OPEN ACCESS

University of Dundee

University of Dundee

LINE-1 methylation in cleft lip tissues

Khan, Faisel; Little, Julian ; Aleotti, Valentina ; Mossey, Peter; Steegers-Theunissen, Régine P. M.; Autelitano, Luca

Published in:
Oral Diseases

DOI:
10.1111/odi.13136

Publication date:
2019

Document Version
Peer reviewed version

Link to publication in Discovery Research Portal

Citation for published version (APA):
Khan, F., Little, J., Aleotti, V., Mossey, P., Steegers-Theunissen, R. P. M., Autelitano, L., Meazzini, M. C., Ravaei, A., & Rubini, M. (2019). LINE-1 methylation in cleft lip tissues: influence of infant MTHFR c.677C>T genotype. *Oral Diseases*, *25*(6), 1668-1671. https://doi.org/10.1111/odi.13136

General rights
Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain.
• You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 05. Dec. 2022

PROFESSOR MICHELE RUBINI (Orcid ID : 0000-0003-1448-9516)

Accepted Article

Article type : Short Communication

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

Mohammad Faisal J Khan[1, 2], Julian Little[3], Valentina Aleotti[1], Peter A Mossey[4], Régine PM Steegers-Theunissen[5], Luca Autelitano[6], Maria C Meazzini[6], Amin Ravaei[1], Michele Rubini[1]

1. Department of Biomedical and Specialty Surgical Sciences, Section of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.

2. Epidemiology Research Group, Department of Public Health and Primary Care, Faculty of Medicine, KU Leuven - University, 3000 Leuven, Belgium.

3. School of Epidemiology and Public Health, University of Ottawa, Ottawa, Ontario, Canada.

4. Craniofacial Development at the World Health Organization–collaborating Centre for Oral and Craniofacial Research, Dental Hospital and School, University of Dundee, Dundee, Scotland.

5. Department of Obstetrics and Gynaecology, Department of Pediatrics, Division Neonatology Erasmus MC, University Medical Center, Rotterdam, The Netherlands.

6. Smile House, Regional Centre for Orofacial Clefts and Craniofacial Anomalies, Department of Cranio-Maxillo-Facial Surgery, San Paolo Hospital, University of Milan, Milan, Italy.

Corresponding author:

Michele Rubini, Department of Biomedical and Specialty Surgical Sciences, Section of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Via Fossato di Mortara 74 I-44121, Ferrara, Italy. Fax No: +39.0532.236157 Email: rub@unife.it

Keywords: Non-syndromic cleft lip with or without cleft palate, DNA methylation, *MTHFR* c.677C>T, LINE-1.

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

This is the peer reviewed version of the following article: Khan, M.F.J, et al. "LINE-1 methylation in cleft lip tissues: influence of infant *MTHFR* c.677C>T genotype", *Oral Diseases* (2019), which has been published in final form at https://doi.org/10.1111/odi.13136. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.
This article is protected by copyright. All rights reserved.

Date of submission: January 4nd, 2019

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.,

This article is protected by copyright. All rights reserved.

Accepted Article

1995). *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).

This article is protected by copyright. All rights reserved.

Accepted Article

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 a., 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

This article is protected by copyright. All rights reserved.

Accepted Article

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.

Acknowledgements

The authors thank the patients and their families for their participation in the study. We acknowledge the support received from the Department of Biomedical and Specialty Surgical Sciences, Section of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Italy (MFJK, postdoctoral fellowship, n. 62040 & 0039804). Further our sincere thanks to Dr. Houda Oudouche, Department of Humanities, University of Ferrara, Italy and Lab. Group members: Luca Dall'Olio, Ilaria Cestonaro, Simone Ravalli, Giorgia Casagrande, Vincenzo Aiello and Gianni Astolfi for their assistance. JL holds a tier 1 Canada Research Chair.

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

This article is protected by copyright. All rights reserved.

Accepted Article

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.

Funding information

This study was partly supported by FAR-2018 grant from University of Ferrara and from Department of Biomedical and Specialty Surgical Sciences, Section of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Italy, Grant/Award Number: 62040 and 0039804

References

Alvizi, L., Ke, X., Brito, L. A., Seselgyte, R., Moore, G. E., Stanier, P., & Passos-Bueno, M. R. (2017). Differential methylation is associated with non-syndromic cleft lip and palate and contributes to penetrance effects. *Scientific Reports*, 7(1), 2441. doi:10.1038/s41598-017-02721-0

Chen, X., Guo, J., Lei, Y., Zou, J., Lu, X., Bao, Y., & Zhang, T. (2010). Global DNA hypomethylation is associated with NTD-affected pregnancy: A case-control study. *Birth Defects Research (Part A): Clinical and Molecular Teratology*, 88(7), 575-581. doi:10.1002/bdra.20670

Friso, S., Choi, S. W., Girelli, D., Mason, J. B., Dolnikowski, G. G., Bagley, P. J., ... Selhub, J. (2002). A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proceedings of the National Academy of Sciences of the United States of America*, 99(8), 5606-5611. doi:10.1073/pnas.062066299

Jiang, R., Bush, J. O., & Lidral, A. C. (2006). Development of the upper lip: morphogenetic and molecular mechanisms. *Development Dynamics*, 235(5), 1152-1166. doi:10.1002/dvdy.20646

Khan, M. F. J., Little, J., Mossey, P. A., Steegers-Theunissen, R. P., Autelitano, L., Lombardo, I., & Rubini, M. (2018a). Evaluating LINE-1 methylation in cleft lip tissues

This article is protected by copyright. All rights reserved.

Accepted Article

and its association with early pregnancy exposures. *Epigenomics*, 10(1), 105-113. doi:10.2217/epi-2017-0081

McKay, J. A., Groom, A., Potter, C., Coneyworth, L. J., Ford, D., Mathers, J. C., & Relton, C. L. (2012). Genetic and non-genetic influences during pregnancy on infant global and site specific DNA methylation: role for folate gene variants and vitamin B12. *PLoS One,* 7(3), e33290. doi:10.1371/journal.pone.0033290

Rai, V. (2018). Strong Association of C677T Polymorphism of Methylenetetrahydrofolate Reductase Gene With Nosyndromic Cleft Lip/Palate (nsCL/P). *Indian Journal of Clinical Biochemistry,* 33(1), 5-15. doi:10.1007/s12291-017-0673-2

Richmond, R. C., Sharp, G. C., Herbert, G., Atkinson, C., Taylor, C., Bhattacharya, S., ... Relton, C. L. (2018). The long-term impact of folic acid in pregnancy on offspring DNA methylation: follow-up of the Aberdeen Folic Acid Supplementation Trial (AFAST). *International Journal of Epidemiology,* doi:10.1093/ije/dyy032

Richmond, R. C., & Joubert, B. R. (2017). Contrasting the effects of intra-uterine smoking and one-carbon micronutrient exposures on offspring DNA methylation. *Epigenomics*, 9(3), 351-367. doi:10.2217/epi-2016-0135

Rosenquist, T. H. (2013). Folate, homocysteine and the cardiac neural crest. *Developmental Dynamics,* 242(3), 201-218. doi:10.1002/dvdy.23922

Sharp, G. C., Ho, K., Davies, A., Stergiakouli, E., Humphries, K., McArdle, W., ... Relton, C. L. (2017). Distinct DNA methylation profiles in subtypes of orofacial cleft. *Clinical Epigenetics,* 9(63), doi:10.1186/s13148-017-0362-2

Shelnutt, K. P., Kauwell, G. P., Gregory, J. F., 3rd, Maneval, D. R., Quinlivan, E. P., Theriaque, D. W., Bailey, L. B. (2004). Methylenetetrahydrofolate reductase 677C-->T polymorphism affects DNA methylation in response to controlled folate intake in young women. *The Journal of Nutritional Biochemistry*, 15(9), 554-560. doi:10.1016/j.jnutbio.2004.04.003

Sun, Y. V. (2014). The Influences of Genetic and Environmental Factors on Methylome-wide Association Studies for Human Diseases. *Current Genetic Medicine Reports,* 2(4), 261-270. doi:10.1007/s40142-014-0058-2

This article is protected by copyright. All rights reserved.

van der Put, N. M., Steegers-Theunissen, R. P., Frosst, P., Trijbels, F. J., Eskes, T. K., van den Heuvel, L. P., Blom, H. J. (1995). Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. *Lancet,* 346(8982), 1070-1071.

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

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

[*] 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.

This article is protected by copyright. All rights reserved.

Accepted Article

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.

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

[*] 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.

Accepted Article

This article is protected by copyright. All rights reserved.