Korean Society of Maternal Fetal Medicine
Vol. 7 No. 3 September 2011

세포영양막에서 니코틴 노출 후 유전자 발현의 변화에 대한 마이크로어레이 분석

연세대학교 의과대학 산부인과학교실

조희영 · 허혜원 · 손가현 · 권하얀 · 김영한 · 박용원 · 권자영

Microarray Analysis of the Gene Expression Profiling in the Cytotrophoblasts on Nicotine Exposure

Hee Young Cho, M.D., Hyewon Hur, M.D., Ga Hyun Son, M.D., Ha Yan Kwon, M.D.,Ph.D., Young Han Kim, M.D.,Ph.D., Yong Won Park, M.D., Ph.D., Ja Young Kwon, M.D.,Ph.D.

Division of Maternal and Fetal Medicine, Department of Obstetrics and Gynecology, Yonsei University College of Medicine, Yonsei University Health System, Seoul, Korea

Objectives: The aim of this study was to compare the gene expression profiles in the human cytotrophoblast cell line after nicotine exposure in vitro.

Methods: The human cytotrophoblast cell line, 3A was cultured in minimum essential medium supplemented. Cells were seeded onto 6-well plate and cultured overnight until 80-90% confluent. Cells were then treated with nicotine of 1×10^{-6}M concentration. We treated 3A cells, with nicotine, then isolated total RNA at 0, 6, and 24 hours following nicotine treatment and hybridized the RNA to microarrays. Detection of array signals was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the BeadChip manual. Microarray experiments and statistical analysis were carried out by Macrogen Incorportation (Seoul, Korea). The microarray measured the expression level of 16,890 probes. Altered gene expressions were selected by 1.5-fold change between control and nicotine treated group.

Results: The 252 genes were filtered as candidates to identify expression levels. After nicotine exposure, total of 15 genes, among which 13 genes were found to be up-regulated and 2 genes were down-regulated after exposure. Genes identified likely regulate immunity, defense and developmental processes, signal transduction and cell metabolism.

Conclusion: Fifteen genes were identified to show changes in expression level after nicotine exposure. Each gene showed different patterns of change after the exposure. SCL7A1 (solute carrier family 7) and SNCG (synuclein gamma gene) could be possible genes to affect IUGR after nicotine exposure. Understanding the pattern of each gene expression after the nicotine exposure will be able to reveal carcinogenic and teratogenic actions of nicotine in more detail.

Key words: Cytotrophoblast, Nicotine, Microarray

Smoking is a major public health problem and it is especially harmful for pregnant women. Epidemiological studies provide that depending on population, 12-31% of pregnant women smoke,[1] therefore approximately 2 million babies are annually exposed in utero to cigarette smoking.[2] Smoking cigarettes during pregnancy threaten health of both fetus and pregnant women by adversely affecting each trimester, and can cause intrauterine growth retardation, still birth, premature labor and abruption of placenta.[3] Many studies have reported that abnormal placentation could be one of the leading cause in smoking-associated pregnancy complications.[4-6] Effect of smoking on placenta differs with exposed stages of pregnancy.[7] It is known that especially during early pregnancy, it alters normal placenta's response to oxygen

투고일 : 2011. 8. 10. 수정일 : 2011. 8. 12. 확정일 : 2011. 8. 17.
주관책임자 : 권자영
E-mail : jaykwon@yuhs.ac

tension and hinders proliferation and control of differentiation of cytotrophoblasts.[8-10]

Cigarette smoke contains cyanide, sulphides, cadmium, carcinogenic hydrocarbons, nicotine, and more than 2500 other chemical components, and most of these are low molecular weight and water soluble which passes placenta easily.[11-13] Especially, nicotine and cotinine is found in higher concentration in fetal side than maternal side, and despite activities of placental xenobiotic-metabolizing enzymes which detoxify these chemicals, these cause direct cellular damage to cytotrophoblast cells.[14]

Many researchers have studied to reveal the mechanism that cigarette smoking affect placental function but it is still unclear. There were only several reports that have addressed the gene expression profiles of the cigarette smoke exposed placentas. Moreover, the direct effect of nicotine during placental development in early pregnancy has never been studied.

Therefore, we sought to evaluate the effect of nicotine on the gene expression in the cytotrophoblasts by analyzing transcriptome from the nicotine-treated cytotrophoblasts and comparing it to that from the control. We also analyzed the affected biological processes and signaling pathways that may represent molecular mechanisms of cytotrophoblasts.

MATERIALS AND METHODS

Cells and cell culture

The human cytotrophoblast cell line, 3A, which was transformed by SV40ts30 was purchased from American Type Culture Collection (Manassas, VA, USA). Cell line was cultured in minimum essential medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 33℃ in humidified 5% CO_2/95% air. Briefly, cells were seeded onto 6-well plate and cultured overnight until 80-90% confluent. Cells were then treated with nicotine of 10^{-6}M concentration. Each experiment was duplicated.

RNA preparation and Microarray

Cells were harvested after 6 and 24 hours for RNA preparation. Total RNA was extracted using an RNA extraction kit (iNtRON Biotechnology, Gyeonggi, Korea) according to the manufacturer's instructions. Double-stranded cDNA was prepared from 2 μg of total RNA and was then stored at -20℃ until further use. For quality control, RNA purity and integrity were evaluated by denaturing gel electrophoresis, OD 260/280 ratio, and analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX) to yield biotinylated cRNA according to the manufacturer's instructions.

750 ng of labeled cRNA samples were hybridized to each Sentrix HumanRef-6-V3 Expression BeadChip for 16-18h at 58℃, according to the manufacturer's instructions (Illumina, Inc., San Diego, CA). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the BeadChip manual. Arrays were scanned with an Illumina Bead array Reader confocal scanner according to the manufacturer's instructions. Array data processing and analysis was performed using Illumina BeadStudio. Microarray experiments and statistical analysis were carried out by Macrogen Incorportation (Seoul, Korea).

Statistical analysis

Sentrix RatRef-12 Expression BeadChips (Illumina, San Diego, CA) were used to analyze differential gene expression profiles. The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. The genes were filtered by Detection *P*-value threshold (Detection *P*-value greater than 0.05 in 2 samples). Also, the genes were filtered when genes had negative signal value. Data were extracted using the software provided by the manufacturer and normalized by Quantile normalization. Fold change was applied to determine differentially expressed sets of genes between

the control and the nicotine-treated cells. Protein ANalysis THrough Evolutionary Relationship (PANTHER) classification System (http://www.pantherdb.org/).

RESULTS

To investigate the gene expression between the human cytotrophoblast cell line 3A and nicotine treated cytotrophoblast cell line 3A, we performed cDNA microarray analysis with using a direct comparison method. The microarray measured the expression level of 16,890 probes. The gene list obtained from a class comparison between control and nicotine exposure group was filtered to identify candidates for which the expression levels differed by at least 1.5-fold between groups. From the initial analysis of the fold changes reduced the number to a total of 252 genes.

As shown in Table 1, all the regression coefficients were more than 0.9, indicating that the differentially expressed genes were successfully identified from control cytotrophoblast cell line 3A and nicotine treated cytotrophoblast cell line 3A.

Hierarchical clustering analysis of the subjects group was showed various gene expression patterns (Fig. 1).

Table 2 showed a list of the genes with an increased expression between control cytotrophoblast cell line 3A and nicotine treated cytotrophoblast cell line 3A according to incubation time. Table 3 showed the genes with a decreased expression of nicotine treated cell line groups compare to control groups. Gene expression patterns in nicotine treated cells and control cells have been

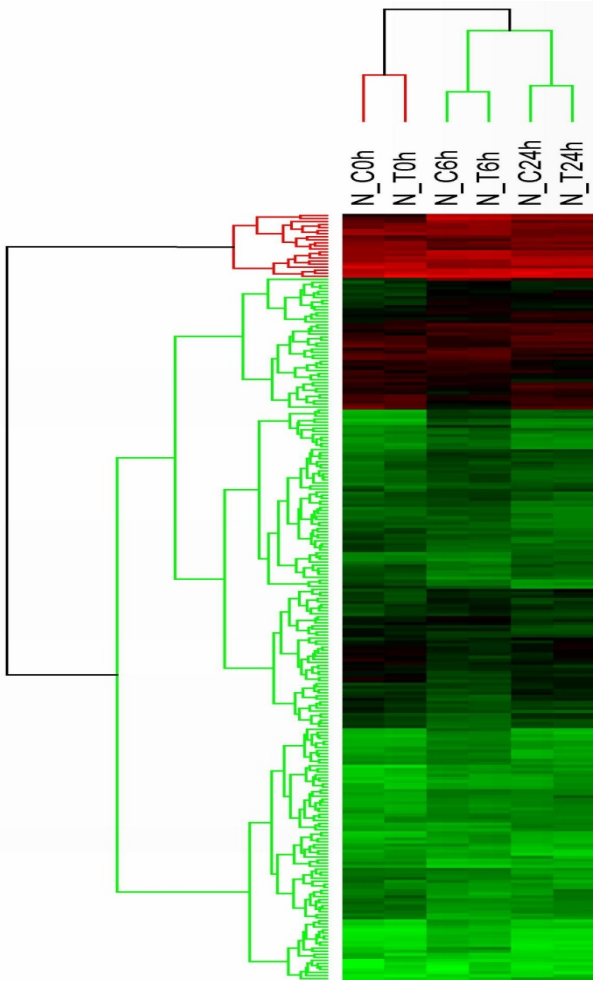

Fig. 1. Hierarchical clustering of genes. Cells were exposed to nicotine for 0, 6, 24 hrs to nicotine (N_T 0 h, 6 h, 24 h) or control (N_C 0 h, 6 h, 24 h). Gene expression changes are indicated by the color bar. The color coding is as follows: red=increased expression; green= repressed expression; and black=no signal detection or unaltered gene expression.

Table 1. The correlation coefficients between control groups and nicotine treated groups

	Control 0 hr	Control 6 hr	Control 24 hr	Nicotine 0 hr	Nicotine 6 hr	Nicotine 24 hr
Control 0 hr	1.000	0.994	0.994	0.995	0.993	0.994
Control 6 hr	0.994	1.000	0.995	0.994	0.997	0.995
Control 24 hr	0.994	0.995	1.000	0.996	0.995	0.997
Nicotine 0 hr	0.995	0.994	0.996	1.000	0.995	0.994
Nicotine 6 hr	0.993	0.997	0.995	0.995	1.000	0.994
Nicotine 24 hr	0.994	0.995	0.997	0.994	0.994	1.000

Table 2. The genes with an increased expression of nicotine treated cells compare to control cells

Accession	Definition	Target ID
AD1	Glutamate decarboxylase 1 (brain, 67kDa)	NM_013445.3
LOC653491	PREDICTED: similar to CG12113-PA	XM_927709.1
MVP	Major vault protein (MVP)	NM_005115.3
C20orf107	Chromosome 20 open reading frame 107	NM_001013646.2
TNFRSF8	Tumor necrosis factor receptor super family, member 8	NM_001243.3
CD274	CD274 antigen (CD274)	NM_014143.2
HNRPA3P1	Heterogeneous nuclear ribonucleoprotein A3 pseudogene 1 (HNRPA3P1) on chromosome 10	NR_002726.1
LOC440686	Similar to histone H2B histone family	NM_001025303.1
SLC7A1	Solute carrier family 7 (cationic amino acid transporter, y+system), member 1	NM_003045.3
SNCG	Synuclein, gamma (breast cancer-specific protein 1)	NM_003087.1
TXNDC11	Thioredoxin domain containing 11	NM_015914.5
NIN	Ninein (GSK3B interacting protein)	NM_182944.1
LOC644391	PREDICTED: hypothetical protein LOC644391	XM_932163.1

Table 3. The genes with a decreased expression of nicotine treated cells compare to control cells

Accession	Definition	Target ID
LOC643310	PREDICTED: similar to heat shock 70kD protein binding	XM_926656.1
TMEM85	Transmembrane protein 85	NM_016454.2
RNF122	Ring finger protein 122	NM_024787.2

compared to cells at zero time at two time points (6 hr, 24 hr) and alteration of gene expression is showing using fold change compared to zero time signal.

Additionally, to further investigate the biological processes, we performed the gene ontology analysis (Table 4, 5). Each gene showed different patterns of change after the exposure. Genes identified likely regulate immunity, defense and developmental processes, signal transduction and protein and nucleic acid metabolism. In respect to molecular function, genes that showed changes seemed to be related nucleic acid binding.

DISCUSSION

Smoking significantly increases the rate of low birth weight infants and intrauterine growth restriction.[15] Moreover, there is a report that the amount of cigarette smoked by a pregnant woman is correlated to the higher risk of intrauterine growth restriction. These correlation was consistent in second-hand smoking.[16] These effects are not just limited at birth but have persistent effects which increase childhood mortality and morbidity, and increased risk of hypertension, coronary heart disease, and diabetes mellitus after becoming adult.[17]

We processed nicotine to cytotrophoblast 3A cell line to find out the effect of smoking on transcriptome of early placental development and investigated change in gene expression. We could confirm increased gene expression in 13 genes and decreased gene expression in 2 genes from cytotrophoblast 3A cell line after nicotine processing.

Table 4. Gene ontology of significantly increased gene numbers after nicotine exposure: The biological processes and molecular functions among the significantly regulated genes

Gene ontology category	Number of genes
Biological process	
Protein metabolism and modification	3
Immunity and defense	2
Signal transduction	2
Apoptosis	1
Nucleoside, nucleotide and nucleic acid metabolism	1
Amino acid metabolism	1
Cell structure and motility	1
Cell proliferation and differentiation	1
Oncogenesis	1
Biological process unclassified	3
Molecular func tion	
Nucleic acid binding	3
Receptor	1
Lyase	1
Signaling molecule	1
Isomerase	1
Molecular function unclassified	3

Table 5. Gene ontology of significantly decreased gene numbers after nicotine exposure: The biological processes and molecular functions among the significantly regulated genes

Gene ontology category	Number of genes
Biological process	
Protein metabolism and modification	1
Amino acid metabolism	1
Amino acid transport	1
Molecular function	
Tranporter	1
Chaperone	1

In IUGR fetus, several metabolite transporter genes (SLC-family facilitated diffusion channels) were differentially expressed, with some family members over-expressed and others under-expressed.[18] SCL7A1 (solute carrier family 7) from this SCL-family gene was up-regulated, and we also detected up-regulated expression of same gene. Therefore, it is possible to postulate that smoking affects SCL-family gene expression and this becomes one of causes of IUGR.

SNCG (synuclein gamma gene), one of up regulated gene is known to be related to apoptosis. Previous studies have shown the trophoblast apoptotic activity to be more pronounced in the placentas from IUGR.[19,20] In addition, Vogt. et al identified apoptosis was increased in the placentas of smoking mothers with growth-restricted infants. It is possible that this could be one of the mechanisms playing a role in the growth restriction of smoking mothers.[21] Therefore, SNCG gene is a candidate gene for mechanism of IUGR caused by smoking.

Other than that, TNFRSF8 (tumor necrosis factor receptor superfamily, member 8) and CD 274 (CD274 antigen) are genes related to immune and defense, and they can be crucial in unveiling mechanisms of cigarette smoke-induced immune-suppression.

Recently, Huuskonen et al. have investigated on the difference in the expression of genes specific for metabolizing genes in the placenta from the smokers in comparison to that of non-smokers at term and reported an alteration in the steroid hormone-metabolizing enzymes genes such as CYP1A1, CYP19A1, and HSD3B1.[22] Other study by Bruchova et al. identified high up-regulation of xenobiotic genes (CYP1A1,CYP1B1, CYB5A, COX412), collagen genes, coagulation genes as well as thrombosis-related genes in placenta of smoking mothers.[23] However, some limitations lie in these studies that only genes involved in metabolizing enzymes were looked at, and that clinical significance of such finding is unclear since most of the pregnancy had an uncomplicated outcome regardless of cigarette smoking, and that this finding can not specify the mechanism as to which compound triggers which cells. Also, both studies showed genes affected by smoking at term placenta, and they did not show change of genes in early placental development period by smoking as current study.

To our knowledge, this study reports the largest data set of gene expression profiles detected in cytotrophoblast 3A cell line exposed to tobacco smoke and control group. The molecular and biological function analysis suggested processes and pathways affected by cigarette smoke exposure that may represent molecular mechanism of smoke-induced cytotrophoblast cell. However, there are potential limitations with the present study. First, human cytotrophoblast cell line 3A used in this study is an immortalized cell line derived from tsA mutant of simian virus 40. Therefore, this cell line cannot fully substitute for in vivo syncytiotrophoblast cell. Second, current study is in vitro study and this cannot reflect in vivo interaction among cytotrophoblast cells. Third, cigarette smoking involves numerous chemical compounds other than nicotine. However, current study can be meaningful in that it showed the direct effect of nicotine, the major compound in cigarette smoking, to cytotrophoblast cell. The results of current study can be a foundation to find out changes in transcriptome in cytotrophoblast cell level caused by cigarette smoking.

Acknowledgements

This study was supported by a faculty research grant of Yonsei University College of Medicine for 2008 (6-2008-0116) .

참고문헌

1. Ebrahim SH, Floyd RL, Merritt RK, Decoufle P, Holtzman D. Trends in pregnancy-related smoking rates in the United States, 1987-1996. JAMA (Chicago, Ill.) 2000; 283: 361-6.
2. Singh SP, Razani-Boroujerdi S, Pena-Philippides JC, Langley RJ, Mishra NC, Sopori ML. Early postnatal exposure to cigarette smoke impairs the antigen-specific T-cell responses in the spleen. Toxicol Lett 2006; 167: 231-7.
3. Jauniaux E, Burton GJ. Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. Early Hum Dev 2007; 83: 699-706.
4. Jauniaux E, Burton GJ. The effect of smoking in pregnancy on early placental morphology. Obstet Gynecol 1992; 79: 645-8.
5. Salafia C, Shiverick K. Cigarette smoking and pregnancy II: vascular effects. Placenta 1999; 20: 273-9.
6. Genbacev O, McMaster MT, Zdravkovic T, Fisher SJ. Disruption of oxygen-regulated responses underlies pathological changes in the placentas of women who smoke or who are passively exposed to smoke during pregnancy. Reprod Toxicol 2003; 17: 509-18.
7. Genbacev O, Bass KE, Joslin RJ, Fisher SJ. Maternal smoking inhibits early human cytotrophoblast differentiation. Reprod Toxicol 1995; 9: 245-55.
8. Zdravkovic T, Genbacev O, McMaster MT, Fisher SJ. The adverse effects of maternal smoking on the human placenta: a review. Placenta 2005; 26 Suppl A: S81-6.
9. Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. Science 1997; 277: 1669-72.

10. Genbacev O, McMaster MT, Lazic J, Nedeljkovic S, Cvetkovic M, Joslin R, et al. Concordant in situ and in vitro data show that maternal cigarette smoking negatively regulates placental cytotrophoblast passage through the cell cycle. Reprod Toxicol 2000; 14: 495-506.
11. Hansen C, Srensen LD, Asmussen I, Autrup H. Transplacental exposure to tobacco smoke in human-adduct formation in placenta and umbilical cord blood vessels. Teratogen Carcin Mut 1992; 12: 51-60.
12. Eskenazi B, Prehn AW, Christianson RE. Passive and active maternal smoking as measured by serum cotinine: the effect on birthweight. Am J Public Health 1995; 85: 395-8.
13. Jauniaux E, Gulbis B, Acharya G, Thiry P, Rodeck C. Maternal tobacco exposure and cotinine levels in fetal fluids in the first half of pregnancy. Obstet Gynecol 1999; 93: 25-9.
14. Zhang J, Ratcliffe JM. Paternal smoking and birthweight in Shanghai. Am J Public Health 1993; 83: 207-10.
15. Dejmek J, Solansk y I, Podrazilova K, Sram RJ. The exposure of nonsmoking and smoking mothers to environmental tobacco smoke during different gestational phases and fetal growth. Environ Health Persp 2002; 110: 601-6.
16. Horta BL, Victora CG, Menezes AM, Halpern R, Barros FC. Low birthweight, preterm births and intrauterine growth retardation in relation to maternal smoking. Paediatr Perinat Ep 1997; 11: 140-51.
17. Barker DJ, Osmond C, Kajantie E, Eriksson JG. Growth and chronic disease: findings in the Helsinki Birth Cohort. Ann Hum Biol 2009; 36: 445-58.
18. McMinn J, Wei M, Schupf N, Cusmai J, Johnson EB, Smith AC, et al. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. Placenta 2006; 27: 540-9.
19. Levy R, Smith SD, Yusuf K, Huettner PC, Kraus FT, Sadovsky Y, et al. Trophoblast apoptosis from pregnancies complicated by fetal growth restriction is associated with enhanced p53 expression. Am J Obstet Gynecol 2002; 186: 1056-61.
20. Erel CT, Dane B, Calay Z, Kaleli S, Aydinli K. Apoptosis in the placenta of pregnancies complicated with IUGR. Int J Gynecol Obstet 2001; 73: 229-35.
21. Vogt Isaksen C. Maternal smoking, intrauterine growth restriction, and placental apoptosis. Pediatr Devel Pathol 2004; 7: 433-42.
22. Huuskonen P, Storvik M, Reinisalo M, Honkakoski P, Rys J, Hakkola J, et al. Microarray analysis of the global alterations in the gene expression in the placentas from cigarette-smoking mothers. Clin Pharmacol Ther 2008; 83: 542-50.
23. Bruchova H, Vasikova A, Merkerova M, Milcova A, Topinka J, Balascak I, et al. Effect of maternal tobacco smoke exposure on the placental transcriptome. Placenta 2010; 31: 186-91.

「국문초록」

목적: 본 연구는 생체 외에서 세포영양막 세포주에 니코틴을 노출시킨 후 유전자 발현의 변화를 비교해 보고자 하였다.

연구방법: 인간 세포영양막 세포주 3A 를 최소 영양 배지에서 배양하여 세포를 6-well plate 에 분주하고 80-90% 자랄 때까지 배양하였다. 세포에 1×10^{-6}M 농도의 니코틴을 처리한 후 0시간, 6시간 그리고 24시간 때에 RNA 를 분리하여 마이크로 어레이 분석을 위해 부합하하였다. 어레이 신호를 감지하기 위해서 BeadChip 매뉴얼에 있는 Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) 를 이용하였으며 마이크로 어레이 실험과 통계분석은 Macrogen Incorportation (Seoul, Korea) 를 통하여 시행하였다. 마이크로 어레이 분석은 16,890 개의 탐색자를 이용하여 발현을 측정하였고, 대조군과 니코틴 처리군 사이에 1.5 배 이상의 변화를 보이는 유전자를 선별하였다.

결과: 총 252 개의 유전자가 발현 정도를 비교하기 위한 후보유전자로 선별되었고 이 중 니코틴 처리 후 15개의 유전자가 대조군과 비교해 유전자 발현의 변화가 있었다. 13개의 유전자는 발현이 증가되었고, 2개의 유전자는 니코틴 노출 후 발현이 감소되었다. 발견된 유전자들은 면역체계, 방어, 발달과정, 신호전달 및 세포대사에 관여하였다.

결론: 각각의 15개 유전자는 니코틴 노출 후 다른 양상의 유전자 발현 변화를 보여주었다. 이 중 SCL7A1(solute carrier family 7) 과 SNCG(synuclein gamma gene) 유전자는 니코틴 노출 후 자궁 내 태아발육 지연에 영향을 줄 가능성이 있는 유전자들이었다. 니코틴 처리 후 생기는 각 유전자들의 발현 양상의 분석은 니코틴의 발암 물질로서의 작용과 기형유발 작용을 밝히는 기초 자료가 될 수 있을 것이다.

중심 단어: 세포영양막, 니코틴, 마이크로어레이