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Microarray analysis of nicotine-induced changes in gene expression in a macrophage-like human cell line

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Background and Objective: Cigarette smoking has been suggested as a risk factor for periodontitis. Thousands of components are present in cigarette smoke, including nicotine, which may play an important role in the observed effects of smoking on cell metabolism. However, the mechanisms underlying these effects are unclear. Using DNA microarrays, we monitored differentially expressed genes, responsive to nicotine, in a macrophage-like human cell line.

Material and Methods: Human U937 cells were treated for 1 h, with or without 1.0 μg/ml of nicotine. For differentiation, cultures were incubated with 10 nM phorbol myristate acetate for 48 h. Analysis of gene expression was performed using a DNA microarray of 8500 genes.

Results: The expression of 4914 genes was detected. Screening was carried out on those genes whose expression in three separate experiments showed an average change of twofold or greater, and 118 up-regulated genes and 97 down-regulated genes were identified. Among these were genes related to inflammation and other immune responses, such as phospholipase A_2 and interferon. Consistent with the array findings, we found similar changes in mRNA expression after analysis using the real-time polymerase chain reaction.

Conclusion: The results suggest that nicotine causes excess inflammation and disturbs host defense mechanisms against pathogens.

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Increasing evidence indicates that tobacco and its smoke constitute a major cause of preventable death and disease. In addition, tobacco smokers are known to have increased risks of cardiovascular disease, peptic ulceration and chronic obstructive pulmonary disease (1–3). Of diseases seen in dentistry, the components of tobacco smoke are associated with malignant oral cancers (4,5), oral mucosal lesions (white lesions and erythroplakia) (6–10) and periodontal disease (11–16). Recent work has indicated that exposure to environmental tobacco smoke, also known as passive smoking, may also be an important risk factor for various diseases (17–19). More than 4000 chemicals are present in cigarette smoke, and approximately 200 components are known to be harmful, including some carcinogens (18). Nicotine, one of the few natural liquid alkaloids, is a colorless, volatile base (pK = 8.0–8.5) that turns brown and produces the characteristic odor of tobacco upon exposure to air (20,21). Nicotine is readily absorbed from the respiratory tract, buccal mucosa, gingiva and skin (20,22). Therefore, it is probable that the various cells within the periodontal tissues are exposed to these higher levels of nicotine during

acute exposure to smoke (23). Of these cells, macrophages play a central role in the immune response against invading pathogens and in wound healing (24–29). Therefore, there is considerable interest in identifying the biological pathways and mechanisms involved in macrophage activation. The purpose of this study was to identify, by using DNA microarrays, genes responsive to nicotine in a macrophage-like human cell line.

Material and methods

Cells and cell culture

U937, a macrophage-like human cell line, was used in this study. Cells were maintained in RPMI-1640 medium (Gibco, BRL Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (BioSource, Rockville, MD, USA), 1% (v/v) penicillin-streptomycin solution (100 U/ml of penicillin and 0.1 μg/ml of streptomycin; Sigma, St Louis, MO, USA), and L-glutamine (2 mM), in 75-cm^2 tissue culture flasks (Falcon; Becton Dickinson, Lincoln Park, NY, USA) at 37°C in 5% CO_2 in air, with high humidity. To stimulate the cells to differentiate into macrophage-like cells, logarithmic cultures, at 1×10^6 cells/ml, were incubated with 10 nM phorbol myristate acetate (Sigma) for 48 h. Nicotine (purity 98%; Sigma-Aldrich Japan, Tokyo, Japan) was freshly prepared and used at a final concentration of 1.0 μg/ml. Cells were treated for 1 h, with or without 1.0 μg/ml of nicotine. Total RNA was isolated from cells using an RNeasy® Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

DNA microarray gene expression analysis

The total RNA collected from replicate plates was pooled and analyzed using a GeneChip Human Genome Focus Array (Affymetrix, Santa Clara, CA, USA). Biotinylated cDNA probe generation, as well as array hybridization, washing and staining, was carried out according to the manufacturer's instructions. Fluorescence intensities for each chip were captured on a GeneChip Scanner 3000 (Affymetrix), and the data obtained were processed and analyzed using GeneChip Operating software, version 1.1 (Affymetrix). Additional data analyses were performed with the bioinformatics algorithms of GENESPRING software (Silicon Genetics, Redwood City, CA, USA). The data presented show the average of three separate experiments.

Real-time polymerase chain reaction (PCR) analysis

Total RNA (3 μl per reaction) was reverse-transcribed at 42°C for 60 min, using a first-strand synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Following cDNA synthesis, 5 μl of each reaction was used as a template for PCR. Real-time PCR was conducted using primers and probe sets from Assay-on-Demand™ Gene Expression Products (Applied Biosystems, Foster City, CA, USA). PCR amplifications for the target genes and for the internal control (glyceraldehyde-3-phosphate dehydrogenase) were performed in capped 96-well optical plates. The reaction conditions were as follows: 5 min at 50°C (one cycle), 10 min at 95°C (one cycle), 15 s at 95°C and 1 min at 60°C (40 cycles). Gene-specific PCR products were continuously measured by means of an ABI PRISM 7700 detection system (Applied Biosystems). Sample results were normalized to an internal control and expressed as relative fold increase. Results shown represent the mean value of two independent experiments, with samples in each experiment run in triplicate.

Statistical analysis

The microarray data were analyzed statistically using GENESPRING software (Silicon Genetics). Statistical significance was determined using Welch's *t*-test. Values of $p < 0.05$ were considered statistically significant.

The real-time PCR data were analyzed statistically using SPSS® software (SPSS, Chicago, IL, USA). All data are presented as mean ± standard deviation. Statistical significance was determined using the Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

We monitored differentially expressed genes, responsive to nicotine, in U937 cells by using a microarray hybridized to dye-labeled cDNA probes synthesized from total RNA of cells incubated with or without 1.0 μg/ml of nicotine. Figure 1 illustrates the overall genetic profile in a scatterplot. Of the 8500

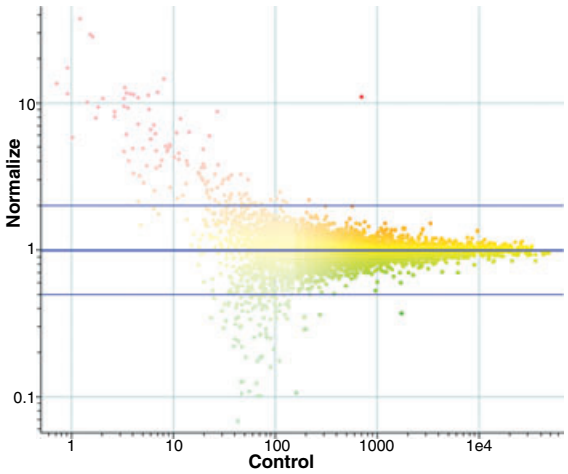

Fig. 1. Scatterplot of the global effects of nicotine treatment on U937 cells. Of the 8500 genes examined, 4914 were detected. Significant changes in mRNA levels were observed for 215 genes: 118 were up-regulated and 97 were down-regulated. The upper line represents twofold up-regulation, the lower line denotes twofold down-regulation and the middle line indicates no change in expression with nicotine treatment.

Table 1. Genes up-regulated in U937 cells treated with nicotine

Molecular functional class	Gene title	Gene symbol	Fold change	GenBank
Binding	**Syndecan-binding protein (syntenin)**	**SDCBP**	**12.68**	**NM_005625**
	Plastin 3 (T isoform)	PLS3	13.61	NM005032
	Eukaryotic translation initiation factor 4E	**EIF4E**	**2.02**	**AW268640**
	MADS box transcription enhancer factor 2, polypeptide D (myocyte enhancer factor 2D)	MEF2D	2.07	AL530331
	Notch homolog 3 (Drosophila)	**NOTCH3**	**2.99**	**NM_000435**
	Hairy and enhancer of split 1 (Drosophila)	**HES1**	**5.23**	**NM_005524**
	Midline 1 (Opitz/BBB syndrome)	**MID1**	**10.86**	**NM_000381**
	Nebulette	**NEBL**	**5.67**	**AL157398**
	Nucleoporin-like 2	**NUPL2**	**5.92**	**NM_007342**
	Myeloid cell nuclear differentiation antigen	**MNDA**	**2.23**	**NM_002432**
	2′-5′-oligoadenylate synthetase 2, 69/71 kDa	**OAS2**	**2.39**	**NM_016817**
	Transgelin	TAGLN	2.64	NM_003186
	Homeobox B5	HOXB5	2.03	NM_002147
	Werner syndrome	WRN	2.40	NM_000553
	Zinc finger protein 588	ZNF588	7.90	NM_016220
	Protein kinase D1	PRKD1	2.34	NM_002742
	Zinc finger protein 225	ZNF225	3.32	NM_013362
	Ret finger protein-like 2	**RFPL2**	**2.01**	**NM_006605**
	Cell division cycle 42 (GTP-binding protein, 25 kDa)	**CDC42**	**4.88**	**NM_015858**
	Paired box gene 3 (Waardenburg syndrome 1)	**PAX3**	**3.28**	**NM_000438**
	Bone morphogenetic protein 8b (osteogenic protein 2)	**BMP8B**	**6.21**	**NM_001720**
	Zinc finger protein, subfamily 1 A, 4 (Eos)	ZNFN1A4	2.80	NM_022465
	Homeobox A3	HOXA3	2.93	NM_030661
	Zinc finger and BTB domain-containing 22	ZBTB22	2.08	AL523144
	DIRAS family, GTP-binding RAS-like 3	**DIRAS3**	**5.09**	**AK021882**
	BRCA2 and CDKN1A interacting protein	**BCCIP**	**11.33**	**NM_016567**
	Serum deprivation response (phosphatidylserine-binding protein)	**SDPR**	**28.18**	**NM_004657**
	Polymerase (RNA) I polypeptide E, 53 kDa	**POLR1E**	**3.01**	**NM_022490**
	CD248 molecule, endosialin	**CD248**	**2.06**	**NM_020404**
	RAB, member of RAS oncogene family-like 2B, RAB, member of RAS oncogene family-like 2A	RABL2B RABL2A	2.54	NM_007081
	Zinc finger protein 14	**ZNF14**	**6.24**	**NM_021030**
Catalytic activity	**Fas-activated serine/threonine kinase**	**FASTK**	**2.16**	**AK023141**
	Cytochrome P450, family 2, subfamily E, polypeptide 1	CYP2E1	8.13	J02843
	Protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	**PPM1G**	**2.45**	**NM_002707**
	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 1	**B3GNT1**	**2.14**	**NM_006876**
	Malic enzyme 1, NADP(+)-dependent, cytosolic	ME1	3.59	NM_002395
	Alpha-2-HS-glycoprotein	AHSG	3.75	NM_001622
	Dickkopf homolog 1 (*Xenopus laevis*)	**DKK1**	**9.01**	**NM_012242**
	Calpain 5	**CAPN5**	**3.61**	**NM_004055**
	CHK1 checkpoint homolog (*S. pombe*)	**CHEK1**	**11.43**	**NM_001274**
	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	**PLA2G7**	**4.29**	**NM_005084**
	Tolloid-like 1	**TLL1**	**10.68**	**AI769310**
	Matrix metallopeptidase 16 (membrane-inserted)	MMP16	3.19	U79292
	Phospholipase A2, group X	**PLA2G10**	**5.05**	**NM_003561**
	Aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	AKR1C3	2.12	AB018580
	Alcohol dehydrogenase IB (class I), beta polypeptide	**ADH1B**	**11.58**	**M21692**
	Regucalcin (senescence marker protein-30)	RGN	4.89	D31815
	Phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)	PDE4D	2.23	AF012074
	Phosducin	PDC	7.14	M33478
	Serine palmitoyltransferase, long chain base subunit 2	SPTLC2	2.55	U15555
	Kinesin family member 15	**KIF15**	**29.33**	**NM_020242**
	Sulfotransferase family 1E, estrogen-preferring, member 1	SULT1E1	2.85	NM_005420
	Kallikrein 5	**KLK5**	**3.26**	**AF243527**
	Tripartite motif-containing 15	**TRIM15**	**2.35**	**U34249**

Table 1. (*Continued*)

Molecular functional class	Gene title	Gene symbol	Fold change	GenBank
Cell adhesion molecule activity	**Integrin, beta 7**	**ITGB7**	**11.88**	**NM_000889**
	Tetraspanin 2	**TSPAN2**	**17.35**	**BF129969**
Defense immunity protein activity	**Golgi autoantigen, golgin subfamily a, 1**	**GOLGA1**	**2.05**	**BG111661**
	CD3e molecule, epsilon (CD3–TCR complex)	CD3E	2.17	NM_000733
	Sperm-associated antigen 8	**SPAG8**	**3.25**	**NM_012436**
	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 5	**LILRB5**	**2.29**	**NM_006840**
	Interleukin-18 receptor accessory protein	**IL18RAP**	**2.07**	**NM_003853**
	G antigen 2	GAGE2	2.97	NM_001474
	G antigen 4	GAGE4		
	G antigen 5	GAGE5		
	G antigen 6	GAGE6		
	G antigen 7	GAGE7		
	G antigen 7B	GAGE7B		
	G antigen 8	GAGE8		
	CUB and zona pellucida-like domains 1	CUZD1	9.52	NM_022034
Motor activity	**Dynein, axonemal, light polypeptide 4**	**DNAL4**	**4.84**	**NM_005740**
	Echinoderm microtubule-associated protein-like 1	EML1	4.06	NM_004434
Signal transducer activity	**Protein kinase, cAMP-dependent, catalytic, beta**	**PRKACB**	**9.37**	**NM_002731**
	FK506-binding protein 5	**FKBP5**	**14.64**	**NM_004117**
	Oncostatin M receptor	OSMR	2.53	NM_003999
	SH3 and cysteine rich domain	**STAC**	**2.10**	**NM_003149**
	Parathyroid hormone receptor 1	**PTHR1**	**2.21**	**NM_000316**
	Regulator of G-protein signalling 7	RGS7	4.03	NM_002924
	5-hydroxytryptamine (serotonin) receptor 2 A	**HTR2A**	**3.07**	**NM_000621**
	Neurotrophic tyrosine kinase, receptor, type 2	**NTRK2**	**2.20**	**NM_006180**
	Interferon, alpha 4	IFNA4	2.03	NM_021068
	G protein-coupled receptor 35	**GPR35**	**2.18**	**AF089087**
	G protein-coupled receptor kinase interactor 1	**GIT1**	**8.72**	**NM_014030**
	Purinergic receptor P2Y, G-protein coupled, 5	**P2RY5**	**9.25**	**NM_005767**
	Interleukin 20 receptor, alpha	**IL20RA**	**11.57**	**NM_014432**
	C-type lectin domain family 2, member D	**CLEC2D**	**2.78**	**NM_013269**
	Growth differentiation factor 9	**GDF9**	**2.41**	**NM_00526**0
Structural molecule activity	**NEL-like 2 (chicken)**	**NELL2**	**7.79**	**NM_006159**
	Tubulin, gamma 2	TUBG2	4.66	NM_016437
	Keratin 8	KRT8	2.34	U76549
	Collagen, type X, alpha 1 (Schmid metaphyseal chondrodysplasia)	**COL10A1**	**37.33**	**X98568**
	ARP3 actin-related protein 3 homolog B (yeast)	**ACTR3B**	**4.13**	**NM_020**455
Transcription regulator activity	Zinc finger protein 136 (clone pHZ-20)	ZNF136	2.80	NM_003437
	POU domain, class 4, transcription factor 2	POU4F2	8.76	NM_004575
	Short stature homeobox 2	**SHOX2**	**7.07**	**AF022654**
	PR domain-containing 13	**PRDM13**	**4.53**	**NM_021620.1**
Transporter activity	**Sodium channel, voltage-gated, type I, beta**	**SCN1B**	**2.01**	**NM_001037.1**
	Chloride channel, calcium activated, family member 2	**CLCA2**	**11.70**	**NM_006536.2**
	Cytochrome P450, family 7, subfamily B, polypeptide 1	**CYP7B1**	**10.70**	**NM_004820.2**
	Peptidylglycine alpha-amidating monooxygenase COOH-terminal interactor	PAMCI	4.71	AF056209
	Glutamate receptor, ionotropic, kainate 1	**GRIK1**	**3.35**	**U16125**
	InaD-like (Drosophila)	INADL	4.68	AJ001306
	Isovaleryl Coenzyme A dehydrogenase	IVD	2.27	AK022777
	ATP-binding cassette, subfamily A (ABC1), member 7	**ABCA7**	**3.22**	**NM_019112**
	Solute carrier family 13 (sodium/sulfate symporters), member 1	SLC13A1	5.82	NM_022444
	ATPase (Na+)/K+ transporting, beta 4 polypeptide	**ATP1B4**	**6.11**	**NM_012069**
Unclassified	Uracil-DNA glycosylase	UNG	2.41	NM_003362
	Transducer of ERBB2, 1	**TOB1**	**2.22**	**AA675892**
	Timeless homolog (Drosophila)	TIMELESS	3.83	NM_003920
	Fibrinogen-like 2	**FGL2**	**6.56**	**NM_006682**
	Retinoblastoma-like 1 (p107)	**RBL1**	**3.75**	**AL365505**
	Apolipoprotein C-IV	**APOC4**	**5.17**	**NM_001646**
	Serum amyloid A4, constitutive	**SAA4**	**3.12**	**NM_006512**
	Down syndrome critical region gene 6	**DSCR6**	**2.11**	**NM_018962**

Table 1. (*Continued*)

Molecular functional class	Gene title	Gene symbol	Fold change	GenBank
Unclassified	**Histone 1, H4g**	**HIST1H4G**	**2.65**	**NM_003547**
	Histone 1, H3e	**HIST1H3E**	**3.80**	**NM_003532**
	Transmembrane 7 superfamily member 3	**TM7SF3**	**3.62**	**NM_016551**
	DnaJ (Hsp40) homolog, subfamily C, member 12	**DNAJC12**	**3.03**	**NM_021800**
	KIAA1704	**KIAA1704**	**2.07**	**NM_018559**
	Purine-rich element binding protein G	**PURG**	**10.15**	**NM_013357**
	Surfactant, pulmonary-associated protein B	**SFTPB**	**3.77**	**J02761**
	EST	EST	11.07	M10098
	EST	**EST**	**6.30**	**AF209975**
	Nyctalopin	**NYX**	**4.01**	**AF254868**
	Chromosome 3 open reading frame 40	C3orf40	4.84	AI141670

Text in bold indicates genes whose expressions are statistically significant.

genes on the chip, 4914 were detectable. Genes were considered to be up- or down-regulated if the average fold change in expression was ≥2.0 in three different experiments. Such changes in mRNA levels were detected in 215 genes; 118 were up-regulated and 97 were down-regulated. Genes differentially expressed are listed in Tables 1 and 2. The bold text shows genes whose expressions are statistically significant. Several genes, such as phospholipase A_2 group VII and phospholipase A_2 group X, related to the inflammatory response and to immune responses, were included in the up-regulated group, whereas complement component receptor 1, interferon-γ and complement factor I were in the down-regulated group. To verify the results of the microarrays, we performed real-time PCR. Again, the expression levels of matrix metalloproteinase 16, phospholipase A_2 group VII and phospholipase A_2 group X were significantly up-regulated in the cells exposed to nicotine, to levels 2.87-, 6.46-, and 3.92-fold higher, respectively, compared with the control cultures ($p < 0.05$; Table 3). Fibroblast growth factor 6, fibroblast growth factor 7 and interferon-γ were significantly down-regulated in the cells exposed to nicotine, to levels 0.29-, 0.17-, and 0.09-fold lower, respectively, compared with the control cultures ($p < 0.05$; Table 3).

Discussion

Smoking is a major risk-preventable factor that affects the incidence and severity of periodontal disease (11–16) and a variety of systemic diseases (1–3). Nicotine, a component of tobacco smoke, has been shown to be present at a high concentration in the saliva of smokers (30) and to be absorbed rapidly through the skin and mucous membranes (20,22). Therefore, it is probable that various cells within the periodontal tissues are exposed to these higher levels of nicotine during acute exposure to smoke. Monocytes and macrophages are key members of the innate immune system and are present in greater numbers in active periodontal lesions than in inactive sites (25–27). Considering the multifunctional roles of monocytes and macrophages, they are likely to play an important role in the initiation and maintenance of the inflammatory processes and alveolar bone loss observed in chronic periodontitis (24,31). Therefore, we investigated the effects of nicotine on a macrophage-like human cell line using DNA microarrays. The plasma levels of nicotine in smokers have been reported to be ≈30–40 ng/ml (20). However, the concentrations of nicotine in the saliva of smokers were ≈0.6–1.3 μg/ml (30,32). Therefore, the nicotine concentrations used in this study were similar to the salivary levels of nicotine in smokers. In addition, this level of nicotine was used in previously reported studies (33,34). In this study, changes in mRNA levels were detected in 215 genes, with 118 genes up-regulated and 97 down-regulated. Macrophages exposed to lipopolysaccharide produce several cytokines, including interferon-γ, tumor necrosis factor-α, interleukins (such as interleukin-1α and -β, and interleukin-6), matrix metalloproteinases and prostaglandin E_2 (25,34–36). In this study, phospholipase A_2 group X and phospholipase A_2 group VII were up-regulated, and phospholipase A_2 group XIIA was down-regulated. Prostaglandin E_2 mediates alveolar bone destruction, whereas nicotine up-regulates the lipopolysaccharide-mediated monocyte secretion of prostaglandin E_2 (34,37). Eicosanoids, such as prostaglandin and leukotrienes, are derived from the hydrolysis of membrane phospholipids. Phospholipase A_2 cleaves its substrate to generate arachidonic acid, a precursor of the eicosanoids, which act as inflammatory agents (38–40). Therefore, nicotine might affect the secretion of prostaglandin E_2 by lipopolysaccharide-treated macrophages via the arachidonic acid cascade. In addition, matrix metalloproteinase 16 was up-regulated. Matrix metalloproteinase 16, which is also called MT-MMP3, induces the activation of pro-gelatinase A (41) and decomposes collagen type III (42). Matrix metalloproteinases mediate the destruction of the extracellular matrix of the gingival and periodontal ligament. Interferon-γ, which is produced by activated T lymphocytes and natural killer cells, is a major cytokine involved in the activation of macrophages (43). Recent studies have shown that interferon-γ is also produced by peritoneal macrophages in response to interleukin-12, and by bone marrow-derived macrophages in response to a combination

Table 2. Genes down-regulated in U937 cells treated with nicotine

Molecular functional class	Gene title	Gene symbol	Fold change	GenBank
Apoptosis regulator activity	Caspase 2, apoptosis-related cysteine peptidase (neural precursor cell expressed, developmentally down-regulated 2)	CASP2	0.49	U13022
Binding	Insulin-like growth factor-binding protein 4	IGFBP4	0.18	NM_001552
	LSM5 homolog, U6 small nuclear RNA associated (*S. cerevisiae*)	LSM5	0.46	NM_012322
	Fibulin 1	FBLN1	0.42	NM_006486
	Polymerase (DNA-directed), delta 4	POLD4	0.36	NM_021173
	Amiloride-binding protein 1 [amine oxidase (copper-containing)]	**ABP1**	**0.10**	**NM_001091**
	Origin recognition complex, subunit 1-like (yeast)	ORC1L	0.24	NM_004153
	Runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	RUNX1T1	0.22	NM_004349
	Coronin, actin-binding protein, 2A	**CORO2A**	**0.34**	**NM_003389**
	Primase, polypeptide 2A, 58 kDa	PRIM2A	0.43	NM_000947
	Fibroblast growth factor 7 (keratinocyte growth factor)	FGF7	0.34	NM_002009
	Plasminogen-like B2	PLGLB2		
	Plasminogen-like B1	PLGLB1	0.07	BC005379
	Plasminogen-like A1	PLGLA1		
	Zinc finger protein 134 (clone pHZ-15)	ZNF134	0.49	NM_003435
	Sal-like 1 (Drosophila)	SALL1	0.29	NM_002968
	Prospero-related homeobox 1	PROX1	0.10	NM_002763
	T-box 6	**TBX6**	**0.41**	**NM_004608**
	Fibroblast growth factor 6	**FGF6**	**0.29**	**NM_020996**
	T-cell leukemia homeobox 3	TLX3	0.18	NM_021025
	Homeobox A6	HOXA6	0.31	NM_024014
	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	SMC2L1	0.38	AU154486
	LIM and cysteine-rich domains 1	LMCD1	0.25	NM_014583
	Polypyrimidine tract-binding protein 2	PTBP2	0.43	NM_021190
	Checkpoint with forkhead and ring finger domains	**CHFR**	**0.48**	**NM_018223**
	Zinc finger protein 180 (HHZ168)	**ZNF180**	**0.36**	**NM_013256**
	Delta-like 3 (Drosophila)	DLL3	0.49	NM_016941
	C-type lectin domain family 4, member E	**CLEC4E**	**0.26**	**NM_014358**
	Neurogenic differentiation 4	NEUROD4	0.25	NM_021191
	RAB11B, member RAS oncogene family	RAB11B	0.41	X79780
	Zinc finger protein 142 (clone pHZ-49)	ZNF142	0.50	D87073
Catalytic activity	Nicotinamide N-methyltransferase	NNMT	0.27	NM_006169
	Deiodinase, iodothyronine, type II	DIO2	0.43	NM_013989
	Complement factor I	**CFI**	**0.11**	**NM_000204**
	TAO kinase 2	TAOK2	0.47	NM_004783
	Klotho	KL	0.25	NM_004795
	Protein kinase, Y-linked	**PRKY**	**0.19**	**NM_002760**
	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 4, pseudogene	PSMD4P	0.31	NM_015887
	Aldehyde dehydrogenase 7 family, member A1	**ALDH7A1**	**0.50**	**BC002515**
	Glutathione transferase zeta 1 (maleylacetoacetate isomerase)	**GSTZ1**	**0.16**	**BC001453**
	Serpin peptidase inhibitor, clade B (ovalbumin), member 3	SERPINB3	0.19	BC005224
	Thioredoxin reductase 2	TXNRD2	0.38	AF201385
	Chymotrypsin-like	CTRL	0.35	BF508685
	Cytochrome P450, family 11, subfamily B, polypeptide 2	**CYP11B2**	**0.11**	**X54741**
	3′(2′), 5′-bisphosphate nucleotidase 1	**BPNT1**	**0.11**	**NM_006085**
	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	AKT3	0.49	NM_005465
	Glucosaminyl (N-acetyl) transferase 3, mucin type	GCNT3	0.44	NM_004751
	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A	MGAT4A	0.48	NM_012214
	Cytidine and dCMP deaminase domain-containing 1	CDADC1	0.43	NM_030911
	Phospholipase A2, group XIIA	PLA2G12 A	0.39	NM_030821
	Cysteine sulfinic acid decarboxylase	CSAD	0.43	NM_015989
Cell adhesion molecule activity	Multimerin 1	MMRN1	0.38	NM_007351
	Neural cell adhesion molecule 2	NCAM2	0.31	NM_004540
	Membrane-associated guanylate kinase, WW and PDZ domain-containing 1	MAGI1	0.41	NM_004742
	Selectin E (endothelial adhesion molecule 1)	SELE	0.35	NM_000450
	Tetraspanin 6	TSPAN6	0.50	AF053453

Table 2. (Continued)

Molecular functional class	Gene title	Gene symbol	Fold change	GenBank
Cell adhesion molecule activity	Plakophilin 3	PKP3	0.29	AF053719
	Melanoma cell adhesion molecule	MCAM	0.50	M28882
	Myelin-associated glycoprotein	MAG	0.41	X59350
Defense immunity protein activity	Interleukin-8 receptor, beta	IL8RB	0.25	NM_001557
	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain (semaphorin) 4F	SEMA4F	0.43	NM_004263
	Complement component (3b/4b) receptor 1 (Knops blood group)	CR1	0.21	X14362
	Squamous cell carcinoma antigen recognized by T cells 2	SART2	0.39	NM_013352
Signal transducer activity	Frizzled homolog 6 (Drosophila)	FZD6	0.13	NM_003506
	Killer cell lectin-like receptor subfamily A, member 1	KLRA1	0.38	NM_006611
	Insulin receptor substrate 4	IRS4	0.47	NM_003604
	Muscle, skeletal, receptor tyrosine kinase	MUSK	0.43	NM_005592
	Nuclear receptor subfamily 2, group E, member 3	NR2E3	0.30	NM_014249
	Interferon-γ	IFNG	0.40	M29383
	RAR-related orphan receptor A	RORA	0.44	L14611
	Neuropeptide Y receptor Y2	NPY2R	0.35	U36269
	5-Hydroxytryptamine (serotonin) receptor 2C	HTR2C	0.41	M81778
	Cannabinoid receptor 1 (brain)	CNR1	0.43	U73304
	Bone morphogenetic protein receptor, type IA	BMPR1A	0.43	AI678679
	Glutamate receptor, metabotropic 8	GRM8	0.45	AC000099
	Gonadotropin-releasing hormone receptor	GNRHR	0.50	Z81148
	Olfactory receptor, family 12, subfamily D, member 2	OR12D2	0.29	NM_013936
Transcription regulator activity	von Hippel-Lindau tumor suppressor	VHL	0.12	NM_000551
	Zinc finger protein 165	ZNF165	0.22	NM_003447
Transporter activity	Solute carrier family 29 (nucleoside transporters), member 1	SLC29A1	0.50	AF079117
	Solute carrier family 16 (monocarboxylic acid transporters), member 5	**SLC16A5**	**0.34**	**AA705628**
	Solute carrier family 35, member E3	SLC35E3	0.45	NM_018656
	Aquaporin 3 (Gill blood group)	AQP3	0.44	N74607
Unclassified	Stathmin 1/oncoprotein 18	STMN1	0.43	NM_005563
	MAD2 mitotic arrest deficient-like 1 (yeast)	MAD2L1	0.34	NM_002358
	Transcobalamin I (vitamin B12-binding protein, R binder family)	TCN1	0.41	NM_001062
	Troponin I type 3 (cardiac)	TNNI3	0.42	NM_000363
	Arginase, liver	ARG1	0.19	NM_000045
	Brain-specific protein p25 alpha	TPPP	0.50	NM_007030
	Histone 1, H4f	HIST1H4F	0.13	NM_003540
	EST	**EST**	**0.35**	**NM_015870**
	Histone 1, H3b	HIST1H3B	0.27	NM_003537
	Collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)	COL2A1	0.47	X06268
	X (inactive)-specific transcript	XIST	0.33	AV699347
	Chromosome 21 open reading frame 7	C21orf7	0.48	NM_020152
	EST	EST	0.37	NM_013307
	Dentin sialophosphoprotein	DSPP	0.34	AF094508
	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	CCL18	0.50	Y13710
	Ankyrin repeat and SOCS box-containing 1	ASB1	0.48	AF055024

Text in bold indicates genes whose expressions are statistically significant.

Table 3. Comparison of the gene expression levels determined by array hybridization and real-time polymerase chain reaction (PCR) analysis

Gene title	Microarray	Real-time PCR
Fibroblast growth factor 6 (FGF6)	0.29	0.29
Fibroblast growth factor 7 (FGF7)	0.34	0.17
Interferon-γ (IFNG)	0.40	0.09
Matrix metalloproteinase 16 (MMP16)	3.19	2.87
Phospholipase A_2, group VII (PLA2G7)	4.29	6.46
Phospholipase A_2, group X (PLA2G10)	5.05	3.92

of interleukin-12 and interleukin-18, suggesting the presence of an autocrine activation pathway (44,45). In our study, interferon-γ was down-regulated. By reducing the secretion of interferon-γ by macrophages, nicotine might block the activation of macrophages. In addition, complement component (3b/4b) receptor 1 and complement factor 1, which are involved in

the complement reaction (24,26,46), were down-regulated. Nicotine might inhibit the complement cascade, reducing the obstacle of complement to bacteria and opsonin activity. Two members of the fibroblast growth factor family – fibroblast growth factor 6 and fibroblast growth factor 7 – were down-regulated, and klotho was also down-regulated. The fibroblast growth factor family influences the proliferation and differentiation of various cell types and wound healing (47). Klotho is related to the aging or differentiation of osteoblasts, osteoclasts and B lymphocytes (48,49). These observations suggest that nicotine also influences wound healing and bone metabolism.

In summary, our studies indicate that nicotine alters the gene expression related to inflammation, immune responses, the arachidonic acid and complement cascades, and wound healing. Therefore, nicotine might alter the secretion of chemical mediators of inflammation and reduce macrophage activity. This mechanism might be related to insufficiency of the immune response and a delay in wound healing, found in smokers.

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