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Smoking-related microRNAs and mRNAs in human peripheral blood mononuclear cells

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ABSTRACT

Teenager smoking is of great importance in public health. Functional roles of microRNAs have been documented in smoke-induced gene expression changes, but comprehensive mechanisms of microRNA-mRNA regulation and benefits remained poorly understood. We conducted the Teenager Smoking Reduction Trial (TSRT) to investigate the causal association between active smoking reduction and whole-genome microRNA and mRNA expression changes in human peripheral blood mononuclear cells (PBMC). A total of 12 teenagers with a substantial reduction in smoke quantity and a decrease in urine cotinine/creatinine ratio were enrolled in genomic analyses. In Gene Set Enrichment Analysis (GSEA) and Ingenuity Pathway Analysis (IPA), differentially expressed genes altered by smoke reduction were mainly associated with glucocorticoid receptor signaling pathway. The integrative analysis of microRNA and mRNA found eleven differentially expressed microRNAs negatively correlated with predicted target genes. CD83 molecule regulated by miR-4498 in human PBMC, was critical for the canonical pathway of communication between innate and adaptive immune cells. Our data demonstrated that microRNAs could regulate immune responses in human PBMC after habitual smokers quit smoking and support the potential translational value of microRNAs in regulating disease-relevant gene expression caused by tobacco smoke.

1. Introduction

The onset of active tobacco smoking begins primarily in youngsters during teenage years. Active smoking may cause some adverse health consequences (Bodner et al., 1998; Vork et al., 2007; Tsai et al., 2010), such as pulmonary function deterioration and occurrence/exacerbation of asthmatic symptoms (Openshaw, 2001). Many studies have shown that tobacco smoke toxicity impacted numerous cell types of the immune system, such as bronchial epithelial cells (Laan et al., 2004), alveolar macrophages (McCrea et al., 1994; Ouyang et al., 2000), natural killer cells (Newman et al., 1991), dendritic cells (Robbins et al., 2004; Vassallo et al., 2005), and B and T lymphocytes (Kalra et al., 2000; van der Strate et al., 2006). In murine models, tobacco smoke reduced dendritic cell antigen-presenting and co-stimulatory molecules, and also reduced capacity for antigen uptake and production of T-cell stimulatory cytokines (Robbins et al., 2008). In asthmatic smokers, reduced number of CD83 + mature dendritic cells and B lymphocytes in bronchial biopsy specimens contributed further evidence for immune dysregulation. Human monocytes may sense tobacco smoke as an injurious stimulus (Walters et al., 2005), and influence gene expression in immune, inflammatory and oxidative stress pathways (Henderson et al., 2008). Another in vitro study also revealed that tobacco smoke exposure in peripheral blood mononuclear cells (PBMC) would induce the production of cytokines through TLR4-dependent signaling (Karimi et al., 2006). These studies indicated that tobacco smoke toxicity in PBMC might alter gene expression in human immune and inflammatory pathways. However, the genetic mechanism and functional regulation of smoke reduction remained under investigated.

MicroRNAs are small non-coding RNAs that regulate gene expression by suppression of translation or mRNA degradation (Esteller, 2011). The target genes are negatively regulated by microRNA. Approaches for identification of microRNA-mRNA regulatory pairs have been introduced by calculating correlation coefficient of the paired microRNA-mRNA and predicting putative target genes of microRNA (He and Hannon, 2004). Although regulatory roles of some microRNAs have been documented in smoke-induced gene expression changes of human bronchial airway epithelial cells (Schembri et al., 2009), the significance of microRNAs in regulating the physiological response of smoke reduction in human PBMC was still unclear.

In current study, we aim to investigate the smoke reduction benefit on immune and inflammatory pathways through microRNA-mRNA regulation. Gene Set Enrichment Analysis (GSEA) and Ingenuity Pathway Analysis (IPA) were used to gain more biological insight into meaningful regulatory pathways of smoke reduction-induced gene expression

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change. The underlying algorithms are novel and valuable tools for data mining in genomics. Based on whole-genome microRNA and mRNA expression data from human subjects, we could extensively investigate the potential microRNA–mRNA regulated network by smoke reduction.

2. Materials and methods

2.1. Study population

TSRT (Teenager Smoking Reduction Trial) is a single-arm community-based intervention program aiming to evaluate the health benefit of active smoking reduction among teenagers. The inclusion criteria were (1) aged between 12 and 16 years old, and (2) having active smoking habits during the past 6 months and smoking >10 cigarettes per week. The active smoking intervention program was based on school-level incentive and was conducted between April and July 2011. The intervention period was three months. Teenagers received a four-time face-to-face health education program weekly in first month. Telephone counseling was performed at least 15–20 min to reduce the child's tobacco smoke exposure, occurring weekly after the face-to-face health education program for two months. The interventionist and parents jointly selected behavioral goals for reducing tobacco smoke exposure for the parent to work toward. The goals will be formalized as a written smoke exposure reduction plan for smoking cessation among the teenagers. The counseling intervention components will be guided by the social cognitive based model of health behavioral change (Patten et al., 2008).

Before and after the intervention program, participants were evaluated their smoking status by urine cotinine/creatinine ratio (CCR) and self-reported number of cigarette consumption per day. Pulmonary function was also measured simultaneously. A total of 100 teenagers with active smoking habits completed the whole trial. All participants with their parents have provided written informed consents. The study protocol was approved by the institutional review board of National Taiwan University Hospital and complied with the principles outlined in the Helsinki Declaration (1994) [Clinical Trial #NCT01317628].

2.2. Urine and blood sample collection

For cotinine analysis, a previously reported protocol of the LC-MS/MS was modified and used throughout (Ceppa et al., 2000). The positive multiple reactions monitoring mode is used for quantitation. Data acquisition and quantitative processing were accomplished using AnalystTM v1.1. Creatinine in each urine sample was analyzed using the levels of the creatinine–picrate complex by means of a routine procedure using a spectrophotometer with the wavelength set at 520 nm. The urine CCR (μg/g) was calculated as 100 x cotinine (ng/ml)/creatinine (mg/dl).

Venous blood sample was drawn in the morning after 8 h fast and 15 min post-physical exercise. PBMC was isolated immediately by Ficoll density centrifugation applying standard procedures (Bach and Brashler, 1970). In order to identify differentially expressed microRNAs and mRNA association with tobacco smoke exposure, we extracted total RNAs from PBMC before and after smoke reduction by standard protocol of TRIzol reagent (Haimov-Kochman et al., 2006).

2.3. mRNA microarray procedure

Total RNAs were hybridized with Illumina Human HT-12 v4.0 Expression BeadChip containing coding transcripts, non-coding transcripts, and experimentally confirmed mRNA sequences aligning with EST cluster. Illumina microarray data was input, quality control, variance stabilization, normalization, and gene annotation using Lumi, a Bioconductor (Seattle, WA, USA) package (Smyth, 2004). We conducted pair t-statistics derived from an empirical Bayesian framework to identify differentially expressed genes before and after smoke reduction, as implemented in Limma Bioconductor package. To correct the associated *p*-values for multiple testing, significant differentially expressed genes were identified based on a false discovery rate (FDR) using the Benjamini and Hochberg approach (Benjamini and Hochberg, 1995).

2.4. MicroRNA expression profiling

Total RNAs were hybridized on Affymetrix GeneChip microRNA array according to the manufacturer's recommendations (Affymetrix, Santa Clara, CA, USA). These data was firstly normalized using the robust multiarray average (RMA) algorithm (Irizarry et al., 2003). The default option of RMA (with background correction, quantile normalization, and log transformation) was used to generate the normalized intensity for each probe set. After RMA, the data was analyzed by pair *t*-statistics derived from within an empirical Bayesian framework to identify differentially expressed microRNAs. Supervised hierarchical clustering of microRNAs was performed using normalized data with a Pearson's correlation similarity metric and average linkage clustering using Genesis software to visualize the expression of microRNAs (Sturn et al., 2002).

2.5. Gene set enrichment analysis of Gene Ontology biological process

In order to access the relative merits of smoke reduction-induced biological process, we performed Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) of Gene Ontology (GO) biological process. A gene set is a group of genes collected by similar biological function, pathway, chromosomal localization or other features. The GSEA algorithm uses predefined gene sets from the Molecular Signatures Database (MolSigDB v3.0). Genes were ranked by the log fold change from the paired samples, and enrichment score (ES) was computed by a running sum statistic to reflect the degree of a gene set over-represented at the extremes (top or bottom) of the entire ranked list. The ES for each gene set was normalized by the size of the set, yielding a normalized enrichment score (NES). Statistical significances (nominal *p*-values) of ES were estimated by empirical permutation tests. The enriched gene sets, or "leading edge genes", were genes that contribute most to the annotated gene sets. In our study, the gene set annotations were based on the GO biological process annotations.

2.6. Ingenuity pathway analysis

The biological networks, functional analyses were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®QIAGEN Redwood City, www.qiagen.com/ingenuity). The canonical pathways in Ingenuity Pathway Analysis (IPA) summarizing the biological function of differential expressed genes were identified by Fisher's exact tests. The significance of the association between the differentially expressed genes and the canonical pathway was referenced in a ratio of the number of genes differentially expressed after smoke reduction that map to the canonical pathways divided by the total number of molecules in certain pathway.

2.7. Integrative analysis of microRNA and mRNA expression

In the current study, we adapted the Pearson's correlation coefficient for the integrative analysis of microRNA and mRNA expression by testing whether the expression of mRNA was related to the microRNA expression. Numerous algorithms have been developed based on sequence alignment, conservation of sequence among species and/or free energy calculation of the microRNA–mRNA complex to identify where the mRNA is a predicted target of microRNA (Yue et al., 2009). Pairs of microRNA-mRNA with statistically significant negative correlations were cross-referenced with TargetSan (Garcia et al., 2011) and miRanda (John et al., 2004) to elucidate the target genes of microRNAs. IPA