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ORIGINAL PAPER

Genomic-based identification of novel potential biomarkers and molecular signaling networks in response to diesel exhaust particles in human middle ear epithelial cells

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Abstract Otitis media (OM) is the most common inflammatory disease of the middle ear cavity. Several factors including viral and bacterial infection, biofilm formation, congenital anomalies, and environmental factors have been recognized as the main causes of OM. Recent epidemiological studies showed that children living in areas with high concentrations of air pollutant including particulate matter and SO_2 have significantly higher rates of OM compared with those in the control area. Another study reported that air pollutant exposure results in significant increases in pediatric OM. A large cohort study in Germany suggested that the prevalence of OM is related to air quality. Diesel exhaust particles (DEPs) are among the major toxic air pollutants of motor vehicle emissions. Hence, identifying the biomarkers of a signaling network for air pollutant (particularly DEPs)-mediated inflammatory responses would be meaningful. In this study, we identified novel biomarkers and potential molecular signaling networks induced by DEPs in human middle ear epithelial cells (HMEECs). Genomic expression analysis via microarray was used to discover novel biomarkers. A total of 254 genes were differentially expressed in DEPs-exposed HMEECs; 86 genes and 168 genes were up-and down-regulated, respectively. To verify reliable biomarkers and define meaningful signaling networks in the entire genome profiling, the *in silico* approach was applied. Based on genomic profiling analysis, we found several novel key molecular biomarkers, including *SRC*, *MUC5AC*, *MUC2*, *MMP14*, *EIF1AK3*, *KITLG*, *NOD1*, and *TP53*. Our findings suggested novel biomarkers for DEPs-responsive genes in HMEECs. Furthermore, we provided scientific evidence for the establishment of novel molecular signaling pathway associated with DEPs exposure in HMEECs.

Keywords Diesel exhaust particles, Gene expression profile, Human middle ear epithelial cells, Molecular signaling network

Rapid industrialization and urbanization have induced a significant increase in the prevalence of respiratory or rhinopharyngitis diseases including allergic rhinitis, asthma, and otitis media (OM)[1,2]. Indeed, the incidence of allergic respiratory disease has increased primarily in urban communities. Furthermore, a strong relevance between air pollution and detrimental health effects was suggested in recent epidemiologic and clinical investigations[3]. OM is one of the most common infections in young children. Exposure to environmental smoke is well known as the main risk factor for OM; however, recent studies have suggested that air pollution is also a potential risk factor[4].

In both developed and rapidly industrializing countries, the major air pollution problem has typically been high levels smoke and sulfur dioxide (SO_2) arising from the combustion of sulfur-containing fossil fuels, such as coal used for domestic and industrial

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purpose. The major threat to clean air is now posed by traffic emissions. Gasoline and diesel-engine motor vehicles emit a wide variety of pollutants, principally carbon monoxide (CO), oxides of nitrogen (NOx), volatile organic compounds (VOCs), and particulates (PM_{10}), which has an increasing impact on urban air quality[1]. Diesel exhaust particles (DEPs) which are generated during the combustion of diesel fuel, are a major component of air pollution[5,6]. Diverse compounds such as elemental or organic carbon, small amounts of sulfate, nitrate, metals, and other trace elements are included in DEPs[6]. Recent investigations reported inflammation in the airways of healthy individuals after DEPs exposure. Inflammatory mediators were also increased in the respiratory tract after DEPs exposure[7].

Although there is relevance between the incidence of OM and DEPs, investigations of the biological effect of DEPs are currently insufficient. Alteration of miRNA expression that may lead to disease pathogenesis has been reported in airway epithelial cells exposed to DEPs[8]. In addition, the inhalation of DEPs caused a hypertensive-like cardiac gene expression pattern associated with mitochondrial oxidative stress in rats[9]. Another investigator reported that DEPs induced a lipopolysaccharide-related gene expression profile in the murine lung[10]. However, most studies focused on bronchial tubes or the respiratory system.

Our previous study showed that DEPs decreased cell viability. Furthermore, inflammation and mucin producing gene expression were induced in human middle ear epithelial cells (HMEECs)[5]. However, the biological effect of DEPs on HMEECs has not yet been elucidated. Analysis of gene expression profiling has been used to discover shared biomarkers or target molecules. Analysis of signaling network among differentially expressed genes enables the identification of significant novel biomarker or target molecules. Furthermore, it can provide relevant biological processes associated with specific diseases or chemical exposure. In this study, we identified novel biomarkers and molecular signaling network in HMEECs exposed to DEPs.

Identification of differentially expressed genes in HMEECs exposed to DEPs

Gene expression microarray was conducted to analyze the genome-wide transcription pattern. A total of 254 genes were regulated by urban particulate matter. Among them, 86 genes were up-regulated whereas 168 genes were down-regulated. To classify these genes according to cellular process, we categorized these genes using Pathway Studio 9.0 software (Ariadne Genomics, Rockville, MD, USA). Up-regulated genes were mainly involved in cellular processes including apoptosis, cell death, cell differentiation, cell migration, and vascularization (Figure 1). Down-regulated genes affected several cellular process including apoptosis, cell cycle, cell differentiation, cell growth, cell survival, DNA replication, and vascularization (Figure 2).

Prediction of potential signaling networks associated with exposure to DEPs in HMEECs

To investigate the molecular signaling networks associated with DEPs exposure, Pathway Studio 9.0 software (Ariadne Genomics) was applied. Based on a number of reliable studies, relevant components in the putative signaling pathways were chosen and incorporated into the established networks. A total of 16 genes were discovered as crucial components in potential signaling networks containing 1.5-fold up regulated genes (Table 2 and Figure 1). Figure 1 showed that diverse cell processes including apoptosis, cell death, cell differentiation, cell proliferation, cell migration, and vascularization were affected by DEPs exposure. Additionally this cell process might lead to cancer, neoplasm, and neoplasm metastasis. Four genes - *MMP14*, *MUC2*, *MUC5AC*, and *SRC* - had numerous connectivity with several genes and diverse cellular processes. Hence, it would be considered as key mediator genes among the up-regulated genes altered by urban particulate matter exposure. A total of 13 genes were revealed as key modulators in the signaling pathway associated with 1.5-fold down regulated genes (Table 3 and Figure 2). Down-regulated gene sets showed unique signaling pathway related to apoptosis, cell cycle, cell differentiation, cell growth, cell survival, DNA replication, and vascularization which can induce inflammation and neoplasm (Figure 2). Particularly, four genes including *EIF1AK3*, *KITLG*, *NOD1*, and *TP53* were identified as main modulator genes due to relatively high relevance among numerous genes and cellular processes.

Validation of the novel biomarkers identified in HMEECs exposed to DEPs

The significant genes identified in the signaling network were verified by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). The expression pattern was similar between gene expression microarray and real-time qRT-PCR (Figure 3).

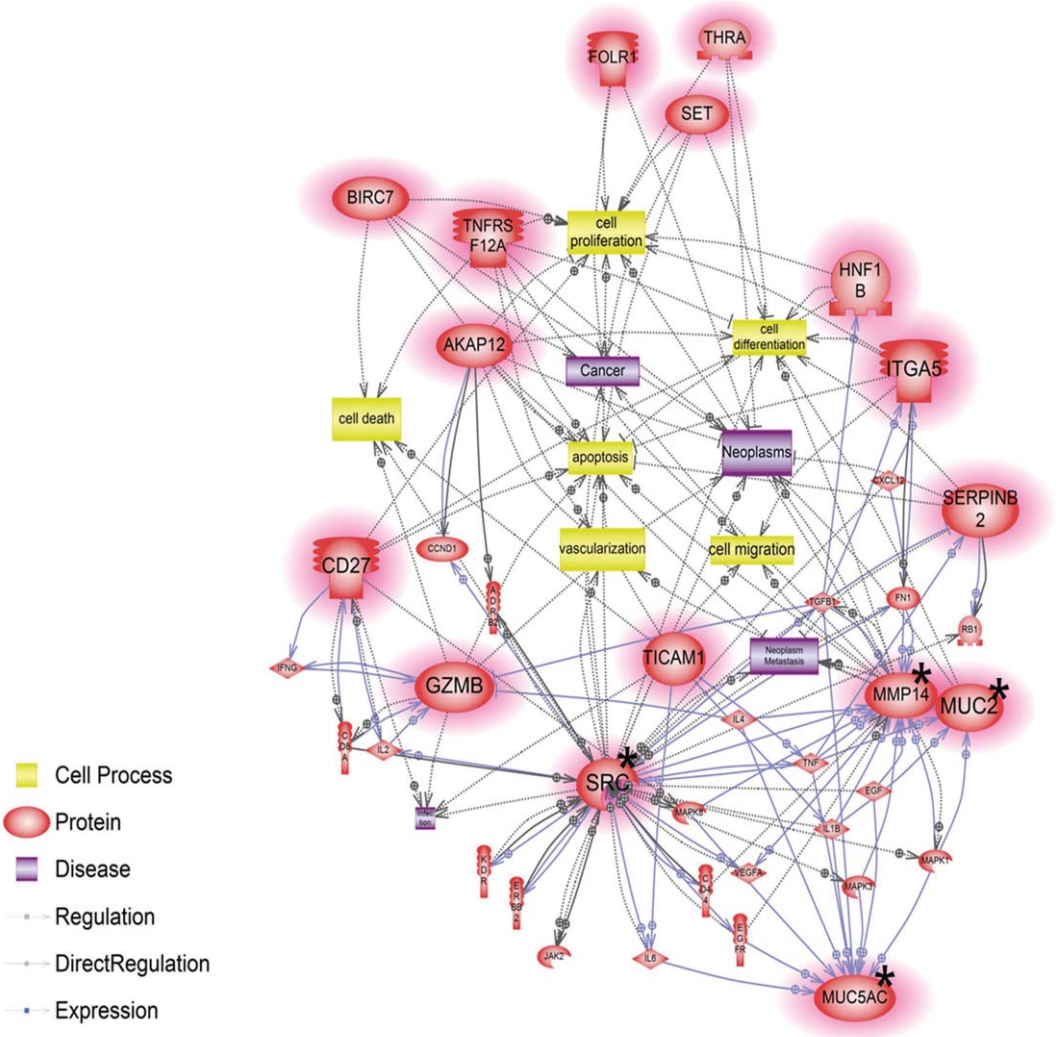

Figure 1. Potential molecular signaling network among up-regulated genes triggered by diesel exhaust particles exposure in human middle ear epithelial cells. Responsive molecular network of up-regulated genes was predicted using Pathway Studio software. Magnified red circles indicate up-regulated genes in our gene expression microarray data.

Discussion

The deleterious effect of DEPs has been reported consistently over the past 20 years. However, studies concerning the molecular effects of DEPs on upper respiratory mucosa of the ear and nose are insufficient. Only a few investigations focused on the relevance of air pollutant-induced biological effects including cytotoxicity, inflammation, and altered gene expression in HMEECs[7,11,12]. Identification of novel potential biomarkers in HMEECs exposed to DEPs could facilitate our understating of significant biological changes via the discovery of molecular biomarkers and the analysis of its associated signaling pathways. Here we analyzed DEPs-induced gene expression profiles of HMEECs for the identification of novel biomarkers and the associated molecular signaling networks.

DEPs induced the alteration of total 254 genes. Among them, 86 genes were significantly up-regulated and 168 genes were down-regulated. In the up-regulated genes, cellular processes including apoptosis, cell death, cell differentiation, cell migration, cell proliferation, and vascularization were exhibited as the main signaling processes. These cellular processes could affect cancer, neoplasm, and neoplasm metastasis. Of the 86 up-regulated genes, 16 genes including *BIRC7*, *TNFRSF12A*, *AKAP12*, *CD27*, *GZMB*, *TICAM1*, *SRC*, *MUC5AC*, *MMP14*, *MUC2*, *SERPINB2*, *ITGA5*, *HNF1B*, *FOLR1*, *SET*, and *THRA* were predicted as

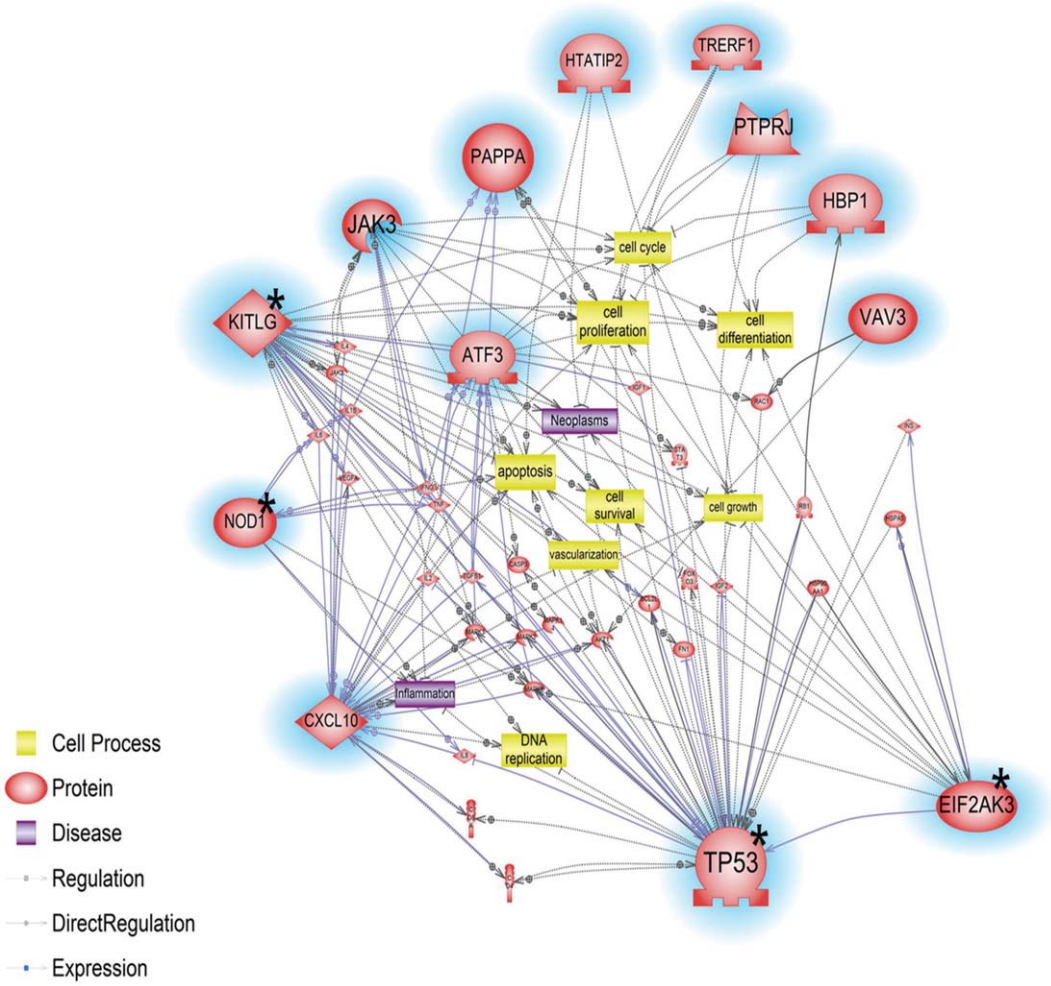

Figure 2. Potential molecular signaling network among down-regulated genes triggered by diesel exhaust particles exposure in human middle ear epithelial cells. Responsive molecular network of down-regulated genes was predicted using Pathway Studio software. Magnified blue circles indicate down-regulated genes in our gene expression microarray data.

Table 1. Primer sequences used for real-time RT-PCR.

Gene name	Accession no.	Forward primer sequence	Reverse primer sequence	Size (bp)
MUC5AC	XM_003960483	CAGCACAACCCCTGTTTCAAA	GCGCACAGAGGATGACAGT	99
SRC	NM_198291	TGGCAAGATCACCAGACGG	GGCACCTTTCGTGGTCTCAC	100
MMP14	NM_004995	CGCTGCCATGCAGAAGTTTT	TGTCTGGAACACCACATCGG	98
MUC2	NM_002457	CGACTACTACAACCCTCCGC	CTCCAGGTAGGACACGGAGA	121
TP53	NM_001126118	AGACCTGTGGGAAGCGAAAA	TCATCCATTGCTTGGGACGG	106
EIF2AK3	NM_004836	ACGATGAGACAGAGTTGCGAC	ATCCAAGGCAGCAATTCTCCC	80
KITLG	NM_000899	GCCAGCTCCCTTAGGAATGAC	TAAGGCTCCAAAAGCAAAGCC	138
NOD1	NM_006092	TGGGGTAAAGGTGCTAAGCG	TTTGGTGACGTACCTGGCTC	106
GAPDH	NM_002046	GAGTCCACTGGCGTCTTCAC	TTCACACCCATGACGAACAT	119

key modulator genes in response to DEPs exposure (Figure 1). Four genes - *SRC*, *MUC5AC*, *MUC2*, and *MMP14* - were validated by real-time qRT-PCR (Figure 3). *SRC*, the V-Src avian sarcoma (Schnidt-Ruppin A-2) vial oncogene, is highly similar to the v-src gene of the Rous sarcoma virus. This proto-oncogene encodes tyrosine-protein kinase whose activity can be inhibited by c-SRC kinase phosphorylation. A recent

Table 2. List of 1.5 fold up-regulated genes against DEPs exposure in HMEECs.

Gene symbol	Gene name	Fold change	*P*-value
ACPT	acid phosphatase, testicular	1.80	0.0074
ACR	acrosin	1.68	0.0070
AHSG	alpha-2-HS-glycoprotein	1.54	0.0009
AKAP12	A kinase (PRKA) anchor protein 12	1.83	0.0001
ALDH3B1	aldehyde dehydrogenase 3 family, member B1	1.55	0.0007
ALOXE3	arachidonate lipoxygenase 3	1.71	0.0006
ARGLU1	arginine and glutamate rich 1	1.67	0.0042
ARHGAP29	Rho GTPase activating protein 29	1.60	0.0012
BIRC7	baculoviral IAP repeat containing 7	1.62	0.0009
C2orf70	chromosome 2 open reading frame 70	1.64	0.0105
CASZ1	castor zinc finger 1	1.53	0.0019
CD27	CD27 molecule	1.84	0.0030
CDSN	corneodesmosin	1.59	0.0012
CELA2B	chymotrypsin-like elastase family, member 2B	1.87	0.0004
CGB	chorionic gonadotropin, beta polypeptide	1.62	0.0110
COL10A1	collagen, type X, alpha 1	1.62	0.0016
DENND1C	DENN/MADD domain containing 1C	1.70	0.0005
DFNB31	deafness, autosomal recessive 31	1.55	0.0290
ELF1	E74-like factor 1 (ets domain transcription factor)	1.50	0.0012
FAM83A	family with sequence similarity 83, member A	1.56	0.0000
FERMT1	fermitin family member 1	1.50	0.0003
FOLR2	folate receptor 2 (fetal)	1.62	0.0062
FOLR4	folate receptor 4, delta (putative)	1.67	0.0183
FOXA3	forkhead box A3	1.58	0.0368
FOXI3	forkhead box I3	1.67	0.0019
FOXN1	forkhead box N1	1.51	0.0143
G0S2	G0/G1switch 2	1.68	0.0001
GLTPD2	glycolipid transfer protein domain containing 2	1.88	0.0040
GLTSCR1	glioma tumor suppressor candidate region gene 1	1.86	0.0013
GPR135	G protein-coupled receptor 135	1.59	0.0043
GPR172B	Uncharacterized	1.50	0.0021
GPR45	G protein-coupled receptor 45	1.54	0.0041
GPR62	G protein-coupled receptor 62	1.82	0.0246
GPSM3	G-protein signaling modulator 3	1.53	0.0003
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	1.69	0.0001
HNF1B	HNF1 homeobox B	1.68	0.0047
IGLON5	IgLON family member 5	1.51	0.0023
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	1.68	0.0004
KANK3	KN motif and ankyrin repeat domains 3	1.72	0.0238
KLK5	kallikrein-related peptidase 5	1.58	0.0110
KRTAP2-1	keratin associated protein 2-1	1.54	0.0007
LCE3B	late cornified envelope 3B	1.52	0.0273
LCE3D	late cornified envelope 3D	1.52	0.0001
LILRA1	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 1	1.73	0.0012
LINC00315	long intergenic non-protein coding RNA 315	1.57	0.0008
LMF1	lipase maturation factor 1	1.66	0.0005
LOC157627(LINC00599)	long intergenic non-protein coding RNA 599	1.57	0.0008
LOC646513	VLGN1945	1.50	0.0000
LOC730755(KRTAP2-3)	keratin associated protein 2-3	2.46	0.0003
LRRC16B	leucine rich repeat containing 16B	1.58	0.0033
MMP14	matrix metallopeptidase 14 (membrane-inserted)	1.53	0.0106
MRGPRE	MAS-related GPR, member E	1.54	0.0059
MTMR6	myotubularin related protein 6	1.51	0.0089
MUC2	mucin 2, oligomeric mucus/gel-forming	1.57	0.0008
MUC5AC	mucin 5AC, oligomeric mucus/gel-forming	1.74	0.0044
OR4X2	olfactory receptor, family 4, subfamily X, member 2	1.54	0.0001
OR6W1P	olfactory receptor, family 6, subfamily W, member 1 pseudogene	1.54	0.0279
PBOV1	prostate and breast cancer overexpressed 1	1.75	0.0004
PCSK6	proprotein convertase subtilisin/kexin type 6	1.62	0.0251
PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent)	1.53	0.0217

Table 2. Continued.

Gene symbol	Gene name	Fold change	*P*-value
PLA2G4F	phospholipase A2, group IVF	1.59	0.0019
PPAN-P2RY11	PPAN-P2RY11 readthrough	1.60	0.0056
RGS11	regulator of G-protein signaling 11	1.51	0.0190
RIMBP2	RIMS binding protein 2	1.73	0.0071
ROGDI	rogdi homolog (Drosophila)	1.50	0.0094
RTN4RL2	reticulon 4 receptor-like 2	1.66	0.0011
RUNDC3A	RUN domain containing 3A	1.53	0.0003
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	1.65	0.0004
SET	SET nuclear oncogene	1.75	0.0042
SPDYE2	speedy homolog E2 (Xenopus laevis)	1.80	0.0185
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	1.59	0.0328
SRPK3	SRSF protein kinase 3	1.67	0.0001
SYCP1	synaptonemal complex protein 1	1.69	0.0044
THRA	thyroid hormone receptor, alpha	1.61	0.0176
TICAM1	toll-like receptor adaptor molecule 1	1.54	0.0038
TMEM86B	transmembrane protein 86B	1.57	0.0000
TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	1.57	0.0003

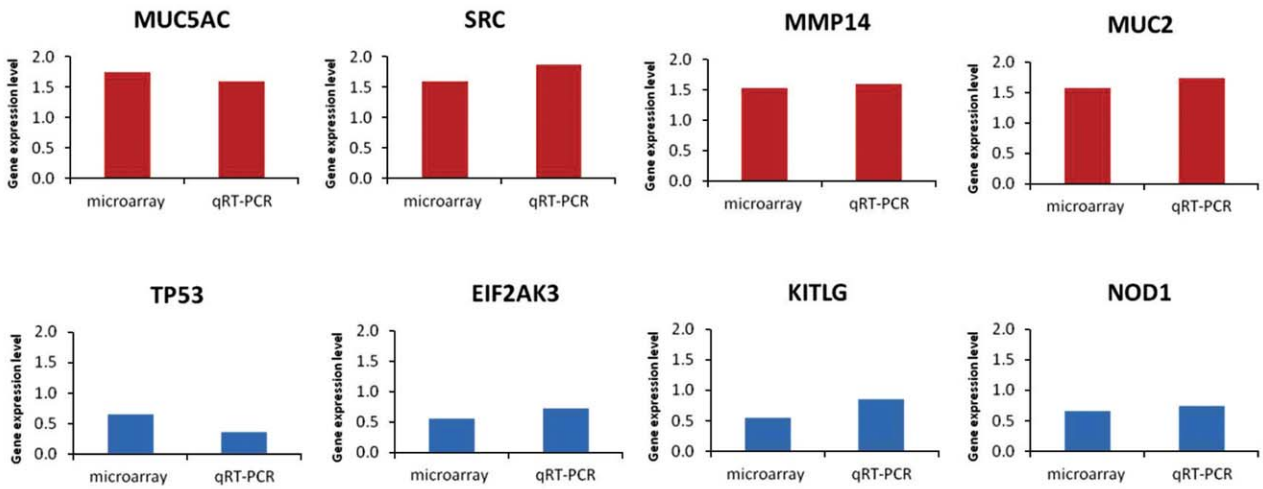

Figure 3. Validation of novel biomarker for diesel exhaust particles exposure in human middle ear epithelial cells. Identified novel biomarkers were verified by real-time reverse transcription polymerase reaction (RT-PCR). Similar expression pattern was exhibited in both gene expression microarray data and real-time RT-PCR data.

study demonstrated that DEPs-induced transcriptional expression of *p21* involves *SRC* activation[13] and suggested novel insights into the potential mechanism of toxicity, including inhibition of proliferation and apoptosis balance in the airway epithelium, resulting from human inhalation of DEPs[13]. In our study, *SRC* up-regulation was also observed in DEPs exposed HMEECs. This result indicated that *SRC* could be a significant novel biomarker for air pollutants, particularly DEPs, in the airway epithelium. *MUC5AC* and *MUC2* belong to the mucin family of glycoproteins[14]. At least 20 unique mucin genes have been identified and shown to be expressed in ear, lung, nose, salivary glands, and gastrointestinal and genitourinary tracts tissues[15-21]. They play a protective role the underlying epithelium. They also maintain mucociliary clearance, and interact with pathogens to affect adherence and host invasion[22,23]. In particular, *MUC5AC* and *MUC2* have been identified as major gel-forming mucins in the middle ear[24]. Significantly increased *MUC5AC* and *MUC2* expressions were observed in the human middle ear epithelium of patients with OM[25,26]. Additionally, *MUC5AC* polymorphisms were detected in patients with OM[24]. Another investigation demonstrated that treatment of cigarette smoking in HMEECs increased the expression of *MUC5AC* mRNA and proteins which play a major role in OM with effusion[27]. Our data also showed up-regulation of *MUC5AC*

Table 3. List of 1.5 fold down-regulated genes against DEPs exposure in HMEECs.

Gene symbol	Gene name	Fold change	*P*-value
ABCC6	ATP-binding cassette, sub-family C (CFTR/MRP), member 6	0.67	0.00971
ACVR2A	activin A receptor, type IIA	0.58	0.01306
AGFG2	ArfGAP with FG repeats 2	0.66	0.00004
ALPK1	alpha-kinase 1	0.55	0.00977
ALS2	amyotrophic lateral sclerosis 2 (juvenile)	0.65	0.00156
ALX1	ALX homeobox 1	0.65	0.00496
ANK3	ankyrin 3, node of Ranvier (ankyrin G)	0.38	0.00702
ANO8	anoctamin 8	0.50	0.00041
ARHGEF15	Rho guanine nucleotide exchange factor (GEF) 15	0.65	0.00025
ARL17B	ADP-ribosylation factor-like 17B	0.61	0.03482
ATF3	activating transcription factor 3	0.61	0.00012
ATG4C	autophagy related 4C, cysteine peptidase	0.62	0.01545
ATG4D	autophagy related 4D, cysteine peptidase	0.65	0.03070
BACE1	beta-site APP-cleaving enzyme 1	0.64	0.00870
BPNT1	3′(2′), 5′-bisphosphate nucleotidase 1	0.61	0.01371
BSPRY	B-box and SPRY domain containing	0.62	0.00028
BTN2A2	butyrophilin, subfamily 2, member A2	0.62	0.00039
C10orf10	chromosome 10 open reading frame 10	0.53	0.00002
C17orf108(LYRM9)	LYR motif containing 9	0.44	0.01434
C2orf63(CLHC1)	clathrin heavy chain linker domain containing 1	0.64	0.00841
C5orf34	chromosome 5 open reading frame 34	0.66	0.00032
C7orf60	chromosome 7 open reading frame 60	0.55	0.00561
CA12	carbonic anhydrase XII	0.58	0.00385
CALCOCO1	calcium binding and coiled-coil domain 1	0.61	0.01070
CAPRIN1	cell cycle associated protein 1T	0.65	0.02307
CCDC121	coiled-coil domain containing 121	0.62	0.03223
CCNT2	cyclin T2	0.60	0.00271
CENPI	centromere protein I	0.60	0.00018
CEP44	centrosomal protein 44 kDa	0.63	0.00838
CEP97	centrosomal protein 97 kDa	0.59	0.00497
CHPT1	choline phosphotransferase 1	0.62	0.03394
CKLF	chemokine-like factor	0.66	0.00014
CPD	carboxypeptidase D	0.63	0.03039
CREBZF	CREB/ATF bZIP transcription factor	0.67	0.01656
CXCL10	chemokine (C-X-C motif) ligand 10	0.62	0.00623
DENND1B	DENN/MADD domain containing 1B	0.62	0.00309
DHFRL1	dihydrofolate reductase-like 1	0.48	0.02430
DISP1	dispatched homolog 1 (Drosophila)	0.59	0.03323
DLX4	distal-less homeobox 4	0.60	0.01806
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	0.52	0.00798
DNAJC25	DnaJ (Hsp40) homolog, subfamily C, member 25	0.66	0.00412
DNER	delta/notch-like EGF repeat containing	0.65	0.02653
DOCK11	dedicator of cytokinesis 11	0.58	0.00374
EIF1AY	eukaryotic translation initiation factor 1A, Y-linked	0.66	0.00846
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	0.55	0.00816
ELOVL4	ELOVL fatty acid elongase 4	0.64	0.00364
ENPP4	ectonucleotide pyrophosphatase/phosphodiesterase 4 (putative)	0.63	0.00911
ERCC4	excision repair cross-complementing rodent repair deficiency, complementation group 4	0.61	0.00854
FAM114A2	family with sequence similarity 114, member A2	0.65	0.04274
FKSG2	tumor protein, translationally-controlled 1 pseudogene	0.64	0.00027
FUT2	fucosyltransferase 2 (secretor status included)	0.61	0.00089
GIPR	gastric inhibitory polypeptide receptor	0.41	0.00003
GNL1	guanine nucleotide binding protein-like 1	0.58	0.00601
GOLGA6C	golgin A6 family, member C	0.60	0.00378
HBP1	HMG-box transcription factor 1	0.58	0.01149
HBS1L	HBS1-like (S. cerevisiae)	0.66	0.00591
HCFC1	host cell factor C1 (VP16-accessory protein)	0.61	0.02634
HCN3	hyperpolarization-activated, cyclic nucleotide-gated K+ 3	0.57	0.01885
HECTD2	HECT domain containing E3 ubiquitin protein ligase 2	0.63	0.00240

Table 3. Continued.

Gene symbol	Gene name	Fold change	*P*-value
HELB	helicase (DNA) B	0.63	0.00395
HERC6	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6	0.65	0.00777
HIST1H2AC	histone cluster 1, H2ac	0.59	0.00504
HIST4H4	histone cluster 4, H4	0.56	0.01654
HNRPLL	Uncharacterized	0.62	0.00087
HRASLS5	HRAS-like suppressor family, member 5	0.58	0.00004
HSDL1	hydroxysteroid dehydrogenase like 1	0.58	0.00764
HSF2BP	heat shock transcription factor 2 binding protein	0.63	0.02057
HSPA6	heat shock 70 kDa protein 6 (HSP70B')	0.66	0.00016
HTATIP2	HIV-1 Tat interactive protein 2, 30kDa	0.55	0.00146
INADL	InaD-like (Drosophila)	0.65	0.00828
ITPK1	inositol-tetrakisphosphate 1-kinase	0.65	0.01448
JAK3	Janus kinase 3	0.63	0.00801
JAZF1	JAZF zinc finger 1	0.61	0.00229
KCNMB4	potassium large conductance calcium-activated channel, subfamily M, beta member 4	0.64	0.00234
KIAA1009	KIAA1009	0.66	0.02357
KIF21A	kinesin family member 21A	0.53	0.00394
KITLG	KIT ligand	0.55	0.00021
KLHL3	kelch-like family member 3	0.61	0.00012
KRT32	keratin 32	0.62	0.00140
LDHC	lactate dehydrogenase C	0.63	0.00003
LINC00087	long intergenic non-protein coding RNA 87	0.52	0.00165
LOC100270746	Uncharacterized	0.65	0.00217
LOC400027	long intergenic non-protein coding RNA 938	0.61	0.00139
LOC401431	Uncharacterized	0.62	0.00155
LOC401588 (ZNF674-AS1)	ZNF674 antisense RNA 1 (head to head)	0.66	0.00611
LRRC37BP1	leucine rich repeat containing 37B pseudogene 1	0.63	0.00088
MAN1C1	mannosidase, alpha, class 1C, member 1	0.60	0.01717
MAP4K3	mitogen-activated protein kinase kinase kinase kinase 3	0.55	0.02131
MCM9	minichromosome maintenance complex component 9	0.51	0.03386
MCTP2	multiple C2 domains, transmembrane 2	0.65	0.00139
MDFIC	MyoD family inhibitor domain containing	0.60	0.00133
MDM1	Mdm1 nuclear protein homolog (mouse)	0.65	0.02187
MKRN7P	makorin ring finger protein 7, pseudogene	0.57	0.01024
MOSPD2	motile sperm domain containing 2	0.65	0.00785
MTHFR	methylenetetrahydrofolate reductase (NAD(P)H)	0.64	0.01174
MYO5C	myosin VC	0.64	0.00257
NOD1	nucleotide-binding oligomerization domain containing 1	0.65	0.02367
NPAT	nuclear protein, ataxia-telangiectasia locus	0.53	0.00497
NTN4	netrin 4	0.60	0.02386
NUP62CL	nucleoporin 62 kDa C-terminal like	0.57	0.01238
NYNRIN	NYN domain and retroviral integrase containing	0.61	0.01044
OPN1MW	opsin 1 (cone pigments), medium-wave-sensitive	0.63	0.00830
OR7C2	olfactory receptor, family 7, subfamily C, member 2	0.65	0.00386
ORAI3	ORAI calcium release-activated calcium modulator 3	0.61	0.00191
ORAOV1	oral cancer overexpressed 1	0.65	0.00002
OSTM1	osteopetrosis associated transmembrane protein 1	0.53	0.01762
OXNAD1	oxidoreductase NAD-binding domain containing 1	0.62	0.01865
PACSIN1	protein kinase C and casein kinase substrate in neurons 1	0.62	0.00086
PAG1	phosphoprotein associated with glycosphingolipid microdomains 1	0.66	0.00014
PARD6B	par-6 partitioning defective 6 homolog beta (C. elegans)	0.65	0.00580
PCGF5	polycomb group ring finger 5	0.58	0.00181
PIKFYVE	phosphoinositide kinase, FYVE finger containing	0.66	0.00037
PLA2G16	phospholipase A2, group XVI	0.65	0.00791
PLEKHA6	phospholipase A2, group XVI	0.65	0.00025
PRSS36	protease, serine, 36	0.66	0.00039
PTGR2	prostaglandin reductase 2	0.64	0.01575
PTPRJ	protein tyrosine phosphatase, receptor type, J	0.62	0.02127
QKI	QKI, KH domain containing, RNA binding	0.62	0.00490

Table 3. Continued.

Gene symbol	Gene name	Fold change	*P*-value
RASD1	RAS, dexamethasone-induced 1	0.59	0.00055
RASGRP2	RAS guanyl releasing protein 2 (calcium and DAG-regulated)	0.59	0.01078
RBM43	RNA binding motif protein 43	0.63	0.01003
RFXAP	regulatory factor X-associated protein	0.63	0.00487
RGMA	RGM domain family, member A	0.60	0.03964
RNF32	ring finger protein 32	0.54	0.02817
RPS2	ribosomal protein S2	0.65	0.00055
RPS21	ribosomal protein S21	0.55	0.00005
RPS8	ribosomal protein S8	0.65	0.00013
RUFY2	RUN and FYVE domain containing 2	0.57	0.00484
SALL2	sal-like 2 (Drosophila)	0.66	0.00028
SASH1	SAM and SH3 domain containing 1	0.57	0.00590
SEC61A2	Sec61 alpha 2 subunit (S. cerevisiae)	0.59	0.01204
SEMA3D	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	0.52	0.00084
SFXN1	sideroflexin 1	0.63	0.00169
SGOL1	shugoshin-like 1 (S. pombe)	0.62	0.01492
SHQ1	SHQ1, H/ACA ribonucleoprotein assembly factor	0.64	0.00909
SLC10A7	solute carrier family 10, member 7	0.66	0.00107
SLC35E2	solute carrier family 35, member E2	0.66	0.00291
SYNE1	spectrin repeat containing, nuclear envelope 1	0.61	0.01726
SYNJ1	synaptojanin 1	0.65	0.00228
SYT14	synaptotagmin XIV	0.63	0.00619
TBC1D12	TBC1 domain family, member 12	0.66	0.00445
TCF15	transcription factor 15 (basic helix-loop-helix)	0.66	0.00112
TECPR2	tectonin beta-propeller repeat containing 2	0.64	0.00748
TIGD7	tigger transposable element derived 7	0.63	0.01990
TMEM201	transmembrane protein 201	0.65	0.00009
TMEM217	transmembrane protein 217	0.64	0.00828
TMEM33	transmembrane protein 33	0.56	0.01290
TP53	tumor protein p53	0.65	0.01494
TRERF1	transcriptional regulating factor 1	0.58	0.00061
TRIM2	tripartite motif containing 2	0.43	0.00924
UBAP2L	ubiquitin associated protein 2-like	0.56	0.00317
UBE3B	ubiquitin protein ligase E3B	0.57	0.02590
UBR3	ubiquitin protein ligase E3 component n-recognin 3 (putative)	0.61	0.00303
VAV3	vav 3 guanine nucleotide exchange factor	0.56	0.00031
WASF3	WAS protein family, member 3	0.64	0.04057
ZADH2	zinc binding alcohol dehydrogenase domain containing 2	0.61	0.00028
ZBTB41	zinc finger and BTB domain containing 41	0.65	0.01289
ZCCHC2	zinc finger, CCHC domain containing 2	0.63	0.01370
ZCWPW1	zinc finger, CW type with PWWP domain 1	0.64	0.00009
ZNF107	zinc finger protein 107	0.65	0.01428
ZNF14	zinc finger protein 14	0.60	0.01077
ZNF25	zinc finger protein 25	0.62	0.02880
ZNF319	zinc finger protein 319	0.60	0.00194

and *MUC2* in HMEECs treated with DEPs. This result implicated that *MUC5AC* and *MUC2* are important novel biomarkers for DEPs on HMEECs.

A total of 168 genes were down-regulated by DEPs exposure in HMEECs. Among them, 13 genes including *KITPG*, *JAK3*, *PAPPA*, *HTATIP2*, *ATF3*, *TRERF1*, *PTPRJ*, *HBP1*, *VAV3*, *NOD1*, *CXCL10*, *TP53*, and *EIF2AK3* were identified as main regulators (Figure 2). To validate mRNA expression, four genes - including *NOD1*, *TP53*, *KITPG*, and *ELF2AK3* were selected and their mRNA expression levels were analyzed by real-time qRT-PCR (Figure 3). *NOD1*, nucleotide-binding oligomerization domain containing 1, is a cytosolic protein. Regarding OM, the level of *NOD1* mRNA was significantly lower in the otitis-prone than in the non-otitis-prone group[28]. In our data, NOD1 down-regulation was observed in the gene expression microarray experiment. Furthermore, this gene was examined using real-time qRT-PCR. Based on our data, *NOD1* might be a novel biomarker for DEPs ex-

posure in HMEECs. Down-regulation of *TP53* was observed in HMEECs against DEPs exposure. *TP53*, tumor protein p53, is well-known tumor suppressor protein containing transcriptional activation, DNA biding, and oligomerization domains. This gene modulates diverse cellular responses including apoptosis, cell cycle arrest, and DNA repair. A recent study reported that *TP53* overexpression was observed in airway epithelial cells under serum-free conditions[29]. Although the expression level was not same as that of our result, they showed p53-mediated cellular response was induced by DEPs exposure. This result indicated that *TP53* also can be a novel biomarker for HMEECs under DEPs exposure. *KITLG* and *EIF2AK3* were first identified here as novel potential biomarkers against DEPs in HMEECs.

In conclusion, we identified differential expression gene profiling against DEPs in HMEECs. Our data provided 29 novel potential biomarkers; among them, eight genes - *SRC*, *MUC5AC*, *MUC2*, *MMP14*, *KITLG*, *EIF1AK3*, *NOD1*, and *TP53* - were validated using real-time qRT-PCR. Furthermore, a molecular signaling network associated with differentially expressed genes was predicted using in silico software. These findings might provide a useful clue for the understanding of air pollutant-induced biological signaling effects, particularly that of DEPs.

Materials & Methods

Cell culture and exposure of DEPs

HMEECs were maintained in a mixture of Dulbecco's Modified Eagle Medium (DMEM) and bronchial epithelial cell basal medium (BEBM) (1 : 1) supplemented with bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, triiodothyronine, retinoic acid, gentamycin, and amphotericin-B. DEPs exposure condition was decided based on our previous study[5]. HMEECs were seeded in 10-cm dishes containing 1×10^5 cells/mL. After overnight culturing, the culture medium was replaced by medium with DEPs concentrations of 40 μg/mL for 6 h.

Gene expression microarray

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA quality was analyzed by Agilent Bioanalyzer Nano Chip 2100 (Agilent Technologies, CA, USA). The RNA samples were then labeled using a Low Input Quick Amp Labeling Kit (Agilent Technologies), in accordance with the manufacturer's protocol. Labeled cRNA was hybridized on GE 4 × 44K v2 microarray (ID G2519-026652; Agilent Technologies) followed by manual washing, according to the manufacturer's procedures. The array was scanned using the Agilent DNA MicroArray Scanner and probe signals were quantified using Feature Extraction software (version 10.10.1.1; Agilent). Normalized data were analyzed using Subio platform v1.16.4376 (Subio Inc. Japan).

Pathway analysis

The molecular pathways of the differentially expressed genes identified by the microarray were dissected using Pathway Studio 9.0 software (Ariadne Genomics). This program integrates relevant information among imported genes, consequently allowing for the identification of biological pathways, gene regulation networks and protein interaction maps.

Real-time reverse transcription polymerase chain reaction

cDNA was synthesized by AccuPower RT premix (BIONEER, KOREA). 8 genes, *SRC*, *MUC5AC*, *MUC2*, *MMP14*, *KITLG*, *EIF1AK3*, *NOD1*, *TP53* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed using an LightCycler 480 Real-Time PCR System (Roche). Each reaction mixture contained 10 μL LightCycler 480 SYBR Green I Master (Roche), 4 pmol each of sense and antisense primers, and 0.4 μL of cDNA in a final volume of 20 μL. Reaction mixtures were incubated at 95°C for 5 minutes to activate FastStart Taq DNA Polymerase, followed by amplification for 50 cycles (10 s at 95°C [denaturation] and 15 s at 60°C [annealing]) for 8 genes and *GAPDH*. Data was analyzed using LightCycler 480 software 1.5 (Roche, UK). Data was analyzed using LightCycler 480 software 1.5 (Roche). Amounts of target mRNA were normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression, and target mRNA expressions in the experimental groups were calculated, relative to the control group.

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