Research Networking Programmes

Short Visit Grant ☐ or Exchange Visit Grant ☒

(please tick the relevant box)

Scientific Report

The scientific report (WORD or PDF file – maximum of eight A4 pages) should be submitted online within one month of the event. It will be published on the ESF website.

***Proposal Title**:* The importance of sub-phenotyping in the genetic of Non-Syndromic Orofacial cleft

Application Reference N°: 5149

1) Purpose of the visit

This project has been in a continuation of the experimental plan that I've carried out in the frame of a previous Exchange Visit at the University of Dundee (Application reference n.5110).

Non-syndromic orofacial clefts (especially cleft lip with/without cleft palate, CL/P, and cleft palate only, CP) are common birth defects with a complex multifactorial aetiology (Mossey et al. 2009. Nowadays, several gene-association studies, linkage studies, and genome-wide association studies, have led to the identification of several susceptibility loci containing candidate genes playing a role in the malformation development. However, the causal variants are still unknown, and there is little information about how the genetic risk carried by unaffected relatives can be inherited by children with overt oral clefts. Recent evidence suggests that unaffected family members present a range of subclinical features, including distinct facial morphology and specific dental & soft tissue features, craniofacial measures, dental anomalies, microforms of clefts, defects of the orbicularis oris muscle and bifid uvula (Weinberg et al. 2006; Neiswanger et al. 2007; Vieira et al. 2008; Weinberg et al. 2013). These subclinical phenotypes may help to explain the incomplete penetrance or apparent lack of Mendelian inheritance patterns observed in families with cases of overt clefts (Leslie & Marazita 2013). Discerning the spectrum of subphenotypic expression of this malformation in unaffected close relatives, who share the same genome portion of individuals presenting

over clefts, may represent a key to deepen understanding the genetic aetiology of non-syndromic OFC (Miller et al. 2014).

This project aimed to investigate the phenotype-genotype correlation in unaffected parents of children with overt orofacial clefts, looking for an association between facial, dental and soft dental tissue features with genetic variants in OFC candidate genes, in order to increases the power of gene-mapping efforts and provide more effective genetic counselling, particularly recurrence risk expectation.

2) Description of the work carried out during the visit

70 controls were recruited at the University of Dundee. 80 parents (40 mothers, 40 fathers) were recruited through cleft clinics in Scotland.

Subjects had two-dimensional (2D) photographs, upper and lower dental impressions, an upper lip ultrasound scan, and lip prints taken. The photographs and dental models have been analysed with traditional morphometrics (size-based) and geometric morphometric (shape-based) techniques, whilst the upper lip ultrasound scans and lip prints are in the process of being analysed by the craniofacial research centre in Pittsburgh. The data have been compared between the controls and the parents and between the CL(P) and CP groups to determine morphogenetic differences between the groups.

The Genomic DNA was purified from saliva samples (using the prepIT-L2P kit, DNA Genotek, Inc. Canada) and quantitated (using a GE Nanoview, GE Healthcare, UK) from the Dundee Dental School research group.

Afterwords, during my visit, the task I performed are:

- gDNA Cleanup: the gDNA from the initial isolation phase was purified using QIAamp DNA Micro kit (Qiagen, UK). Purification was performed according to the manufacturer's instructions, using 100µl of gDNA. gDNA was then assessed for concentration and purity using a GE Nanoview and subsequently stored at -20°C.

- Bioinformatic selection of candidate gene variants: Considering the small sample set, we decided to restrict the analysis just to specific candidate regions, in order to avoid some of the difficulties presented by multiple testing; candidate gene variants for the genetic association study were selected using a bio-informatics approach (reviewing publish studies in Pubmed and using tools as GenomeBrowser, dbSNP) focusing on genes and loci that are known in literature as associated loci with NS CL/P risk and having a role in craniofacial embryogenesis. 30 genes and 62 genetic variants were selected for the genetic analysis:
14 genes (IRF6, 8q24 region, NOG, VAX1, GREM1, SPRY2, THADA, ARHGAP29, MAFB, PAX7, NTN1, EPHA3, TMP1, 8q21.23 region) were selected in the present studies because previously identified within CL(P) susceptibility loci in recent genome-wide association studies (Birnbaum et al. 2009; Grant et al. 2009; Beaty et al. 2010; Ludwig et al. 2012; Böhmer et al. 2013; Beaty et al. 2013).
The FOXE1 is the only gene identified as associated to CL/P risks in a meta-analysis of 13 genome-wide linkage studies, and was subsequently confirmed as associated with both

CL(P) and CP (Marazita et al. 2004; Moreno et al. 2009; Marazita et al. 2009; Ludwig et al. 2014).
The role of BMP4, FGFR2, TGFA, TGFB3, ADAMTS20, MSX1, FAF1, MMP17, SFSWAP and SOX9 in face morphogenesis is well known thanks to several studies in animal models (Suzuki et al. 2009; Bachler & Neubüser 2001; Rice et al. 2004; Stanier & Pauws 2012; Rice et al. 2004; Dixon & Ferguson 1992; Kaartinen et al. 1997; Rao et al. 2003; Enomoto et al. 2010; MacKenzie et al. 1991; Zhang et al. 2002; Ghassibe-Sabbagh et al. 2011; Lee & Saint-Jeannet 2011), and genetic variants in the neighbour of this gene have been related to OFC by several gene-association studies (Suzuki et al. 2009; Riley et al. 2007; Osoegawa et al. 2008; Ardinger et al. 1989; Jugessur et al. 2003; Lidral et al. 1998; Jugessur et al. 2003; Vieira et al. 2003; Wolf et al., 2015; Lidral et al. 1998; Blanco et al. 1998; Romitti et al. 1999; Blanco et al. 2001; Beaty et al. 2001; Mitchell et al. 2001; Jezewski et al. 2003; Jugessur et al. 2003; Vieira et al. 2003; Moreno et al. 2004; Ghassibe-Sabbagh et al. 2011; Desmyter 2012).
Finally, In a recent study performed by Miller et al., genetic variants in LEFTY1, LEFTY2, PITX2, ISL1 and SNA1 genes, related to left-right body pattering, were associated to facial asymmetry (Miller et al. 2014).

- Genetic Analysis using hight-density Genome-Wide human arrays:
The DNA samples have been processed (from 8 to 24 samples at a time) using the the Affymetrix Cytoscan Assay kit. The protocol consists in:
1) Restriction Enzyme Digestion (samples are digested by the Nsp I restriction enzyme)
2) Ligation (the digested samples are ligated using the Nsp I Adaptor)
3) PCR and product check with agarose gel electrophoreris run
4) PCR product purification and quantitation (Nanodrop)
5) Fragmentation (in order to obtain DNA fragment of roughly 25bp), check with agarose electrophoresis run
6) Labelling of the fragmented samples
7) Hybridization of the product on Cytoscan 750K_array, Affymetrix
8) Wash and Stain of the Arrays
9) Scan of the array.

The scan results have been analysed using the software ChAS (chromosome Analysis suite), capturing genomewide data for over 200,000 gene-centric SNPs as well as 946,000 probes designed to detect copy number variation for each sample.
The results have been exported as excel files, containing both CNVs and SNPs data.

- Data analysis:
Among the 200,000 SNPs genotyped using the arrays, we select just those within our 30 candidate genes, in order to perform a gene-candidate case-control association study. 179 SNPs have been selected and analysed comparing allele frequency between cases and controls (using the chi squared test for trend) and calculating the Odds Ratio.
Subsequently, the genotypic data have been analysed toghether with the phenotypic, in order to identify ant genotype-phenotype correlations.

3) Description of the main results obtained

The facial shape and dental features statistically differed between the groups and the Orbicularis oris muscle (OOM) defect rate was higher in the parental group in comparison to the controls, however no lip print differences were detected.

The genetic analysis showed some interesting difference between cases and controls; The most interesting result was observed for the SNP rs6657063 C>G, an intron variant within the gene ARPHAG29. The allelic frequency of the variant allele (G) is major in cases rather than in controls (82/57 p = 0.016), and the odds ratio showed an inverse association of the homozygous variant genotype compared with the homozygous wt (OR GG/CC = 0.21, 95%CI: 0.05-0.83, P=0.02). The locus containing ABCA4 and ARHGAP29 genes was identified as NS CL/P risk locus associated in the GWA studies and ARPHAG29 is expressed in the developing lip and palate in murine embryos.
Other interesting results have been observed for the SNP rs3901678 A>C, an intron variant in THADA gene (OR CC/A- = 0.36, 95%CI: 0.14-0.89, P=0.025), and for rs10956463 A>C, located in 8q21.13 (ORAC/AA = 0.31, 95%CI: 0.109-0.902, P=0.02)

Unfortunately, no genotype-phenotype correlations was identified using selected candidate gene polymorphisms. Despite this, these preliminary results demonstrate how unaffected relatives present variations in genes thought to be involved in cleft lip and palate development and suggest the possibility of a phenotype-genotype correlation in presence of facial cleft-related subclinical features.

4) Future collaboration with host institution (if applicable)

5) Projected publications / articles resulting or to result from the grant *(ESF must be acknowledged in publications resulting from the grantee's work in relation with the grant)*

6) Other comments (if any)

During my visit at the University of Dundee, I also collaborated in writing the research project named "Genotype / Phenotype correlation in the field of orofacial clefts: identification of novel genes using HD array technology" that was successfully applied to the Tattersall Scholarships Comittee.