POST-GENOMICS IN COELIAC DISEASE: EPIGENETIC CHANGES IN INTESTINAL EPITHELIUM AND LAMINA PROPRIA, LONG NON CODING RNA AND SERUM METABOLOMICS

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Background

Celiac Disease (CeD) is associated with HLA DQ2/DQ8, but HLA variants are necessary but not sufficient to develop CeD. To date, 57 non-HLA variants have been estimated to account for 15% of CeD heritability, but the remaining 50% of the heritability of CeD remains unexplained.

More than 90% of CeD-associated SNPs are located outside protein-coding genes, suggesting they may have a regulatory role. A fine-mapping study in CeD identified 7 SNPs that affect lncRNA. The analysis of eQTL CeD-associated polymorphisms showed non coding RNA (LncRNA) that they often affect the expression quantitative trait loci (eQTL) of nearby genes in different cells types. However, to date, they have been explored in small intestinal biopsies only, which contain multiple cell types, leading to results that are difficult to interpret. Inasmuch the majority of published eQTL studies largely ignored the effects of SNPs on regulatory LncRNA. To translate genetic association into disease pathogenesis, we need not only information on the genes, but also on the molecular pathways affected by these SNPs. To understand how SNPs affect CeD risk it will be crucial to explore, in a context-dependent manner and over stimulation with gluten, the cell types relevant to CeD as epithelial cells, intraepithelial lymphocytes, T and B-cells.

Finally, the lipid profile of infants has been proposed as a potential biomarker of CeD metabolism that can be measured before they exhibit serological and clinical signs of the disease. We suggest that the state of the host is a main factor for the abnormal

immune response to gluten. Long before any exposure to the offending agent or any production of specific antibodies, several molecular mechanisms are differentially expressed in infants who will develop CeD compared to their peers matched for the same genetic profile.

Main achievements

In our last published paper, we evaluated alterations in candidate gene expression to identify, at the level of distinct cell populations, potential alterations consistent with the gluten-induced damage in CeD; and to describe the mechanisms of epigenetic regulation that underlie these alterations. Epithelium was separated from lamina propria in biopsies of CeD patients and controls (CTRLs) using magnetic beads. Gene-expression was analysed by RT-PC; methylation analysis required bisulfite conversion and NGS. Reverse modulation of gene-expression and methylation in the same cellular compartment was observed for the IL21 and SH2B3 genes in CeD patients compared to CTRLs. Bioinformatics analysis highlighted the regulatory elements in the genomic region of SH2B3 that altered methylation levels. The cREL and TNFAIP3 genes showed methylation patterns that were significantly different between CeD patients and CTRLs. In CeD, the genes linked to inflammatory processes are up-regulated, whereas the genes involved in the cell adhesion/integrity of the intestinal barrier are down-regulated. These findings suggest a correlation between gene-expression and methylation profile for the IL21 and SH2B3 genes, confirming the hypotesis that the abnormal response to dietary antigens in CeD might be related not to abnormalities of gene structure but to the regulation of molecular pathways.

1) Long-Non coding-RNA

Concerning the lncRNA analysis we are now exploring the effect of CeD-associated SNPs, putatively affecting lncRNAs function, in keys CeD cells types. We would observe directly their localization in FFPE sections of CeD small-intestinal biopsies, and then quantify their expression by RealTime-PCR in epithelial and non-epithelial sorted intestinal cells. Then we will evaluate the pathways regulated by the lncRNAs: indeed we are going to analyze the lncRNAs and their target gene expression, the relevant protein synthesis, and their effects on the downstream pathways in both epithelium and lamina propria compartments. In the first phase, we obtained, by immuno-sorting, the separation of the epithelial component magnetically labelled with CD326 (EpCAM) MicroBeads from non-epithelial component of small intestine biopsy of 20 CeD, 20 CTRLs, recruited in our Department. The efficacy of separation between the two compartments, epithelium and lamina propria, was evaluated by real-time PCR. We dosed Epithelial Cell Adhesion Molecule (EpCAM) expression level, as the specific marker of the epithelial cells, in both epithelial and lamina propria cells, normalized

to the expression of an endogenous gene (GUSb) and used as reference sample in the epithelial compartment. A 98% purity of the epithelial compartment was achieved. In particular, the analysis of EpCAM expression generated a selection of 97.8% epithelial cells (CD326+) in celiac biopsies and 97.5% epithelial cells (CD326+) in the biopsies of controls. Then total RNA was extracted from intestinal cells and transcribed into cDNA.
In the next step we proceeded to the design of the custom TaqMan Gene Expression assays for the 7 long non-coding RNA and afterwards we performed gene-expression experiments on control samples to evaluate their efficacy. The gene GUSb was chosen as reference gene after it had been determined as the most stable reference gene out of 5 candidates (β-actin, B2M, GAPDH, GUSb, and HPRT1). To date, on our populations, gene-expression experiments of the 7 long non-coding Rna identified are ongoing.

2) Metabolomic- Phospholipid Profile

In our recent study we explored the serum phospholipid profile in infants at risk for celiac disease, followed up to 8 years of life to monitor the onset of CeD. We compared 30 patients who developed the disease with 20 age- and sex-matched peers with similar genetic profiles who did not develop the disease within 8 years. Serum phospholipids were analyzed at 4 months, before exposure to gluten, and at 12 months of age, when none showed any marker of disease. In the 30 CeD patients, we also analyzed the serum at the time of diagnosis (> 24 months). The serum phospholipid profile was fairly constant across 4 and 12 months of age and, in CeD, up to 24-36 months. The lipidomic profile was dramatically different in infants who developed CeD when compared to control Not Yet-CeD peers. We identified a specific serum phospholipid signature that predicts the onset of celiac disease in HLA at-risk infants years before the appearance of antibodies specific for CeD in the serum and before any clinical symptoms, even before gluten introduction into the diet at 4 months. Specifically, lysophosphatidylcholine, phosphatidylcholine, alkylacyl-phosphatidylcholine, phosphoethanolamines, phosphatidylserines, phosphatidylglycerol and phosphatidylinositol were found to be differentially represented in CeD versus NY-CeD. A set constituted by a limited number of alkylacyl-phosphatidylcholine and lyso-phosphatidylcholine, together with the duration of breast-feeding, allows the discrimination of infants who develop celiac disease before 8 years of age from those at a similar genetic risk who do not develop the disease.

Future perspectives

We plan to complete studies on lnc-RNA in the next 2 years, in particular we would like to continue this project through the following steps:

1. Selection of FFPE small-intestine biopsies from 20 CeD patients, 20 Controls (CTRLs), and 20 positive inflammed controls (IBDs).
2. Mapping lncRNAs expression in specific intestinal cells in situ by immunohistochemistry and RNA in-situ hybridization. We plan to use RNAscope®-assay that allows simultaneous single-molecule visualization while preserving tissue morphology.
3. Bioinformatic prediction to identify the target genes and molecular pathways regulated by the lncRNA.
4. Development of epithelial organoid from 5 CeD patients on a gluten free diet with normal histology, to explore the effect of gluten peptides on the function of the lncRNA through their target pathway.
5. Confirmation of the causal effect of polymorphisms of lncRNAs in specific cell lines, modified by CRISPR/Cas9 technology, that is able to manipulate effectively the non-coding regions of the genomic DNA, to create or correct the specific mutations. Selection of appropriate read-out, among epithelial or immune cells, in relation to the role played by lncRNA and its target genes.

In the contex of lipidomic analysis, we have no clear explanation about why the lipid profile of infants who later develop CeD is so different from their peers with a similar genetic background that do not develop CeD, but these findings give a further strong support that the 'future CeD' infants are born not only with a peculiar genomic background, but also with a specific metabolomics profile, of which lipidomic is a consistent slice. This remarkable difference of serum phospholipids in infants who will develop an auto-immune disease, has to be explored in the domain of T-Cell immunity regulation and a series of epigenetic mechanisms able to drive genetically predisposed infants to their final outcome.

Publications

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