Research Networking Programme

Final Report

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Title: **DNA and Tissue Bio-banking for Ecogenetics and Epigenetics of Orofacial Clefts**

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1. SCIENTIFIC BACKGROUND AND AIM OF THE VISIT

The bio-banks includes wide array of biological specimens in the form of DNA, Cells, tissues etc. which collectively can be considered as library of an individual human. These bio-banks play crucial role in bio-medical research and serve as essential resource for genotyping and next generation sequencing (NGS) based studies. In-line with this, the discovery of genetic factors associated with the development of cleft lip with or without palate (nsCL/P) has opened up opportunities of maintaining a Cleft DNA bank which preserves the opportunity for future research or genetic testing and may benefit the case-parents and cases community as a whole.

During this extended ESF-EuroCleftNet short visiting grant, an effort to manually extract DNA from the frozen blood samples from East EU countries eg. Slovenia, Hungary and Bulgaria was proposed. In order, the pooling of this Eastern - EU samples in the collection of our previous DNA-biobank would provide us with increased sample size resulting in increased statistical power of future gene or gene-environmental studies on nsOFC. In addition, the establishment of Cleft lip tissue bio-bank was part of this visit from which the genomic DNA was extracted and stored. Furthermore, fixation and paraffin embedding of the tissue was included in the proposal. The whole blood DNA bio-bank and tissue specific DNA along with the tissue sections is an open access supplement to every partnered group/members under the aegis of EuroCleftNet network.

The nsOFCs are considered multifactorial condition caused by interactions between genetic factors and the exposure to environmental risk factors. The environmental and nutritional factors [including exposure to folic acid, (Hernandez-Dias et al, 2000; Puh et al, 2007), tobacco smoke (Little et al, 2004), alcohol consumption (Romitti et al, 2007; Grewal et al, 2008) contribute to the development of cleft lip and palate during pregnancy.

Recent genome-wide association studies (GWAS) have identified the major loci associated with increased risk of nsCL/P in Europeans, but these alone can explain only a small fraction of the multifactorial etiology of nsOFCs. It is generally accepted that gene-environment interactions (GxE) could play a major role in the pathogenesis of nsOFCs, and the identification for these interactions could offer a benefit in clinical diagnostics and potentially provide new measures for primary prevention.

Concerning the pipeline, we selected a gene whose deletion has been linked to peri-natal lethality and impaired development of palatal shelves (Zhang et al, 2015). In addition, a missense variant in human LOXL3 has been identified in a family with autosomal recessive Stickler syndrome (Alzahrani et al, 2015). Although, the Stickler syndrome is commonly caused by mutations in different collagen

genes and is characterized by cleft palate, the recent association of LOXL3 in Sticker syndrome indicate a possible link between LOXL3 based collagen remodeling and developing Cleft palate. Therefore, we speculated LOXL3 as possible candidate gene in developing nsCPO. Based on this hypothesis, we scrutinized as many as 25 missense variant in the LOXL3 gene and selected the missense variant with the highest MAF (MAF ≥ 13%, T>A) located on exon 10, with an assigned dbSNP: rs17010021, which convert isoleucine to phenylalanine. We evaluated case-parent trios from Western and Eastern European for its association with nsCPO. Additionally, we factored the influence of parental imprinting.

2. WORK DESCRIPTION AND RESULTS

2.1. Project 1. DNA Bio-banking:

2.1.1. DNA Extraction and reconstitution:

The whole blood DNA was extracted from three Eastern EU countries (Slovenia, Hungary and Bulgaria) from the blood samples collected under EUROCRAN project in order to expand the already existing EU Cleft DNA-biobank. The genomic DNA of case-parent trio was extracted from peripheral blood DNA using Nucleon BACC1 DNA extraction kit according to the manufactures protocol.

2.1.2. DNA titration/quantification:

All EUROCRAN samples from five Eastern-EU countries, including Slovenia, Slovakia, Hungary, Bulgaria and Estonia after extraction of DNA was titrated using 2.0 Qubit Fluorometer and dsDNA BR Assay Kit (Life Technologies) according to the manufactures protocol. The Figure 1 represents the pictorial representation of the assay procedure.

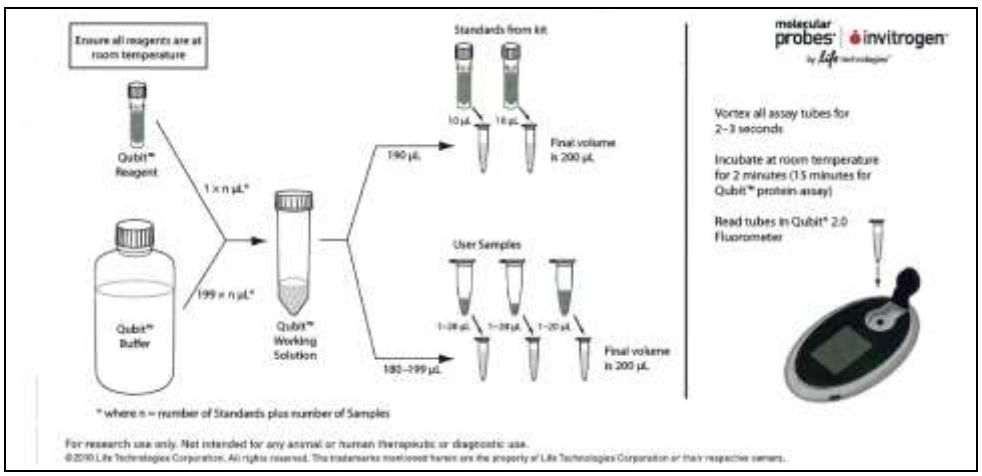

Figure 1. Qubit DNA assay procedure

2.1.3. DNA storage:

The quantified DNA with their specific concentrations were transferred in a 2D-barcoded matrix vials which was further placed in a 96 wells 2D bar coded matrix boxes with unique barcode identifiers and stored at -20^{o}C. These samples are delinked: Biographical data of the case-parent trios is removed and the sample is identifiable by only a unique bar code.

2.2. Project 2. GxE association study

2.2.1. Method

The DNA samples from case-parent nsCPO trios from EUROCRAN were genotyped for a SNP located on exon 10 of LOXL3 gene. The patients with isolated CPO were used for analysis and divided in groups of hard and soft palate. The genetic association of LOXL3 variant was evaluated using transmission disequilibrium test (TDT) (Speilman et al, 1993). The McNamar Chi-squared test, with a significance threshold of $p < 0.05$, was used to justify the deviation from Hardy-Weinberg equilibrium. Using a log linear model we screened for possible parent-of-origin effect which access on risk increment (I_M) in the offspring and entails on the allele transmitted from the mother compared to the father (Weinberg et al., 1998; Wilcox et al., 1998). In addition, the relative risks (RR) associated with the child's genotypes was also calculated using a Log-Linear model.

2.2.2. Result.

The outcome of genetic association revealed no significant segregation of minor allele from the parent to the off-spring. There was increased risk with child having homozygous genotype compared to heterozygous genotype. The result of parental asymmetry showed a significant transmittance of minor allele from the father. Furthermore, on gender stratification, the trend of developing nsCPO was more inclined to females than to males.

2.3. Project 3. Tissue Bio-banking and Epigenetic study:

2.3.1. Lip tissue collection:

The lip tissue samples obtained during first surgery operation from nsCL/P patients was collected at the San Paolo Hospital, Milan, Italy. Samples from either sides of cleft lip are collected in lysis buffer and buffered formalin for DNA extraction and paraffin embedding respectively. Thereafter, samples are transferred to University of Ferrara, where it is processed for DNA extraction and paraffin embedding.

2.3.2. DNA Extraction, reconstitution and Paraffin embedding:

The tissue was homogenized and DNA extracted using Nucleon BACC1 DNA extraction kit according to the manufactures protocol. The resultant DNA was titrated using 2.0 Qubit Fluorometer and dsDNA BR Assay Kit (Life Technologies) according to the manufactures protocol as represented in the figure 1 above.

The formalin fixed tissues were processed using standard protocol and embedded in paraffin blocks.

2.3.3. Tissue DNA and Paraffin block storage:

The tissue extracted DNA was stored in the manner similar to the storage of whole blood extracted DNA as documented in the above section. The paraffin blocks are labelled with specific sample numbers and stored in a box at $4^{o}C$ for future use.

2.3.4. Epigenetic Study:

The possible association of global methylation (LINE-1 sequences) and gene specific methylation (promoters of selected cleft genes) with nsCL/P and nsCPO is underway. The possible outcome could be available in weeks' time and would be made available to the EuroCleftNet board members.

3. FUTURE PLANS AND PUBLICATIONS

3.1. Future plan.

We plan take advantage of the increased pool of DNA bio-bank which now includes both the Western and Eastern European samples to explore gene-environment interactions and gene-gene interactions involving variants located in CPO GWAS-loci recently published by Mangold et al, 2016 and Leslie et al, 2016 and nsCL/P GWAS documented by Leslie et al, 2016.

In addition to the use of DNA bio-bank the newly set tissue bio-bank is been advantaged in exploring the histological differences on either side of the clefted tissue. The work is underway to delineate differences in muscles with a view to ascertain differences in two different cleft phenotypes, CL and CLP.

3.2. Future publication.

The manuscript defining the association of LOXL3 missense variant and risk with maternal and child genotype along with parental asymmetry in nsCPO samples involving both western and Eastern Europe samples in under progress. Along this the differences in muscle arrangement on either side of cleft to ascertain the possibility of different cleft phenotypes is also underway.

REFERENCES:

1. Hernandez-Dias S, Werler M, Walker A. **Folic acid antagonistsduring pregnancy and the risk of birth defects**. *N Eng J Med* 2000; 343: 1608–1614.

2. Puh OE, Szunyogh M, M_Etneki J, Czeizel A. **Drug treatment during pregnancy and isolated orofacial clefts in Hungary.** *Cleft Palate Craniofac J* 2007; 44: 194–202.

3. Little J, Cardy A, Munger R. **Tobacco smoking and oral clefts: a meta-analysis.** *Bull World Health Organ* 2004; 82: 213–218.

4. Romitti PA, Sun L, Honein MA, Reefhuis J, Correa A, Rasmussen SA. **The National Birth Defects Prevention Study.Maternal periconceptional alcohol consumption and risk of orofacial clefts.** *Am J Epidemiol* 2007; 166: 775–785.

5. Grewal J, Carmichael S, MA C, Lammer E, Shaw G**. Maternal periconceptional smoking and alcohol consumption and risk for select congenital anomalies.** *Birth Defects Res A Clin Mol Teratol* 2008; 82: 519–526.

6. Zhang, J., Yang, R., Liu, Z., Hou, C., Zong, W., Zhang, Gao, J. **Loss of lysyl oxidase-like 3 causes cleft palate and spinal deformity in mice**. *Hum Mol Genet* 2015; 24: 6174-6185.

7. Alzahrani F, Al Hazzaa SA, Tayeb H, Alkuraya FS. **LOXL3, encoding lysyl oxidase-like 3, is mutated in a family with autosomal recessive Stickler syndrome**. *Human genetics*. 2015; 134:451-3.

8. Spielman, R. S., McGinnis, R. E., & Ewens, W. J. **Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM)**. *Am J Hum Genet* 1993; 52: 506-516.

9. Weinberg, C. R., Wilcox, A. J., & Lie, R. T. **A log-linear approach to case-parent-triad data: assessing effects of disease genes that act either directly or through maternal effects and that may be subject to parental imprinting**. *Am J Hum Genet* 1998; 62: 969-978.

10. Wilcox, A. J., Weinberg, C. R., & Lie, R. T. **Distinguishing the effects of maternal and offspring genes through studies of "case-parent triads**. *Am J Epidemiol* 1998; 148: 893-901.

11. Mangold E, Bohmer AC, Ishorst N, Hoebel AK, Gultepe P, Schuenke H, et al. **Sequencing the GRHL3 Coding Region Reveals Rare Truncating Mutations and a Common Susceptibility Variant for Nonsyndromic Cleft Palate**. *American journal of human genetics*. 2016; 98:755-62.

12. Leslie EJ, Liu H, Carlson JC, Shaffer JR, Feingold E, Wehby G, et al. **A Genome-wide Association Study of Nonsyndromic Cleft Palate Identifies an Etiologic Missense Variant in GRHL3**. *American journal of human genetics*. 2016; 98:744-54.

13. Leslie EJ, Carlson JC, Shaffer JR, Feingold E, Wehby G, Laurie CA, et al. **A multi-ethnic genome-wide association study identifies novel loci for non-syndromic cleft lip with or without cleft palate on 2p24.2, 17q23 and 19q13**. *Human molecular genetics*. 2016.