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Long noncoding RNA are aberrantly expressed *in vivo* in the cystic fibrosis bronchial epithelium☆

Paul J. McKiernan[a], Kevin Molloy[a], Sally A. Cryan[b], Noel G. McElvaney[a], Catherine M. Greene[a,*]

[a] Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland
[b] School of Pharmacy, Royal College of Surgeons in Ireland, Dublin 2, Ireland

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ABSTRACT

Long non-coding RNAs (lncRNAs) have emerged recently as key regulatory molecules with diverse roles at almost every level of the regulation of gene expression. The roles of these RNAs in the pathogenesis of cystic fibrosis (CF); a lethal multisystem, autosomal recessive disorder have yet to be explored. Our aim was to examine the expression profile of lncRNA, in the airway epithelium of people with CF. We examined the expression of 30,586 lncRNAs by microarray (Human LncRNA Array v3.0, Arraystar, Inc.), *in vivo* in bronchial cells isolated from endobronchial brushings obtained from CF and non-CF individuals. In total, we identified 1,063 lncRNAs with differential expression between CF and non-CF individuals (fold change ≥ 3, $p \leq 0.001$). The microarray also contained probes for ~26,109 protein coding transcripts, of which 720 were differentially expressed between CF and non-CF brush samples (fold change ≥ 3, $p \leq 0.001$). Confirmation of a selection of differentially expressed coding mRNA and lncRNA transcripts such as XIST and TLR8 was achieved using qRT-PCR. Gene ontology bioinformatics analysis highlighted that many processes over-represented in the CF bronchial epithelium are related to inflammation. These data show a significantly altered lncRNA and mRNA expression profile in CF bronchial cells *in vivo*. Dysregulation of some of these lncRNAs may play important roles in the chronic infection and inflammation that exists in the lungs of people with CF.

This article is part of a directed issue entitled: Cystic fibrosis: from o-mics to cell biology, physiology, and therapeutic advances.

1. Introduction

Cystic fibrosis (CF) is a lethal multisystem, autosomal recessive disorder, affecting many organs. Its pulmonary manifestations are responsible for the associated high morbidity and mortality. CF is characterised by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, of which over 1900 mutations have been identified to date. A deletion of phenylalanine at position 508 of the CFTR protein (p.Phe508del) is the most frequent mutation, and accounts for approximately 70% of the alleles in CF worldwide (Sheridan et al., 2011), and 90.2% of alleles in the Republic of Ireland (Cystic Fibrosis Registry of Ireland, 2012). The *CFTR* gene encodes an ATP-regulated chloride channel and is present within the apical surface of epithelial cells. The development of chronic inflammatory lung disease is typically the primary manifestation in people with CF, with additional disease in other organ systems including pancreatic insufficiency, sweat electrolyte imbalance and male infertility (Sheridan et al., 2011). Some of the major determinants of CF lung disease include a decreased airway surface liquid volume, increased mucus viscosity, chronic microbial colonisation, an impaired protease-antiprotease balance and increased pulmonary inflammation. Bronchial epithelial cells with their array of pattern recognition receptors, such as the Toll-like receptors (TLRs), are key contributors to the airway inflammation evident in the CF lung. When stimulated with their cognate agonists, these cells have been shown to promote pro-inflammatory gene expression, within the context of CF (Greene et al., 2005; Carroll et al., 2005). Although advances in recent years have improved treatment and lengthened the median survival of people with CF (Dodge et al., 2007), there is still no effective cure. Therefore, determining the expression and function of long non-coding RNAs (lncRNAs) in CF will illuminate regulatory mechanisms controlling changes in gene expression and could direct the development of future therapeutic approaches.

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* Corresponding author. Tel.: +35318093712.
E-mail address: cmgreene@rcsi.ie (C.M. Greene).

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Non-coding RNAs have emerged recently as key regulatory molecules with diverse roles in fundamental biological processes. These RNA transcripts can be divided into two classes based on length; lncRNAs (>200 nt), or short ncRNAs (<200 nt) such as microRNAs, small nucleolar RNAs and piwi-interacting RNAs. lncRNAs have many diverse functions. They have the ability to bind to RNA, DNA and protein. They can be used in cells as an indicator or signal of transcriptional activity. They can act as decoys by binding to RNA or protein and titrating these away from other molecules; they can act as guides to direct localisation of ribonucleoprotein complexes; and they can act as scaffolds and structural platforms for nuclear processes. Aberrant expression of lncRNAs have been associated with various human conditions, including those as diverse as cancers (Yu et al., 2012), myocardial infarction (Ishii et al., 2006a) and Alzheimer's disease (Faghihi et al., 2008). lncRNAs have been shown to be involved in the regulation of gene expression at almost every level, from nuclear epigenetic modifications to cytoplasmic mRNA translation. Our group was the first to examine the microRNA expression profile of the CF airway epithelium (Oglesby et al., 2010). Performed on endobronchial brushings from people with and without CF, microRNA profiling studies identified various microRNAs that had altered expression in CF. Our group also identified that three of these microRNAs with increased expression *in vivo* (miR-145, miR-223, and miR-494) correlate with decreased Phe508del *CFTR* expression and regulate its expression (Oglesby et al., 2013). What remains to be seen is whether there exists an altered lncRNA profile in the CF airway epithelium.

In this study, we have profiled the expression of lncRNA and mRNA in airway epithelial cells obtained from endobronchial brushings from people with and without CF. We also evaluated differentially expressed lncRNAs in independent patient samples. Our results demonstrate an altered expression profile of lncRNAs in cystic fibrosis bronchial epithelial cells.

2. Materials and methods

2.1. Study populations and bronchial brush sampling

Twenty-two individuals were recruited into this study; 10 had CF (confirmed by sweat testing and/or genotyping) and 12 were non-CF controls, with a mean age of 22.4 ± 4.9 yr and 37.2 ± 15.6 yr, respectively (Table 1). Forced expiratory volume in one second (FEV_1) and colonisation status of CF individuals are shown in Table 2.

All participants were undergoing diagnostic and/or therapeutic fibre-optic flexible bronchoscopy as part of routine care. Non-CF control individuals were undergoing exploratory bronchoscopy in the investigation into idiopathic pulmonary disease. Fully informed consent was obtained before the procedure, and appropriate approval was obtained from our institutional review board. After completion of the bronchoscopy and before the withdrawal of the bronchoscope, an area 2 cm distal to the carina (medially located) in either the right or left main bronchus was selected and washed twice with 10 ml sterile 0.9% NaCl. Next, a sterile 10×1.2-mm bronchial brush (Olympus Medical Systems, Tokyo, Japan) was inserted through the appropriate port on the bronchoscope and the chosen area sampled with two consecutive brushes by scraping the area gently. The brush was withdrawn and immediately placed in 5 ml RPMI + Glutamax supplemented with 10% foetal calf serum and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA).

2.2. Isolation of RNA

Brushes were gently agitated to dislodge cells into the media, which was centrifuged at $300 \times g$ for 5 min, and cell pellets were resuspended in Tri Reagent (Sigma-Aldrich) before RNA extraction as per manufacturer's protocol. The quality and the concentration of the RNA samples were monitored at absorbance ratios of A_{260}/A_{280} and A_{260}/A_{230} using a NanoDrop 8000 spectrophotometer.

2.3. RNA labelling and array hybridisation

Expression profiling studies were performed on RNA from three individuals with CF (one male and two female) and three non-CF controls (one male and two female) by Arraystar, Inc. (Rockville, MD, USA). Total RNA from each sample was quantified by the NanoDrop ND-1000 and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. For microarray analysis an Agilent array platform was employed. mRNA was purified from total RNA after removal of rRNA using the mRNA-ONLY™ Eukaryotic mRNA Isolation Kit (Epicentre Biotechnologies, Madison, WI, USA). Each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3′ bias utilizing a random priming method. The concentration and specific activity of the labelled cRNAs (pmol Cy3/μg cRNA) were measured by NanoDrop ND-1000, 1 μg of each labelled cRNA was fragmented by adding 5 μl 10× blocking agent and 1 μl of 25× fragmentation buffer, heated to 60 °C for 30 min, and diluted with 25 μl 2× GE hybridisation buffer. Fifty microlitres of hybridisation solution was dispensed into the gasket slide and assembled to the Human LncRNA Array v3.0 slide (8×60 K, Arraystar). The slides were incubated for 17 h at 65 °C in an Agilent hybridisation oven then washed, fixed and scanned using the Agilent DNA Microarray Scanner (part number G2505C). Approximately 30,586 lncRNAs and 26,109 coding transcripts collected from the most authoritative databases, such as RefSeq (release 55), UCSC Human (GRCh37/hg19), GENCODE 13, and lncRNAdb (2.0) were detected using microarray. Microarray data from this study has been archived to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo, accession number GSE55146).

2.4. Data analysis

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization was performed using Expander and subsequent data processing was performed using the GeneSpring GX v12.0 software package (Agilent Technologies). Quantile normalisation of the raw data was carried out. This is a transformation to make the distribution of probe intensities identical for all arrays performed. Data was filtered to remove transcripts with unreliable expression measurements (transcripts that at least 1 out of 6 samples have flags in 'present' or 'marginal' were chosen for further analysis), lncRNAs and mRNAs with expression above a set threshold were chosen for further analysis. Differentially expressed lncRNAs and mRNAs between two groups were identified through volcano plot filtering and hierarchical clustering was performed to show the distinguishable lncRNA and mRNA expression patterns among samples.

2.5. LncRNA classification and pathway analysis

Differentially expressed lncRNAs were assigned to different subgroups to aid in the identification of any putative functional relationships between lncRNAs and their associated protein-coding genes. Subgroups included lncRNAs with enhancer-like functions, long intergenic RNAs (lincRNA), human homeobox transcription factors (HOX) cluster profiling, lincRNAs nearby coding genes, and enhancer lncRNAs nearby coding genes. Our microarray enabled the expression analysis of ~26,109 protein coding transcripts,

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