger-scale replication.

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15   **Keywords**: LINE-1, DNA methylation, Cleft lip with or without cleft palate.

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17   **Running title**: LINE-1 methylation in cleft lip tissues

18

## Introduction

Orofacial clefts (OFC) are congenital anomalies affecting the lip, palate or both and categorized in two broad phenotypes, cleft lip with or without cleft palate (CL/P) and cleft palate (CP) [1]. The occurrence of the two phenotypes shows a sex based disparity, with a male predominance in CL/P and a female predominance in CP [2, 3]. About 30% of cases of OFC are syndromic. The non-syndromic cases are thought to be due to multiple genes and environmental factors [4, 5].

Like most other chronic diseases, the heritability of developing non-syndromic OFC is only to a small extent accounted for by the major risk loci so far identified, indicating that the infant's genetic profile alone cannot explain the origin of this malformation. And as the formation of the lip and palate starts early in pregnancy, is vulnerable to perturbation of the maternal nutritional and non-nutritional milieu [6] that can affect its epigenetic programing. Therefore, cleft of the lip and palate can arise as a result of any change that impacts its normal development such as genetic variation [7, 8], and environmental factors including maternal nutrients, smoking and hormones [9, 10, 11, 12], but the role of these factors in etiology is still inconclusive [13, 14].

Numerous studies suggest that the risk for OFC is increased by disturbance of the one-carbon metabolism cycle [15], although the role of specific nutrients such as folate remains controversial [16, 17, 18]. Folate feeds into the one-carbon metabolism cycle that results in the formation of methyl groups [19]. The level of supplementation of methyl donors in pregnancy has been shown to influence the levels of DNA methylation in infants [20], especially in the periconceptional period [21]. Moreover, in a mouse study, an increase in the level of dietary methyl donors has been found to increase genomic DNA methylation levels in the offspring [22].

DNA methylation of the pyrimidine base cytosine in DNA may be one of the mechanisms underlying differential programming of cell lineages in mammalian development, as suggested by the erasing or reshuffling of methylation marks in the early embryo and its reestablishment after implantation [23, 24, 25]. This process establishes basic adult methylation patterns prior to organogenesis. DNA methylation is in a state of flux during gametogenesis and early embryogenesis, which can be modulated by embryonic environmental exposures. Potentially, investigation of the methylation of long interspersed nucleotide element-1 (LINE-1) repetitive elements, generally accepted to be a surrogate measure of global DNA methylation content

51  [26, 27], could provide insight into the role of the environment in regulating whole genome
52  DNA methylation.

53  Numerous studies have shown changes in LINE-1 DNA methylation associated with the
54  onset of specific conditions such as gestational diabetes, preeclempsia, congenital heart
55  diseases; CHD and neural tube defects; NTDs [ 28, 29, 30, 31] and with prognosis of diseases
56  such as several types of cancer [32, 33, 34]. Studies in animals or animal derived tissues have
57  shown the involvement of DNA methylation in the development of OFC, one study involved
58  lip tissue [35], but the majority of investigations are limited to secondary palate tissues with an
59  intact upper lip [36, 37]. Most human OFC cases involve clefting of the upper lip [38, 39, 40].
60  Moreover, the lip and palate have separate embryological origins and therefore may have
61  different etiologies and DNA methylation status [41]. This is supported by recent data showing
62  distinct methylation profile in different cleft subtypes using blood DNA [42]. In addition Alvizi
63  *et al*. observed that DNA methylation correlated with the penetrance of nonsyndromic cleft lip
64  and palate (nsCL/P) [43]

65  There is a lack of epigenetic data on the DNA methylation of cleft tissues in humans. To
66  overcome this shortcoming, we undertook a pilot study of LINE-1 methylation in lip tissue
67  taken from humans undergoing surgical repair of cleft lip (CL) to address epigenetic changes.
68  We also investigated the association of LINE-1 methylation with reported maternal
69  periconceptional folic acid supplementation, sex and cleft subtype.

70  **Materials and methods**
71  **Cases**
72  Infants with non-syndromic cleft lip with or without palate were identified in the context
73  of the ongoing PENTACLEFT project [44]. The PENTACLEFT project protocol includes the
74  recruitment of non-syndromic CL/P cases, their parents and maternal grandparents, and the
75  collection of genomic DNA from peripheral blood or buccal swab samples. The project was
76  approved by local IRB (prot. N.08-2011), and case enrolment required written parental
77  informed consent. Families of consecutive cases were invited to enrol in the study at the
78  Regional Centre for Orofacial Clefts and Craniofacial Anomalies, San Paolo Hospital, Milan,
79  Italy, at the time of the first surgical intervention on the index child. Infants with recognized
80  syndromic clefts or the Pierre Robin sequence were excluded from the study. Parents of
81  included infants were asked to respond to a specific questionnaire that was administered by
82  personal interview when the affected child was brought to the surgical centre to undergo the

83 primary surgery. Information on educational status, ethnic group and family history of both
84 parents was collected, along with data on maternal medical and reproductive history, exposure
85 to environmental risk factors, use of drugs, medications and supplements such as folic acid
86 (FA) during the periconceptional period (from three month before to three months after
87 conception). Lip tissue samples were collected from non-syndromic CL/P cases at the time of
88 first surgery.

**Tissue samples**

90 The lip tissue samples were collected from Twenty-three non-syndromic CL/P cases, with
91 an average age of 6.5 (95% CI 5.0-7.1) months at the time of surgery: 12 males (7 CL, 5 CLP;
92 3 cases with preconceptional FA, preFA, and 9 without preconceptional FA, No-preFA); and
93 11 females (6 CL, 5 CLP; 2 cases with preFA, 7 No-preFA, and 2 with missing preFA data).
94 Samples were collected immediately in lysis buffer (pH7.4) from both lateral and medial side
95 of CL. The samples were then transferred to the laboratory at University of Ferrara where they
96 were processed for epigenetic study.

**DNA extraction and sodium bisulfite treatment**

98 The cases' lateral and medial side cleft lip tissues collected in lysis buffer were
99 homogenized separately, with a view to primarily include the connective tissue portion of the
100 upper lip, with minimum contribution from the epidermis. The genomic DNA was extracted
101 from the homogenate using Nucleon BACC1 kit (Amersham Biosciences, part of GE
102 Healthcare Europe, CH) according to the manufacturer's instructions and quantified using
103 Qubit® dsDNA BR Assay Kit (Life technologies Oregon, USA). The DNA with concentration
104 >10ng on Qubit® instrument was selected and bisulfite converted using EZ-DNA Methylation-
105 Lightning™ Kit (Zymo Research, Irvine, CA, USA).

**Pyrosequencing**

107 The LINE-1 DNA methylation level was measured for all the study samples with
108 pyrosequencing on PyroMarkQ96 ID using PyroMark Gold reagents (Qiagen). LINE-1 region
109 including 4 CpG sites (position 305 to 331 in accession no. X58075) was amplified by PCR
110 using the following primers: 5'-TTTTGAGTTAGGTGTGGGATATA-3' and 5'-Bio-
111 AAATCAAAAAATTCCCTTTC-3'. LINE-1 PCR products represent a pool of approximately
112 15 000 genomic loci interspersed across the whole human genome [45, 46]. PCR reactions
113 were performed in duplicate to achieve precision between runs with total volume of 25µl
114 containing 10X PCR buffer, 50mMMgCl$_2$, 2.5mMdNTPs, 10pM of each primer, 5U Taq

polymerase and 2.5μl of bisulfite modified DNA with the following cycling profile: 27 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s, followed by 72°C for 2 min. The amplicon of 147bp was analyzed on 8% polyacrylamide gel using silver staining. The PCR plate with each well containing 20μl of PCR product, 20μl of RNAse free distilled water, 3μl of sepharose beads containing streptavidin and 37μl of binding buffer; thus a total volume of 80μl of the mixture was placed on the thermo-mixture. Following this, the PCR product was made single-stranded to act as a template in a pyrosequencing reaction by washing with ethanol 70% and denaturation buffer using a Pyrosequencing Vacuum Prep Tool (Bio-Stage).

The pyrosequencing runs were performed to obtain a pyrogram from each PCR reaction, using software for analysis in AQ (allele quantification) mode, in a total volume of 40μl per well, including 38.4μl of annealing buffer and 1.6μl of 10pM sequencing primer with suspended beads containing the sample DNA. The assays was created according to the manufacturer's recommendations and the output of the two pyrosequencing runs was averaged. The nucleotide dispensation order was: ACTCAGTGTGTCAGTCAGTTAGTCTG. LINE-1 DNA methylation values were detected at positions +306, +318, +321 and +328 in Genebank sequence X58075. The CpG site at position +328) was not considered for subsequent analyses, as precision of methylation values was insufficient, probably due to the adjacent CT dinucleotide. Using the combined average data, the overall LINE-1 DNA methylation values was calculated as the mean of the proportions of C (%) at the 3 CpG sites analyzed, (positions +306, +318 and +321) and this indicated the level of methylation of LINE-1 elements [47].

**Statistical analysis**

All the statistical analysis was performed using the IBM SPSS Statistics 21. All p-values were 2-sided, with a threshold for declaring statistical significance of $p<0.05$. The distributions of LINE-1 methylation levels were checked for normality using the Shapiro-Wilk test that is appropriate for small samples; none departed from normality. For within case comparison between lateral and medial cleft side, a paired student's t-test was used. For comparison of 2-level categories of periconceptional use of supplements containing folic acid, sex and cleft subtype, the unpaired student's t-test was performed. In view of possible concerns about multiple comparisons, we also applied the Bonferroni correction to comparisons within and between cases. This was a secondary analysis because of the known limitations of the Bonferroni correction and inapplicability of other forms of adjustment to this study [48, 49]. We adopted the most conservative approach of adjusting for all 13 comparisons reported.

**Results**

147

148 Our results are based on samples that showed a normal distribution of LINE-1 methylation
149 for both medial ($p$=0.124) and lateral ($p$=0.773) sides. Initial analysis using the nominal p<0.05
150 threshold showed that the DNA in tissue taken from the medial side of the cleft lip was found
151 to have 1.8% more methylation compared to DNA in tissue taken from the lateral side
152 ($p$=0.031; Table 1). In analysis stratified on sex, no significant difference in methylation
153 between the sexes for either the lateral (males 71.5±3.1% verses females 72.0±1.2%; $p$=0.748)
154 or medial (males 74.2±3.0% verses females 73.0±2.4%; $p$=0.293) sides was observed (Figure
155 1). However, the methylation levels between lateral and medial sides in males was observed to
156 be significantly different (lateral 71.5±3.10% verses medial 74.2±3.0%; $p$=0.011), (Table 1).
157 When methylation levels within and between CL and cleft lip and palate (CLP) subtype were
158 analyzed, no significant differences were observed (Table 1). To evaluate the role of
159 periconceptional folic acid as an environmental factor affecting the level of global DNA
160 methylation, we calculated the average methylation level on lateral and medial cleft sides in
161 infants born to mothers with and without periconceptional folic acid supplementation.
162 Comparison between these two groups showed no significant difference. However,
163 methylation on the medial side was 2.7% higher than that on the lateral side in the cases whose
164 mothers did not take periconceptional folic acid supplements, ($p$=0.011; Table 1). In secondary
165 analysis using a Bonferroni corrected threshold ($p$=0.004) , this finding was not statistically
166 significant.

**Discussion**

167

168 In this first pilot study using human lip tissue obtained from infants with non-syndromic
169 CL/P, we observed differences in LINE-1 DNA methylation between tissues on the lateral and
170 medial side of the cleft. These differences were apparent in boys but not in girls, and in infants
171 whose mothers did not take supplements containing folic acid in the periconceptional period
172 but not in the offspring of women who took supplements. There were no differences in
173 methylation by sex or cleft subgroup.

174 From our results it appears that the medial side of clefts have higher global methylation
175 levels, especially in male infants. This pattern is also apparent in infants from pregnancies in
176 which supplements containing folic acid were not taken during the periconceptional period, but
177 we acknowledge that numbers are very small. We also recognize that the inability to obtain
178 normal lip tissues with which to compare our lip tissue samples is a shortcoming that prevents

179 the direct determination of whether this epigenetic difference between of the two sides is a
180 pattern present in the general population, rather than being specific to clefting, i.e. a real cause
181 or consequence of clefting. However, we suggest that this difference may reflect the fact that
182 these tissues develop during separate embryonic stages and therefore possibly experience
183 different environmental exposures. The lateral aspects of the upper lip originate from the
184 maxillary process (MxP) during the 4th week of embryonic development, while the medial
185 aspects of the upper lip originates from the medial nasal process (MNP), beginning in the 5th
186 week [4]. It is possible that the two separate windows of origin may have been exposed to
187 different environmental milieus that resulted in differential methylation of the two sides of the
188 cleft, in turn influencing the closure of the two processes and the occurrence of a cleft lip.

189 Another possible explanation for the observed differences in methylation of the two cleft
190 sides could lie in the developmental field concept, and act in a spatial and temporal manner
191 [50]. For example, in normal circumstances, the lateral and medial aspects of the upper lip
192 originate from two different embryonic units that constitute a morphogenetic reactive unit. It
193 is possible that this part of the embryo's reactive unit in the two aspects of the upper lip may
194 have experienced different spatial and temporal forces of organization and differentiation
195 (epimorphic field) leading to dysmorphogenesis of the two sides, reflected as a difference in
196 methylation as observed in our study. Some empirical support is provided by a recent animal
197 study that shows temporal regulation of Sonic Hedgehog (SHH), resulting in down-regulation
198 of *Foxf2* expression and reduced proliferation of medial nasal process mesenchymal cells that
199 are required for upper lip closure [51]. We postulate that differential expression of a single
200 gene on the two sides of the developing lip could be regulated by different epistatic factors,
201 and hence we plan to investigate expression of specific genes implicated in human clefting in
202 future work.

203 An influence of in-utero environment on epigenetic modulation is compatible with previous
204 reports showing associations between DNA methylation changes and neural tube defects [52]
205 and congenital heart defects [53, 54]. Neural tube defects are clearly linked with low folate
206 status [55], and there is some evidence that this is also the case for several types of congenital
207 heart defect [56]. There appears to be no previous reports on the association between folic acid
208 intake during the periconceptional period or pregnancy and global LINE-1 DNA methylation
209 in humans in general, or specific to the development of CL/P [57]. In infants born to mothers
210 who did not use periconceptional folic acid supplementation we found a suggestion of a trend
211 of increased methylation on the medial side.

In the cleft subgroup analysis, we found no significant difference in methylation between the two sides, a result that is compatible with the similar DNA methylation profile of CL and CLP reported by Sharp *et al*. [42]. Similarly, sex subgroup analysis showed no significant difference in methylation between the two sides. However, comparison within males showed a significantly higher methylation for the medial side that may be an outcome of differential developmental programming in males, who have an increased susceptibility to CL/P [2, 3]. This may reflect a role of sex in cleft etiology. We acknowledge that our results are based on small numbers, because collecting tissues from the cleft cases is of great challenge [58]. Statistical power is low, and we note that all nominally significant results in this study were non-significant in the secondary analysis applying the Bonferroni correction. Therefore, we urge that until replication of our results with in a larger sample size, the clear answer to this primarily evidence should be taken with caution.

According to a recent study, infants with cleft lip and cleft lip and palate subtypes may have similar rates of development, suggesting that epigenetic changes associated with development may not be a confounding factor in epigenetic studies of cleft lip, and cleft lip and palate [42]. Children with CL and CLP underwent surgery and thus had samples taken at approximately similar ages. There are reports of the absence of an age effect on LINE-1 methylation [59, 60, 61], and from a study on lip tissues collected from 4-month-old CL and CLP cases that shows an independent expression of genes associated with ageing [60]. Therefore, we consider that our observation is little influenced by ageing. Moreover, the advantage of using tissues derived from same individuals with relatively similar age in our study overcomes the influence of DNA sequence on DNA methylation and possibly the influence of age on DNA methylation, if any. Although the targeting of lip tissues in this study potentially would give the most direct insight into epigenetic changes associated with the occurrence of cleft lip, we are aware that heterogeneity could arise in these tissues from neural-crest derived connective tissue and muscles, and in-situ derived epidermis. However, we sought to overcome this limitation by collecting tissues in a lysis buffer to minimize the contribution from the epidermis and primarily include the connective tissue portion of lip tissues. Moreover, the observation of similar correlations between blood and tissue methylation in nsCL/P epigenetic study of Sharp *et al*. [42] and Alvizi *et al*. [43] suggests that the two tissues can be considered to be exchangeable in nsCL/P methylation studies at least. An aim of our future work in newly recruited cases is to collect blood and investigate correlation between methylation in blood with that in tissue from the lateral and medial sides of clefts.

245    Another potential limitation of our study is that the tissue DNA methylation measurement
246    in infancy may have been indirectly influenced by the presence of a cleft lip and so may differ
247    from that at the time of lip fusion in embryonic development. But for ethical reasons, this is
248    the only accessible, and the closest, tissue associated with OFC that can be studied in humans.
249    It is obviously difficult to collect lip tissue specimens from normal babies and this limits
250    making direct causal inference.

251    Of note, our study is based on small sample size (with possibility of both Type I and Type
252    II errors) and being aware of this limitation, splitting our samples based on factors (sex, cleft
253    subtype and pre-FA) thought to affect methylation, was an attempt to provide preliminary data.
254    Our primary analysis did not include Bonferroni correction because of known limitations
255    including Type II error [48] and the inapplicability of other forms of adjustment [49] in this
256    exploratory study, in view of being cautious of not missing a possible effect worthy of future
257    investigation. Since the recruitment of cleft cases is still ongoing in the PENTACLEFT project,
258    we hope to replicate and better justify our preliminary finding using larger number of cases and
259    to investigate epistatic regulation of genes implicated in OFC.

260    In conclusion, the observed difference in methylation between tissue taken from the lateral
261    and medial sides of a cleft lip may reflect the fact that these tissues develop during separate
262    embryonic stages and therefore possibly experience different environmental exposures that can
263    regulate DNA methylation patterns differently. The finding of a difference in DNA methylation
264    in male but not female infants should be further investigated. Our findings suggest that
265    epigenetic mechanisms may be important in the etiology of OFC, warranting replication in a
266    larger study.

267

268    **Summary points**

269    - The etiology of non-syndromic orofacial cleft (OFC) is only in part explained by
270      genetic variants. We hypothesized the possible role of early pregnancy epigenetic
271      programming in the pathogenesis of OFC.
272    - There is lack in epigenetic data on the DNA methylation of cleft tissues in humans.
273      Therefore, to overcome this shortcoming, this pilot study is the first comparative
274      assessment of long interspersed nucleotide element-1 (LINE-1) methylation between
275      tissues taken from the two sides of infants with cleft lip, and investigate possible
276      association with reported maternal periconceptional environmental exposures.

277     • We show that LINE-1 methylation of tissues from medial side of the lip is higher
278         compared to the lateral side, and that is particularly apparent for male infants. In
279         addition, we show that the medial side methylation is higher for infants whose mothers
280         did not take supplements containing folic acid during periconceptional period.

281     • The observed differences in methylation between tissue taken from lateral and medial
282         sides of cleft lip may reflect the fact that these tissues develop during separate
283         embryonic stages and therefore possibly experience different environmental exposures
284         that can modulate DNA methylation patterns differently.

285     • The differences in methylation between males and females may reflect a play of chance.

286     • This study suggests differential methylation of two cleft side that may impact lip fusion,
287         warranting replication in a larger study.

288

**Financial & competing interests disclosure**

The Authors have no conflicts of interest to declare

**Ethics approval and consent to participate**
Research ethical approval was granted by the local IRB (prot. N.08-2011) and required written
parental informed consent was collected for enrolled case.

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