Direct Effects of Pure Nicotine, Cigarette Smoke Extract, Swedish-type Smokeless Tobacco (Snus) Extract and Ethanol on Human Normal Endothelial Cells and Fibroblasts

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Abstract. *The adverse health effects of cigarette smoking are well established including the increased risk of various types of cancer. In this study, the direct effects of ethanol, pure nicotine, cigarette smoke extract and Swedish type smokeless tobacco (Snus) extract on normal cells were investigated. Materials and Methods: Primary normal adult human endothelial cells and fibroblasts at early passage were used. Upon exposure to pure nicotine, cigarette smoke extract, Snus extract and ethanol, these cells were assessed for DNA synthesis, gene expression profile and cellular morphology. Results: Normal human fibroblasts and endothelial cells have unique gene expression profiles. The effects of treatment with ethanol and nicotine from different sources was more prominent in endothelial cells than fibroblasts. The combination of alterated gene expressions and strongly inhibited DNA synthesis was only detected in cells exposed to smoke extract. In the presence and absence of ethanol, pure nicotine and Snus extract induced abnormalities in the cytoplasm without any significant degree of cell death. With similar doses of nicotine and ethanol, the additional components in smoke extract had a dominant effect. The smoke extract induced vast cellular abnormalities and massive cell death. Conclusion: Cigarette smoke induced massive cell death and various abnormalities at cellular and molecular levels in surviving endothelial cells and fibroblasts. The combination of genomic alterations and the chronic inflammatory microenvironment induced from massive cell death, will potentially promote tumourigenesis and various diseases in cigarette smokers.*

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Despite ongoing efforts to increase public awareness, one sixth of the world's population continue to smoke and half of all smokers will develop a serious tobacco smoking-related disease (1). Individuals exposed to cigarette smoke by passive, second-hand smoking can also be seriously affected by such smoke inhalation (2). These effects include increased risks of cancer, cardiovascular disease, chronic obstruction pulmonary disease and metabolic disorders (3).

Cigarette smoke contains nicotine and other components that are distributed between the particulate and gaseous phases (4). Nicotine is thought to be the major psychoactive and addictive component of tobacco. Despite this, nicotine replacement therapy has been used as a smoking substitute in smokers who cannot give-up the habit unaided (5). Interestingly, using nicotine as a preventive or therapeutic modality for neuropsychiatry and neurodegenerative disorders has even been promoted (6, 7).

Ethanol consumption and smoking are independent habits but both are associated with increased risk for cancer and cardiovascular disease (8). Persistence of cigarette smoking and drinking after treatment of cancer was strongly related to the development of a secondary tumour (9). Furthermore, cessation of smoking was also associated with a reduced risk of cancer (3).

Over time and in a dose-dependent manner, nicotine has been shown to induce the production of inflammatory cytokines and prostaglandins in normal fibroblasts (10,11). Nicotine metabolites have also been suggested to be carcinogens (12-14). Nevertheless, the precise role of nicotine on tumourigenesis has not yet been fully clarified.

Users of traditional smokeless products can be found in several parts of the world. The health risk profile for smokeless tobacco products has been described as being distinctly different from that of smoked products (15, 16). The possibility of preventing the harmful effects of smoking through encouraging smokers who are unable or unwilling to stop smoking to switch to less harmful smokeless products has been discussed.

Recently, the low incidence of tobacco-associated morbidity and mortality in Sweden was suggested to be related to the low incidence of smoking and the habit of using local non-smoking tobacco, known as Snus (17,18). Interestingly, screening across the lifespan of 16,642 Swedish twins does not support there being any strong association between Snus use and the incidence of cardiovascular disease (19).

The aim of our current study was to assess the direct effects of ethanol, pure nicotine, Snus and cigarette smoke extract on adult normal fibroblasts from the oral cavity and adult normal endothelial cells. DNA synthesis, gene expression profiles and alterations in cellular morphology were gathered for this investigation.

Materials and Methods

Normal human cells. Adult normal human endothelial cells, HSAVEC were obtained from PromoCell GmbH (www.promocell.com). These cells were grown in endothelial cell growth medium 2 (EGM-2) and 5% foetal calf serum as described by the manufacturers (PromoCell GmbH, Germany). Normal human fibroblasts, AG09319 (F19) were obtained from the Coriell Institute for Medical Research (www.coriell.org). They were derived from minced gum tissue of a 25-year-old Caucasian female. These fibroblasts were grown in DMEM/Harm's F-12 media with L-glutamine and 10% foetal calf serum (Sigma, USA).

The expanded endothelial cells and fibroblasts were seeded at 0.5×10^5 cells/ml in a 24-well or in a 96-well cell culture plate. After 48-h culture, the cells at passage 6 were used for our investigation. At least two independent experiments were performed.

Ethanol, pure nicotine, Swedish non-smoking tobacco (Snus) extract and cigarette smoke extract. Ethanol (99.9%; Kemetyl AB, Sweden) and pure nicotine (99%; Sigma Chemical Co., USA) were used. Snus extracts were made by shaking 7.5 g portions of Swedish Snus (Ettan; Swedish Match AB, Sweden) with respective media in an orbital shaker at 125 rpm for 16 hours. The slurries were vacuum filtered through glass filters (G3) and the filtered extracts were stored at –80˚C.

The cigarette smoke extract was prepared from filter cigarettes of an American blend type (0.8 mg nicotine, 10 mg tar, and 10 mg CO). Briefly, cigarettes were smoked under standard conditions using a Borgwaldt RM 20/CS-smoking machine. Particulate phase from the cigarettes was collected on a 9-cm Cambridge filter. The filter was weighed before and after smoking to ensure that the amount of collected particulate phase was reproducible between smoking sessions. The smoke components in the Cambridge filter were extracted with ethanol for 20 minutes in an ultrasonic bath. These extracts were concentrated using a rotary evaporator without warming.

The amount of nicotine in Snus and cigarette smoke extracts was measured by LC-MS-MS. These extracts were aliquot, protected from light and stored at –80˚C. The ethanol, nicotine and extracts containing nicotine from the different sources were freshly prepared at the indicated concentration before use in the test system.

DNA synthesis assay. The exponential fibroblast or endothelial cells were cultured in the presence of 0.2% ethanol, and at different concentrations of pure nicotine or the extract containing nicotine with or without 0.2% ethanol. After 24 hours of culture including a 12-h terminal pulse with 1 μCi/well of ^{3}H-thymidine, the cells in triplicate microwells were harvested and radioactivity was measured in a beta counter.

The relative percentage of cellular proliferation was calculated from the mean isotope count per minute (CPM) values in the treated cultures compared to their control cultures without any treatment.

Gene expression profile by c-DNA array. The gene expression profile of normal human endothelial cells and fibroblasts was analysed after 1 h culture in the presence of 0.2% ethanol, 100 μM pure nicotine, 100 μM pure nicotine with 0.2% ethanol alcohol, Snus extract containing 100 μM nicotine , Snus extract containing 100 μM nicotine with 0.2% ethanol and cigarette smoke extract containing 100 μM nicotine and 0.2% ethanol. Briefly, 5 μg RNA from 1 h-treated cultures and non-treated controls were used as template for biotin-labelled cDNA probe synthesis as previously described (20).

The relative expression of 100 well-defined genes was analysed using the GEArray Q series (SuperArray Inc. USA). This array contained 300-600 bp cDNA fragments printed with 1-mm tetra spot format/gene on nylon membrane. Plasmid DNA, pUC18 and blank spots were also included as negative controls to confirm the hybridisation specificity of the test. The UniGene/GenBank accession number and array position of the genes can be accessed at www.superarray.com. In order to minimise experimental variation, the entire array filters and reagents were purchased from the same batch.

The cDNA probes were hybridised to cDNA fragments on GEArray membranes. The chemiluminescence signal intensity of each gene was obtained by exposing the hybridised filter to X-ray film. To obtain comparable values, the signal intensity of each gene was normalized to that of four housekeeping genes, *β-actin*, ribosomal protein L13A (RPL13A), *Cyclophilin A* and *GAPDH* on the same membrane.

All normalized expression values of the treated and non-treated cells were compared. A particular gene was considered as up- or down-regulated if the expression ratio of the gene in the treated cells was two-fold the arbitrary threshold above or below the expression level of the corresponding reference, non-treated cells.

Cellular morphology. After 24-h culture of fibroblasts or endothelial cells under the different treatments, the cell morphology was observed with an Olympus phase-contrast microscope. Several photographs were taken in random areas with a camera mounted on the microscope. The suggestive characteristic of cell death was blebbing of the cell membrane, rounding of cells with nuclear brightness or nuclear shrinkage.

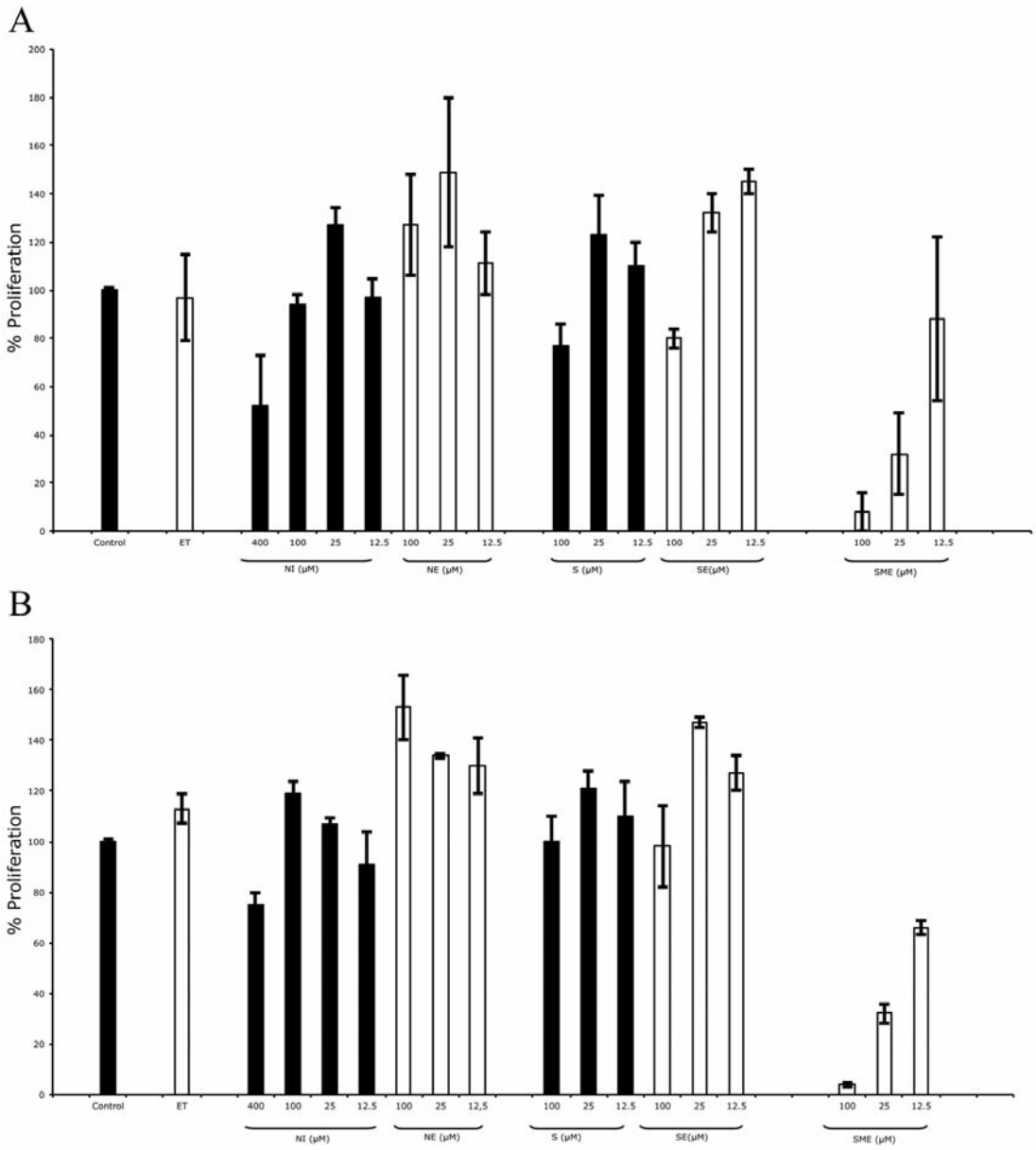

Figure 1. *DNA synthesis (As shown by ^{3}H-thymidine incorporation) of human normal endothelial cells (A) and fibroblasts (B) in 24-h treated cultures, compared with their corresponding control, non-treated cultures. Each bar represents the CPM (mean±SD) of three independent experiments. The cells were treated with 0.2% ethanol (ET), pure nicotine (NI), pure nicotine +0.2% ethanol (NE), Snus extract containing nicotine (S), Snus extract containing nicotine +0.2% ethanol (SE) or smoke extract containing nicotine +0.2% ethanol (SmE), compared with their control culture (Control). Concentrations shown are those of nicotine.*

Results

Cellular proliferation. The mean and standard variations in CPM of the 24-h treated cultures compared with the controls are shown in Figure 1. Using DNA synthesis as an indication of cellular proliferation, similar patterns in treated normal endothelial cells (Figure 1 A) and fibroblasts (Figure 1 B) were observed.

Culturing with 0.2% ethanol had no influence on DNA synthesis. However, increased DNA synthesis was observed in the presence of pure nicotine and Snus extract containing nicotine. The 0.2% ethanol had a marginal influence on pure nicotine- or Snus extract-induced DNA synthesis. In spite of similar nicotine and ethanol concentration, cigarette smoke extract strongly inhibited DNA synthesis by more than 50% and this occurred in a dose-dependent manner.

Gene expression profile. Unique gene expression profiles were seen in normal endothelial cells and fibroblasts prior to the treatments (Figure 2). Among 96 well-defined genes, a higher number of these genes were up-regulated in normal human endothelial cells compared to the normal fibroblasts. Normal endothelial cells and fibroblasts expressed similar levels of the angiogenesis-related gene, *Thrombosporin 1*. Interestingly, neither of these cell types expressed any detectable level of *PDGF, GAP* or *Rb*.

The gene expression profile after 1-h treatment was compared with that of the corresponding, non-treated controls (Figure 3). In the endothelial cells (Figure 3, HSAVEC) treated with ethanol, pure nicotine , Snus extract, pure nicotine and ethanol, Snus extract and ethanol, and cigarette smoke and ethanol, changes in gene expression were detected in 57 (60%), 80 (84%), 77(81%), 81 (85%), 80 (84%) and 48 (51%) genes, respectively. Under similar conditions, treated fibroblasts (Figure 3, F19) displayed an altered expression of 45 (47%), 69 (73%), 74 (78%), 46 (48%), 74 (78%) and 79 (73%) genes, respectively.

Cellular morphology and cell death. Cellular morphology of the endothelial cells (Figure 4A) or fibroblasts (Figure 4B) did not show any influence by 0.2% ethanol up to 24-h *in vitro*. Remarkable changes were observed in the form of prominent cytoplasmic vacuoles in the presence of 100 μM pure nicotine and Snus extract containing 100 μM nicotine with and without 0.2% ethanol. No obvious cell death was detected either in human normal endothelial cell or fibroblast cultures with 0.2% ethanol, 100 μM pure nicotine, Snus extract containing 100 μM nicotine or in any of these combined treatment cultures.

Cigarette smoke extract containing 100 μM nicotine and 0.2% ethanol, significantly induced vast phenotypic abnormalities in the endothelial cells (Figure 4 A) and fibroblasts (Figure 4 B). These treated cells lost their normal cellular integrity and cell-to-cell contact. Within this culture condition, 68±18% and 52±12% death cells were found in endothelial cells and fibroblasts, respectively.

Discussion

To date, the direct effects of cigarette smoking and nicotine have mostly been investigated with established human cell lines (21, 22). Nevertheless, using cell lines as the experimental model may not be entirely appropriate for normal cell responses due to various changes in these immortalized cell lines. Thus, early passage of normal adult fibroblasts and normal adult endothelial cells were used in our investigation.

Normal endothelial cells and fibroblasts differ in their gene expression profile. These differences may be due to the origin of cells, cell cycle stage, and differentiation. Ethanol, pure nicotine and extracts from Snus and cigarette smoke significantly influenced the gene expression patterns in these cells.

The effects of the treatments, as indicated by changes in cellular morphology, cell death or inhibition of cell growth, may not directly correlate to the number of altered genes. The fate of treated cells may be based on the balance among functional genes, not only one particular gene. Our assumption is supported by the investigation of cell death and the balance of oncogene and anti-oncogene expression (23, 24).

By smoking 25 cigarettes per day, a smoker would accumulate approximately 100 μM of nicotine in the saliva (25). At this nicotine concentration, pure nicotine and Snus extract altered gene expression patterns, cellular morphology and cell growth of normal human fibroblasts and endothelial cells. Thus, maintained cessation of smoking by nicotine replacement therapy might induce abnormalities in normal cells (26, 27). The possibility of nicotine-induced abnormalities and harm to normal cells therefore needs further investigation.

The additional components in cigarette smoke extract appear to have a dominant effect over those of nicotine and ethanol. The smoke extract components strongly induced massive cell death in normal human cells, as indicated in our investigation. It is well established that the dead cells release various toxic substances and thereby create inflammatory conditions. The higher levels of inflammatory proteins in the urine of healthy smokers compared to non-smokers reflects this phenomenon (28).

Surviving cells that have acquired abnormalities in their gene expression patterns and cellular morphology due to cigarette smoke could then potentially continue to accumulate genetic abnormalities (29, 30). Thus, our investigation suggests that smoking potentially induces changes that lead to various diseases, including cancer due to the toxic effects of certain components in smoke, not to nicotine per se. With the combination of vast phenotypic abnormalities and a chronic inflammatory microenvironment due to massive cell death, these abnormal cells have great potential to become the progenitors of malignant cells.

In summary, pure nicotine and Snus extract alone, or in combination with ethanol, altered gene expression profiles, cellular morphology and cell growth of normal adult human endothelial cells and fibroblasts. Moreover, cigarette smoke extract appears to strongly induce cell death and abnormalities in surviving cells. Cigarette smoke clearly contains other components, besides nicotine, capable of causing chronic inflammation that results from massive cell death. In addition, abnormal cells within a chronic inflammatory microenvironment may develop genomic instability, potentially promoting tumourigenesis.

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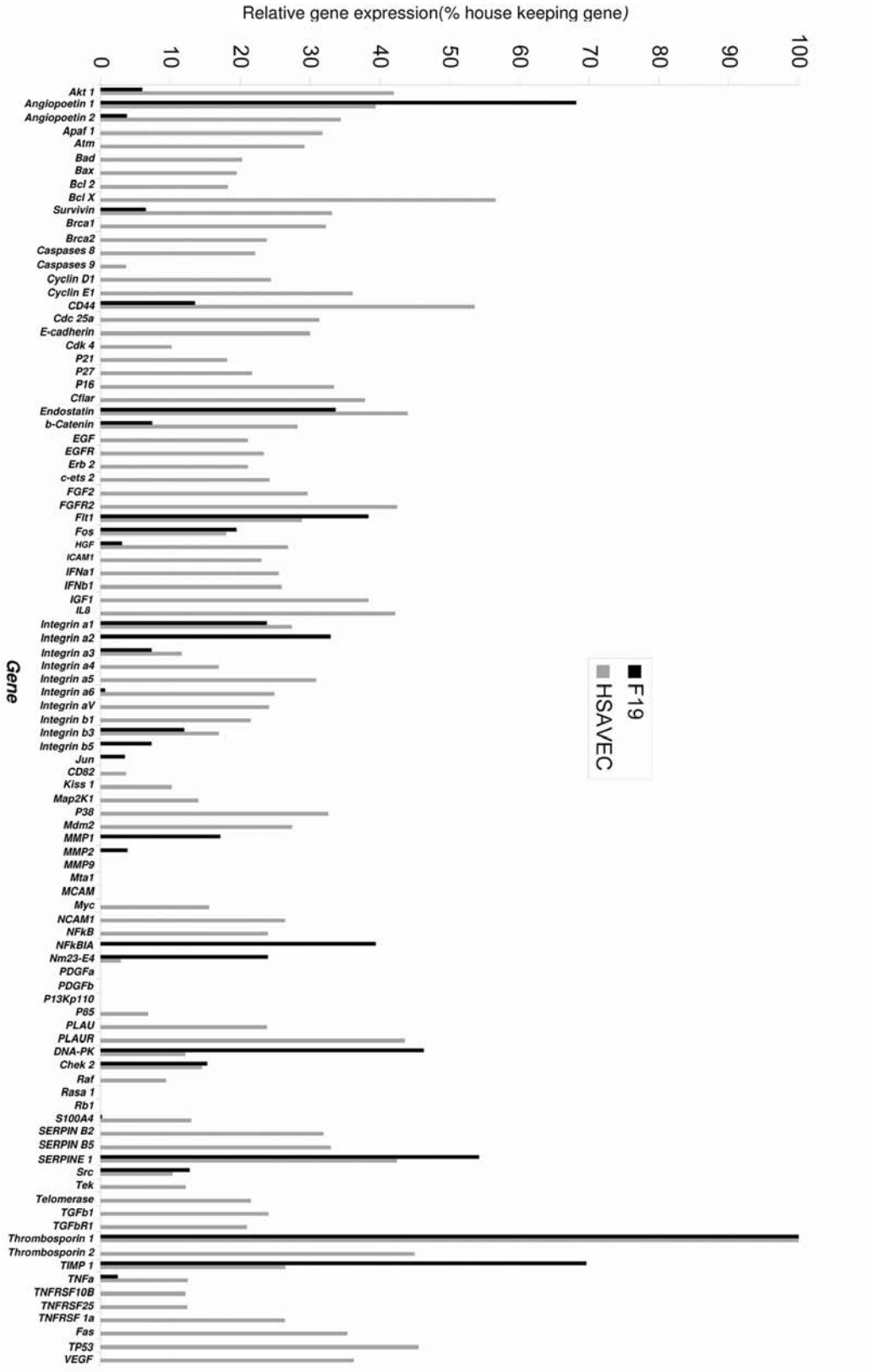

Figure 2. *The gene expression profile of the endothelial cells (HSAVEC) and fibroblasts (F19). The means of two independent experiments after normalization with four housekeeping genes, β-actin, GAPDH, cyclophilin A, RPL13A are shown.*

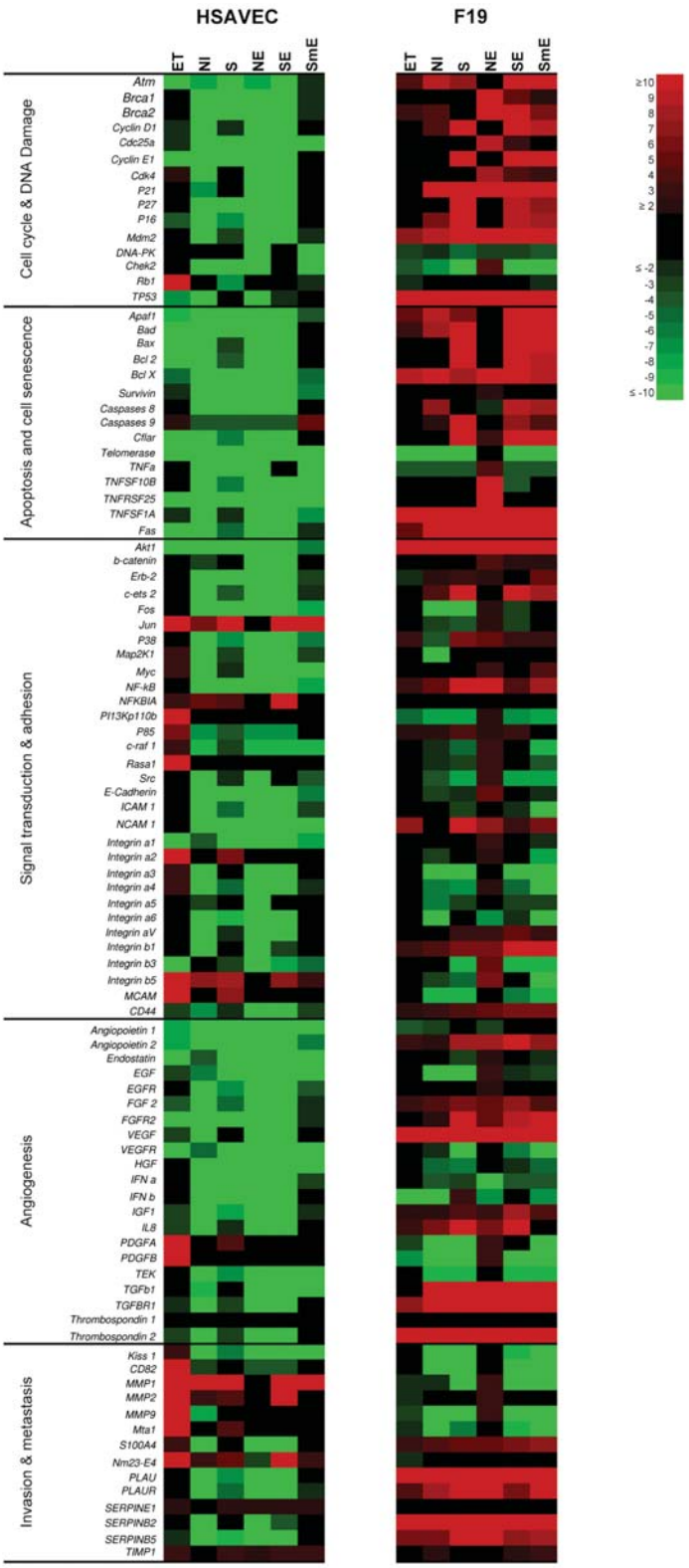

Figure 3. *Gene expression in human normal endothelial cells (HSAVEC) and fibroblasts (F16) at 1-h treatment relative to that of their corresponding control cells. The treatment were 0.2% ethanol (ET), 100 μM pure nicotine (NI), 100 μM pure nicotine +0.2% ethanol (NE), Snus extract containing 100 μM nicotine (S), Snus extract containing 100 μM nicotine +0.2% ethanol (SE) or smoke extract containing 100 μM nicotine +0.2% ethanol (SmE), respectively. The mean ratios of two independent experiments are shown.*

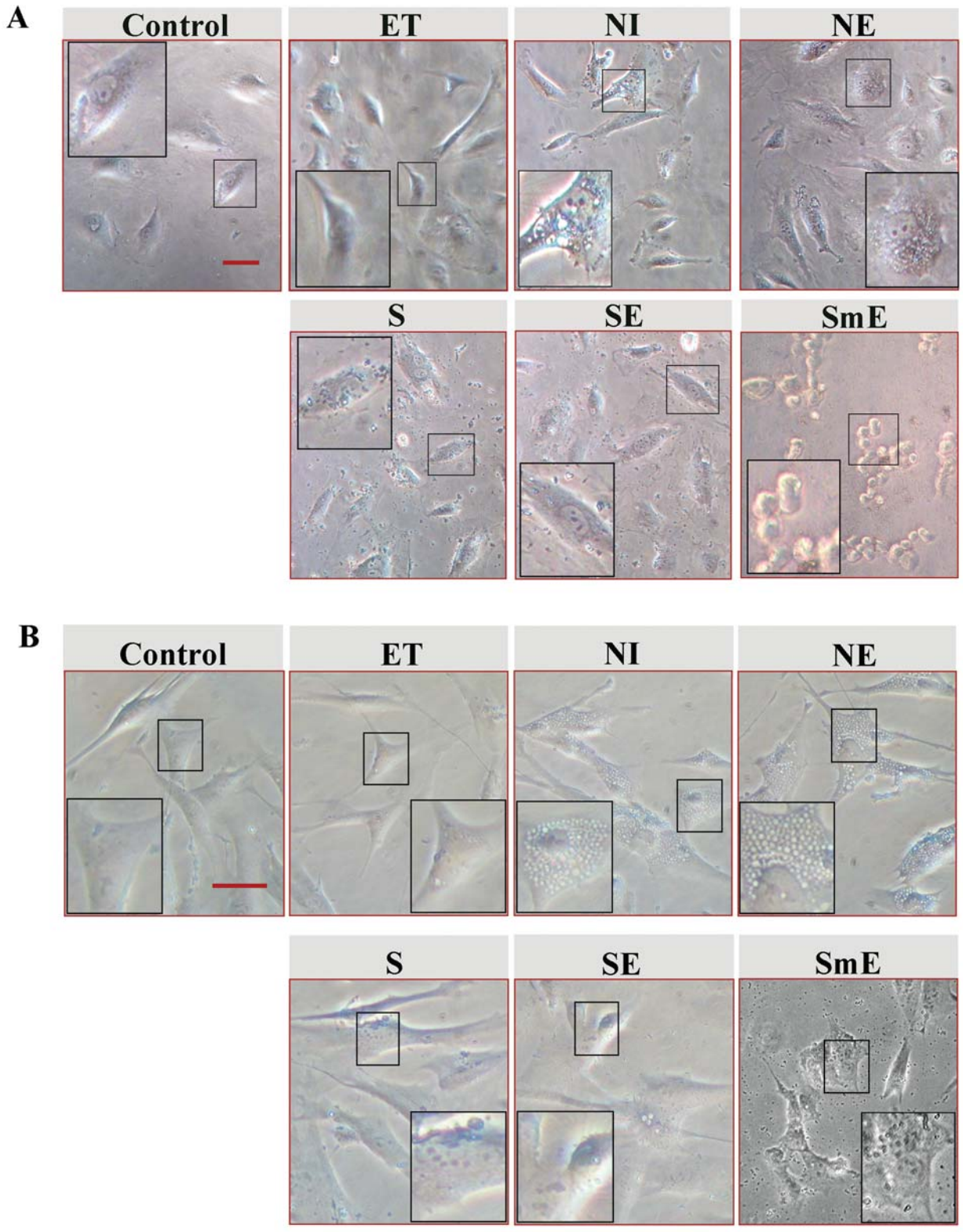

Figure 4. *The morphology of human normal endothelial cells (A) and fibroblasts (B) after 24-h culture in the control media (Control) or in the presence of 0.2% ethanol (ET), 100 μM pure nicotine (NI), 100 μM pure nicotine +0.2% ethanol (NE), Snus extract containing 100 μM nicotine (S), Snus extract containing 100 μM nicotine +0.2% ethanol (SE) or smoke extract containing 100 μM nicotine +0.2% ethanol (SmE), respectively. One representative experiment and 100-μm scale bar is shown.*

References

1 Jha P, Ranson MK, Nguyen SN and Yach D: Estimates of global and regional smoking prevalence in 1995, by age and sex. Am J Pub Health *92*: 1002-1006, 2002.

2 Mao Y, Hu J, Semenciw R and White K: Active and passive smoking and the risk of stomach cancer, by subsite, in Canada. Eur J Cancer Prev *11*: 27-38, 2002.

3 Vineis P, Alavanja M, Buffler P, Fontham E, Franceschi S, Gao YT, Gupta PC, Hackshaw A, Matos E, Samet J, Sitas F, Smith J, Stayner L, Straif K, Thun MJ, Wichmann HE, Wu AH, Zaridze D, Peto R and Doll R: Tobacco and cancer: recent epidemiological evidence. J Natl Cancer Inst *96*: 99-106, 2004.

4 Smith CJ and Fischer TH: Particulate and vapor phase constituents of cigarette mainstream smoke and risk of myocardial infarction. Atherosclerosis *158*: 257-267, 2001.

5 Molyneux A:Nicotine replacement therapy. BMJ *328*: 454-456, 2004.

6 Buckingham SD, Jones AK, Brown LA and Sattelle DB: Nicotinic acetylcholine receptor signalling: roles in Alzheimer's disease and amyloid neuroprotection. Pharmacol Rev *61*: 39-61, 2009.

7 Shim SB, Lee SH, Chae KR, Kim CK, Hwang DY, Kim BG, Jee SW, Lee SH, Sin JS, Bae CJ, Lee BC, Lee HH and KimYK: Nicotine leads to improvements in behavioral impairment and an increase in the nicotine acetylcholine receptor in transgenic mice. Neurochem Res *33*: 1783-1788, 2008.

8 Altieri A, Bosetti C, Talamini R, Gallus S, Franceschi S, Levi F, Dal Maso L, Negri E and La Vecchia C: Cessation of smoking and drinking and the risk of laryngeal cancer. Br J Cancer *87*: 1227-1229, 2002.

9 Do KA, Johnson MM, Lee JJ, Wu XF, Dong Q, Hong WK, Khuri FR and Spitz MR: Longitudinal study of smoking patterns in relation to the development of smoking-related secondary primary tumors in patients with upper aerodigestive tract malignancies. Cancer *101*: 2837-2842, 2004.

10 Almasri A, Wisithphrom K, Windsor LJ and Olson B: Nicotine and lipopolysaccharide affect cytokine expression from gingival fibroblasts. J Periodontol *78*: 533-541, 2007.

11 Nakao S, Ogata Y and Sugiya H: Nicotine stimulates the expression of cyclooxygenase-2 mRNA *via* NFkappaB activation in human gingival fibroblasts. Arch Oral Biol *54*: 251-257, 2009.

12 Hecht SS: Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer *3*: 733-744, 2003.

13 Hecht SS, Hochalter JB, Villalta PW and Murphy SE: 2'-Hydroxylation of nicotine by cytochrome P450 2A6 and human liver microsomes: formation of a lung carcinogen precursor. Proc Natl Acad Sci USA *97*: 12493-12497, 2000.

14 Wu WK and Cho CH: The pharmacological actions of nicotine on the gastrointestinal tract. J Pharmacol Sci *94*: 348-358, 2004.

15 Costea DE, Lukandu O, Bui L, Ibrahim MJ, Lygre R, Neppelberg E, Ibrahim SO, Vintermyr OK and Johannessen AC: Adverse effects of Sudanese toombak vs. Swedish snuff on human oral cells. J Oral Pathol Med *39*: 128-140, 2010.

16 Idris AM, Ibrahim SO, Vasstrand EN, Johannessen AC, Lillehaug JR, Magnusson B, Wallstrom M, Hirsch JM and Nilsen R: The Swedish snusand the Sudanese toombak: Are they different? Oral Oncol *34*: 558-566, 1998.

17 Lewin F, Norell SE, Johansson H, Gustavsson P, Wennerberg J, Biorklund A and Rutqvist LE: Smoking tobacco, oral snuff, and alcohol in the etiology of squamous cell carcinoma of the head and neck: a population-based case-referent study in Sweden. Cancer *82*: 1367-1375, 1998.

18 Rodu B and Cole P: Lung cancer mortality: comparing Sweden with other countries in the European Union. Scandn J Public Health *37*: 481-486, 2009.

19 Hansson J, Pedersen NL, Galanti MR, Andersson T, Ahlbom A, Hallqvist J and Magnusson C: Use of snus and risk for cardiovascular disease: results from the Swedish Twin Registry. J Int Med *265*: 717-724, 2009.

20 Laytragoon-Lewin N, Lagerlund M, Lundgren J, Nordlander B, Elmberger G , Sodergren T, Lagerros C, Rutqvist LE and Lewin F: Significance of RNA reference in tumour-related gene expression analyses by cDNA array. Anticancer Res *25*: 1397-1407, 2005.

21 Shin VY, Wu WK, Chu KM, Koo MW, Wong HP, Lam EK, Tai EK and Cho CH: Functional role of beta-adrenergic receptors in the mitogenic action of nicotine on gastric cancer cells. Toxicol Sci *96*: 21-29, 2007.

22 Ye YN, Liu ES, Shin VY, Wu WK, Luo JC and Cho CH: Nicotine promoted colon cancer growth *via* epidermal growth factor receptor, c-Src, and 5-lipoxygenase-mediated signal pathway. J Pharmaco Exp Thera *308*: 66-72, 2004.

23 Ramqvist T, Magnusson KP, Wang Y, Szekely L, Klein G and Wiman KG: Wild-type p53 induces apoptosis in a Burkitt lymphoma (BL) line that carries mutant p53. Oncogene *8*: 1495-1500, 1993.

24 Wang Y, Ramqvist T, Szekely L, Axelson H, Klein G and Wiman KG: Reconstitution of wild-type p53 expression triggers apoptosis in a p53-negative v-myc retrovirus-induced T-cell lymphoma line. Cell Growth Differ *4*: 467-473, 1993.

25 Feyerabend C, Ings RM and Russel MA: Nicotine pharmacokinetics and its application to intake from smoking. Br J Clin Pharmacol *19*: 239-247,1985.

26 Soma T, Kaganoi J, Kawabe A, Kondo K, Imamura M and Shimada Y: Nicotine induces the fragile histidine triad methylation in human esophageal squamous epithelial cells. Int J Cancer *119*: 1023-1027, 2006.

27 West KA, Brognard J, Clark AS, Linnoila IR, Yang X, Swain SM, Harris C, Belinsky S and Dennis PA: Rapid Akt activation by nicotine and a tobacco carcinogen modulates the phenotype of normal human airway epithelial cells. J Clin Invest *111*: 81-90, 2003.

28 Airoldi L, Magagnotti C, Iannuzzi AR, Marelli C, Bagnati R, Pastorelli R, Colombi A, Santaguida S, Chiabrando C, Schiarea S and Fanelli R: Effects of cigarette smoking on the human urinary proteome. Biochem Biophys Res Comm *381*: 397-402, 2009.

29 Gemenetzidis E, Bose A, Riaz AM, Chaplin T, Young BD, Ali M, Sugden D, Thurlow JK, Cheong SC, Teo SH, Wan H, Waseem A, Parkinson EK, Fortune F and Teh MT: FOXM1 upregulation is an early event in human squamous cell carcinoma and it is enhanced by nicotine during malignant transformation. PLoS One *4*, e4849-4854, 2009.

30 Wu HT, Ko SY, Fong JH, Chang KW, Liu TY and Kao SY: Expression of phosphorylated Akt in oral carcinogenesis and its induction by nicotine and alkaline stimulation. J Oral Path Med *38*: 206-213, 2009.

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