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Gene expression profiles in HPV-immortalized human cervical cells treated with the nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

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Abstract

Human papillomavirus (HPV) infection is an established etiological factor for cervical cancer. Epidemiological studies suggest that smoking in combination with HPV infection plays a significant role in the etiology of this disease. We have previously shown that the tobacco carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), is present in human cervical mucus. Here, we hypothesized that treatment of HPV 16-immortalized human ectocervical cells (Ecto1/E6E7) with NNK would alter the expression of genes involved in cellular transformation. Ecto1/E6E7 cells were treated with water (vehicle control) alone or with 1, 10, and 100 μ M of NNK in water for 12 weeks. The colony forming efficiency increased following NNK treatment; the maximum effect was observed after 12 weeks with 100 μ M NNK. Microarray analysis revealed that, independent of the dose of NNK, expression of 30 genes was significantly altered; 22 of these genes showed a dose response pattern. Genes identified are categorized as immune response (LTB4R), RNA surveillance pathway (SMG1), metabolism (ALDH7A1), genes frequently expressed in later stages of neoplastic development (MT1F), DNA binding (HIST3H3, CHD1L), and protein biosynthesis (UBA52). Selected genes were confirmed by qRT-PCR. Western blot analysis indicates that phosphorylation of histone 3 at serine 10, a marker of cellular transformation, was up-regulated in cells treated with NNK. This is the first study showing that NNK can alter gene expression that may, in part, account for transformation of HPV-immortalized human cervical cells. The results support previous epidemiological observations that, in addition to HPV, tobacco smoking also plays an important role in the development of cervical cancer.

Introduction

Infection with human papillomaviruses (HPVs), especially HPV-16 is the most important etiological factor for cervical cancer (1,2); HPV DNA is detected in more than 99% of invasive cervical carcinomas (3). E6 and E7 proteins of HPV-16 bind and inactivate tumor suppressor genes *p53* and retinoblastoma (*Rb*) resulting in cell survival and proliferation. Therefore,

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simultaneous expression of E6 and E7 genes of HPV can induce immortalization of cervical cells (4). However, various epidemiological studies suggest that infection with HPV alone is not sufficient for the development of cervical cancer (5,6). Other factors such as tobacco smoking increase the risk for cervical cancer (5,6). A recent report found that the risk of developing cervical carcinoma in situ in HPV-16 positive smokers at time of first Papanicolaou smear was over 14-fold greater compared to women who were smokers but were HPV-16 negative (7). In addition, smokers with high HPV-16 viral load at the time of first cervical smear were at higher risk (27-fold) of developing cervical cancer compared to smokers without HPV infection (7). In nonsmokers, however, a high HPV-16 load contributed to only a 6-fold increase in cervical cancer. The number of cigarettes per day is also strongly associated with conversion from cervical intraepithelial neoplasia grade 1 (CIN1) to CIN grades 2 and 3 as well as with the extent of cell proliferation assessed by Ki67 staining; the latter was higher in CIN grades 2 and 3 in heavy smokers (8).

Tobacco-derived carcinogens such as tobacco-specific nitrosamines (TSNA) are well established alkylating DNA damaging agents (9,10). We have previously demonstrated the presence of NNK in human cervical mucus and levels of NNK in cigarette smokers were significantly higher than those in nonsmokers (mean $\pm$ standard deviation: 46.9 ± 32.5 ng/g of mucus versus 13.0 ± 9.3 ng/g of mucus, $p=0.004$) (11). Furthermore, we showed that human cervical tissue and human cervical cells can metabolically activate NNK to genotoxic intermediates (12). This genotoxic damage, if not repaired, can alter the expression of various genes that may be critical in the development of cervical cancer.

Changes in gene expression in various grades of cervical neoplasia have been reported (13–18); a recent report elegantly describes genetic changes involved in the progression of cervical neoplasia from normal cervix to squamous cell carcinoma (19). However, these comprehensive changes in gene expression might not necessarily reflect changes induced by tobacco-derived carcinogens such as NNK.

Tobacco smoke condensate has been reported to transform immortalized human cervical cells [20,21] by up-regulating levels of *B-myb*, p53 and WAF1 mRNA (22) and enhancing the expression of antiapoptotic proteins bcl-2, bcl-x_L, and BAG-1 (23). NNK has been shown to induce malignant transformation in human breast epithelial cells (24) and in HPV-immortalized human bronchial epithelial cells (25). The latter report (25) showed elevated levels of p16, β -catenin and PCNA in the lung. Because of the lack of information on the effects of NNK on gene expression in HPV-immortalized human cervical cells, the aim of this investigation was to test our hypothesis that continuous exposure of HPV-immortalized human Ecto1/E6E7 cervical cells to NNK would alter expression of genes involved in cellular transformation.

Materials and methods

Cell lines and treatments

Ectocervical epithelial cells (Ecto1/E6E7), transduced with retroviral vector LXS_N-16E6E7 in the presence of polybrene (26), were purchased from American Type Culture Collection (Manassas, VA). These cells have an epithelial morphology and stable phenotype (26). Keratinocyte serum free media containing L-glutamine, prequalified human recombinant Epidermal Growth Factor 1–53 and Bovine Pituitary Extracts were obtained from Invitrogen (Carlsbad, CA). Cells were continuously treated in duplicate with either vehicle only (water), or 1 μ M, 10 μ M or 100 μ M of NNK in water for 1, 2 and 12 weeks. The latter time point was selected based on previously published studies using human ectocervical, breast and bronchial cells (21–26). Cells were subcultured every week at which time fresh medium containing NNK was added. Additionally, medium containing NNK was replenished every third day. Cells treated with NNK were microscopically examined for any signs of toxicity at every passage.

Anchorage independent colony forming assay

Soft agar colony forming assay was performed on all Ecto1/E6E7 cells treatments after 1, 2 and 12 weeks. Cells were trypsinized and plated in triplicate at a density of 50,000 cells in 2 ml of 0.35% agar over 0.7% agar Noble in 6 well plates. Cultures were covered by 2 ml of medium and incubated in 5% CO₂ at 37°C. Medium was replenished every 3 days for 3 weeks at which time colonies were counted. Colonies with more than 50 cells were considered as positive.

RNA isolation

Total RNA from both vehicle control and from 1, 10 and 100 µM NNK-treated Ecto1/E6E7 cervical cells for 12 weeks was isolated using TRI reagent kit (Molecular Research Inc., Cincinnati, OH). Briefly, TRI reagent was added on top of cells and mixed by pipetting up and down. The resulting mixture was transferred to centrifuge tubes and bromochloropropane was added. The tubes were shaken vigorously and then centrifuged at 12,000 rpm at 4°C for 15 min. The aqueous phase was removed and RNA was precipitated by isopropyl alcohol. Tubes were centrifuged at 12,000 rpm and RNA pellets were re-suspended in diethylpyrocarbonate (DEPC)-treated water. RNA (18S and 28S bands) was visualized using the Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA) and all samples had 28S/18S ratios of between 1.2 and 1.5. The purity of RNA samples were also assessed by spectrophotometry and had OD260/OD280 ratios of greater than 1.8. The concentrations were adjusted to 1 µg/µl for all samples.

Microarray analyses

Microarray analysis was limited to 12 weeks since maximum colony forming efficiency was observed at this time point (cf. Results) and was performed on RNA from controls and from 1 µM, 10 µM, and 100 µM NNK-treated cells in duplicate (n=2) at the PSU-COM Functional Genomics Core Facility using the Codelink Human Whole Genome Bioarray system (GE Healthcare, Piscataway, NJ). The Codelink Human Whole Genome Bioarrays contains over 50,000 probes corresponding to characterized genes and ESTs. cRNA synthesis was performed using 2 µg of RNA according to manufacturer's instructions. Briefly, RNA is reverse transcribed to synthesize first strand cDNA. This step was followed by second strand synthesis and purification using Qiaquick (Qiagen, Valencia, CA) spin columns. Second strand cDNA is added to IVT reaction containing T7 reaction buffer, T7 NTPs, 10 mM biotin-11-UTP, and T7 polymerase and incubated at 37°C for 14 hours. The resulting Biotin-labeled cRNA was then purified using RNEasy columns (Qiagen), quantitated and volume-adjusted for a total of 10 µg, fragmented, and denatured. Bioarrays were hybridized in hybridization reaction containing cRNA and incubated for 18 hours at 37°C in a shaker (300 rpm). Alexa Fluor 647 labeled streptavidin was then added to the slides followed by washings in TNT buffer (0.1 M Tris-HCL, pH 7.6, 0.15 M NaCl, and 0.05% Tween 20). Bioarrays were scanned with an Axon 4000B scanner with GenePix4 v4.0 software at a 5 µm resolution at 635 nm with laser power set at 100%, PMT voltage set at 600V, focus position at 0 µm, and lines to average = 1. Images were imported into Code Link Expression Analysis Software v4.1 (GE Healthcare). Initial quality control (positive and negative controls), exclusion of manufacturing defects (MSR spots), background subtraction, and intra-array normalization was then performed and results exported to GeneSpring 7.3 (Agilent Technologies). Measurements less than 0.01 were then set to 0.01, arrays were normalized to the 50th percentile, and individual genes normalized to the median. Genes targeted for further analysis (Table 1) were limited to those which showed a dose-response pattern and whose expression was altered by ≥2.0-fold or ≤0.5-fold following treatment with 100µM NNK for 12 weeks. The full complement of microarray data has been deposited to the Gene Expression Omnibus (27).

Quantitative real-time PCR

First strand cDNA was produced from 1.0 µg of total RNA using Oligo (dT)_{12–18} primers, and the SuperScript III Reverse Transcription kit (Invitrogen; Carlsbad, CA) by standard methods. The concentration and quality of resulting cDNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Samples were standardized to 10 ng/µl and 20 ng of cDNA per sample were then used as template for real-time PCR using a SYBR Green Master Mix (Qiagen Corp, USA). Samples were normalized to β-actin and validated. QuantiTect Primers Assays (Qiagen) were also used under identical conditions. Reactions were performed with Amperase activation (2 min at 50°C), HotStar Taq DNA Polymerase activation (15 min at 95°C), and then 40 cycles of 15 sec at 95°C, 30 sec at 53°C and 30 sec at 72°C. Quantitation used the $2^{-\Delta\Delta C_t}$ method of crossing thresholds (28) with β-actin as the endogenous control for normalization. Reactions were performed in 384 well optical plates in a 7900HT instrument (Applied Biosystems, Foster City, CA), with 10 µL reaction volumes and analyzing Sequence Detection System 2.2.2 software. To exclude the possibility of genomic DNA contamination, control PCR reactions without cDNA template and without RT control samples were performed for each gene-specific primer set. Amplification data for the genes of interest was normalized to β-actin within each individual PCR reaction. Quadruplicates of each PCR reaction were performed and the resultant data were averaged. qRT-PCR was performed on RNA from water control and from 1 µM, 10, and 100 µM of NNK treatments in duplicate samples.

Western blot analysis

Western blot analysis of histone 3 and phosphorylated histone 3 were performed as previously described (29,30). After 12 weeks of NNK treatment, Ecto1/E6E7 cells were lysed in cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, and 1 mM PMSF for 30 min on ice. Cell suspensions were centrifuged at 25,000 rpm for 10 min at 4°C and supernatants were analyzed for their protein content. Equal quantity of proteins in solubilization buffer containing β-mercaptoethanol were separated on 10% SDS-PAGE. Proteins were electrotransferred onto nitrocellulose (Biorad, Hercules CA), blocked with 5% bovine serum albumin (BSA) in 0.1 M Tris-buffered saline (TBS), pH 7.5, for 1 hr. Rabbit anti-human-Phospho-Histone H3 (Ser10) and Histone H3 (Santa Cruz Biotechnology Inc, Santa Cruz, CA) were added to the blots in 1% BSA, 0.1 M TBS, pH 7.5, and kept overnight at 4°C on a rocker at slow speed. The blots were washed twice in 0.1 M TBS plus 0.05% Tween 20 (TBST), pH 7.5, for 10 min each, followed by incubation with anti-rabbit-horseradish peroxidase labeled antibody at a dilution of 1:2000 in 1% BSA, 0.1 M TBS for 1 hr at room temperature. Blots were washed twice in TBST for 7 min each followed by a final wash in TBS. The antibody reactions were revealed using the chemiluminescence detection reagents according to the manufacturer's recommendations (Amersham Biosciences, Pittsburg PA). β-Actin (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was used as a loading control. The intensity of bands was measured using UVP works 4.0 densitometry analysis software. Samples were normalized with respect to β-actin values. Fold change was calculated for the comparison between groups. The intensities of the reactions were measured using Quick scan densitometer.

Results

Anchorage independent colony forming assay

Colony forming efficiency (Figure 1) was increased from 0.02% (vehicle treated cells) to 0.40% in 1 µM to 0.90% in 10 µM and 100 µM NNK-treated cells in the first passage (1 week), and to 0.45%, 1.04% and 1.84% in cells treated with 10 µM and 100 µM NNK in the second passage (2 weeks), respectively. After 12 weeks of NNK treatment the colony forming efficiency increased from 0.02% (control cells) to 1.6% (1 µM and 10 µM NNK) and to 2.4%

(100 μ M NNK). In general, the number of colonies increased from less than 10 (0.02 %, controls) to more than 1200 (2.4%, 100 μ M NNK-treated cells for 12 weeks). Changes in morphology and cell viability were not observed following microscopic examination and trypan blue exclusion assay (data not shown).

Microarray analysis

Microarray analyses were limited to 12 weeks of NNK treatment. We demonstrated that there were 304 differentially expressed genes in cells treated with 1 μ M NNK as compared to controls; in cells treated with 10 μ M and 100 μ M NNK the number of differentially expressed genes were 272 and 613, respectively (≥ 1.5 -fold difference, $p < 0.05$). Expression of 30 genes was altered ≥ 2.0 or ≤ 0.5 in 100 μ M NNK-treated cells (Figure 2) and 22 of them showed a dose response pattern (Table 1). These 22 genes belong to ontological groupings of immune response (*LTB4R*), RNA surveillance pathway (*SMG1*), metabolism (*ALDH7A1*), genes frequently expressed in later stages of neoplastic development (*MTIF*), DNA binding (*HIST3H3*, *CHD1L*), protein biosynthesis (*UBA52*), and genes of unknown function (e.g. *C21*, *COMMD1*). The primary goal for this study was to identify classes of genes that could play a critical role in cervical carcinogenesis we have decided to perform these analyses using 3 different doses of NNK and to further confirm the identity of selected genes by qRT-PCR analyses.

Quantitative real-time PCR analysis

To further validate gene expression profiling determined by the microarray analyses 4 genes out of 30 from representative categories that are known to be involved in the multistep carcinogenesis process (*SMG1*, *ALDH7A1*, *MTIF*, and *HIST3H3*) were selected for quantitative RT-PCR. PI-3-kinase-related kinase (*SMG1*) was increased 2-fold in microarray analysis and similar increase in qRT-PCR (Figure 3). Aldehyde dehydrogenase 7 family member A1 (*ALDH7A1*) was increased 2-fold at 100 μ M in microarray and qRT-PCR showed slight increase in gene expression at 10 μ M NNK (Figure 3). Metallothionein 1F (*MTIF*) was increased 3–5 fold at 1 μ M, 10 μ M and 100 μ M NNK in microarray analysis, while qRT-PCR showed a 2-fold increase in 10 μ M and 100 μ M NNK treated Ecto1E6/E7 cells (Figure 3). Histone 3 H3 (*HIST3H3*) gene expression was elevated in a dose dependent manner when examined by microarray analysis of 1, 10, and 100 μ M NNK treated Ecto1E6/E7 cells. The qRT-PCR results showed a marginal increase at 1 μ M of NNK and leveled off at higher doses (Figure 3).

Western blot analysis

Phosphorylation of histones at serines 10 and 28 has been linked with neoplastic transformation of cells (29). Since, up-regulation of histone 3 was detected in our microarray analysis as well as by qRT-PCR, we further conducted a Western blot analysis using antibody against phosphorylated histone 3. As compared to water control treated cells the expression of phosphohistone-3 (ser10) increased 1.46-fold in cells treated with 10 μ M NNK and 1.29-fold in cells treated with 100 μ M NNK; NNK at various doses had no effect on the expression of histone 3 protein (Figure 4).

Discussion

Our group reported that NNK, one of the most potent carcinogens found in tobacco smoke, was detected in human cervical mucus of smokers (11). The corresponding levels in smokers' and non-smokers' were 46.9 ± 32.5 ng/g and 13.0 ± 9.3 ng/g, respectively, which represents approximately 0.05–0.23 μ M concentrations of NNK. The doses of NNK used in this study were relatively higher as compared to those detected in human cervical mucus although similar doses were previously used for treatment of various human cells (24,25). We also showed that

human cervical tissue can activate NNK to genotoxic intermediates (12). For this study we have selected a line of pure cervical epithelial cells (Ecto1/E6E7), transduced with retroviral vector LXS_N-16E6E7. These ectocervical cells originate from the transformation zone of the cervix where cancer usually originates (31). Our soft agar colony-forming assay reported here indicates that NNK, even at very low dose (1 μ M), can induce anchorage independence in HPV-immortalized human cervical cells after 12 weeks (Figure 1). Transformed cells acquire the ability to form colonies in agar medium and suppress the growth of normal cells (32). A correlation between colony formation on 0.33% agar medium and tumorigenicity has been reported (33). Prior to the present investigation there were no data available on transformation of human cervical cells by specific tobacco carcinogens such as NNK. However, it has been demonstrated that 100 μ g/ml (approx. 500 μ M) of NNK was sufficient to make human HPV-immortalized bronchial cells (BEP2D) anchorage independent (25); even at a lower dose of NNK (100 pM) anchorage independent growth of non-cancerous immortalized human breast epithelial cells (MCF10A) was demonstrated (24). The colony forming efficiency observed in our study was relatively modest ranging from less than 0.5% for cells treated with 1 μ M NNK for passages 1 and 2 to approximately 2.5% for cells treated with 100 μ M NNK at passage 12 (Figure 1). However, these efficiencies were significantly higher than those previously reported for NNK-treated MCF10A cells (0.05% and 0.08%, respectively) (24) and similar to those reported for BEP2D cells treated with 100 μ g/ml and 400 μ g/ml of NNK (approximately between 0.25% and 0.45%) (25). NNK at 100 μ M also stimulates proliferation of colon cancer cell line HT-29 (34). Collectively, literature data and results reported here indicate that NNK can induce cell transformation in breast, lung, colon and cervical tissue.

Multiple reports addressed the changes in gene expression in different grades of cervical neoplasia and in tumors of the uterine cervix (13–18). Recently, Gius *et al.* (19) have described a comprehensive model examining grade-specific changes in gene expression involved in the entire spectrum of cervical carcinogenesis. They reported that CIN 1 is related to proliferative/immunosuppressive genes, CIN 2 reflects changes in genes with proangiogenic stromal/epithelial signature, while CIN 3 reflects genes with proinvasive genomic signature (19).

However, according to our knowledge there are no published reports addressing global changes in gene expression of HPV immortalized cervical cells induced by tobacco smoke carcinogens (e.g. NNK). Using the Codelink Human Whole Genome Bioarray system, we examined the changes in gene expression after multiple treatments of HPV immortalized cervical cells with NNK. Microarray analysis of cells treated with different doses of NNK showed changes in gene expression involved in many different cellular functions (Table 2). Although, expression of genes detected in our study differ from those reported by Gius *et al.* (19), many of these genes are involved in similar cellular processes. Gius *et al.* (19) did not address the impact of smoking on expression of differentially expressed genes. For example, these authors reported differential expression of immunosuppression genes such as *IFNAR1* (IL receptor 1), *IL1RN* (IL receptor antagonist), *EMPI*, as related to CIN 1 transition. In the present study we report that NNK is capable of altering the expression of certain immune response gene (e.g. *RFX1*, *LTB4R*) (Table 1). *SMG-1* is the most recently identified member of PIKK (PI-3 kinase-related kinase) family. *SMG-1* is involved in regulation of nonsense-mediated mRNA decay (NMD). In addition to its role in nonsense-mediated mRNA decay, *SMG-1* is involved in regulation of genotoxic stress response machinery in mammalian cells. Cellular exposure to ultraviolet light or infrared stimulates *SMG-1* kinase activity (35). In this study, based on microarray analysis, we showed that *SMG1* is up-regulated in Ecto1/E6E7 cervical cells following treatment with NNK; this was further confirmed by qRT-PCR. Taken together, UV light, ionizing radiation, as well as NNK all are capable of up-regulating *SMG1* gene expression.

Microarray analysis revealed that seven out of 22 genes were metabolism related. Four genes (*SOAT2*, *RHCG*, *PGD*, *SLC9A9*) were down-regulated with NNK treatment and 3 genes

(*SELO*, *ALDH7A1*, *ASL*) were up-regulated with NNK treatment. Specific functions of these genes in NNK metabolism remain to be elucidated. It is, however, quite conceivable that genes like aldehyde dehydrogenase (*ALDH7A1*) could influence the balance between NNK and its reduced carcinogenic metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). Five different enzymes responsible for NNAL formation have been identified in humans [36–39]. Two are members of the short-chain dehydrogenase/reductase (SDR) superfamily, namely 11 β -hydroxysteroid dehydrogenase (11 β -HSD) type 1, present in microsomes, and cytosolic carbonyl reductase. Three other cytosolic enzymes, namely AKR1C1, AKR1C2 and AKR1C4, are members of the aldol-keto reductase (AKR) superfamily.

In the present study, 3–5 fold increase in *MTIF* was observed in microarray analysis of cervical cells treated with NNK at 10 μ M and 100 μ M. The qRT-PCR results further confirmed these results. Metallothioneins (MTs), a family of low molecular weight, cysteine-rich proteins, play an important role in maintaining transition metal ion homeostasis and redox balance in cell proliferation and apoptosis (40). Abdel-Mageed and Agrawal have shown that over expression of MT potentiates the growth of MCF7 cells (41). MT isoforms have been associated with aggressive behavior in endometrial adenocarcinomas (42); the levels of *MTIF* isoform correlated with histological grades in breast carcinoma (43). This increased expression of *MTIF* after NNK treatment is indicative of possible involvement of NNK in malignant transformation of cervical cells.

RAD23B plays an essential role in pathway of human nuclear excision repair (NER) system (44). NER plays an important role in the repair of bulky DNA lesions derived from various chemical carcinogens. Bulky pyridyloxobutylated adducts derived from NNK are likely to be repaired by NER (45) although *O*⁶-alkylguanine-DNA alkyltransferase could also be involved in this process (46,47). *RAD23B* was down-regulated as assessed by microarray analysis.

Other genes that may play significant role in tobacco-induced cervical carcinogenesis are those related to carcinogen-DNA binding. Histones are integral and dynamic part of cells responsible for gene transcription. Phosphorylation of serines and threonines of histone 3 can be induced by different stimuli such as UV, arsenite, and EGF. Histone 3 is crucial for chromatin structures and is responsible for neoplastic transformation (30). In our study gene expression of *HIST3H3* is consistently up regulated in Ecto1/E6E7 cells treated with different doses of NNK both by microarray analysis and by qRT-PCR. Furthermore, our Western blot analysis data showed that phosphorylated-histone 3 at Ser10 was up-regulated at 10 μ M NNK and is suggestive of transformation by NNK. Our results are in line with those reported by Choi et al. (29) demonstrating that phosphorylation of histone H3 at serine 10 is indispensable for neoplastic cell transformation.

In summary, continuous treatment of HPV-immortalized cells with NNK, even at a dose as low as 1 μ M for 12 weeks, was sufficient to induce anchorage independence in soft agar. Changes in expression of multiple genes, primarily *SMG1*, *ALDH7A1*, *MTIF*, and *HIST3H3*, by NNK provide some insights into the molecular basis that can account for NNK-induced transformation of HPV immortalized cervical cells. Future studies in our laboratory with a nude mouse model will further confirm this observation. Monitoring expression of genes reported here in Papanicolaou smears of HPV-positive smokers and non-smokers would provide mechanistic information on the role of NNK in cervical cancer development in humans; this will also be the focus of future investigations.

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Abbreviations

BrdU	bromodeoxyuridine
CIN	cervical intraepithelial neoplasia
Ecto1/E6E7	ectocervical epithelial cells
HPV	human papillomavirus
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
qRT-PCR	quantitative real-time PCR
TBS	Tris-buffered saline
TSNA	tobacco-specific nitrosamines

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Figure 1.

Soft agar colony forming assay; size of the colony is increasing with the increasing dose of NNK. ^aSoft agar colony forming assay was performed on all Ecto1/E6E7 cells treatments after 1, 2 and 12 weeks. Colonies with more than 50 cells were counted as positive. Values were based on triplicate determination (mean $\pm$ standard error).

Figure 2.

A cluster based representation of altered genes in 1, 10, and 100 μ M NNK-treated Ecto1/E6/E7 cells from group of 30 selected genes ($n=2$ per group) with intensity provided for each condition, normalized to the control group (vehicle treated). Genes that are up-regulated relative to control are marked in red and genes that are down-regulated are marked in green. Expression of these genes was altered ≥ 2.0 or ≤ 0.5 in 100 μ M NNK-treated cells for 12 weeks as compared to controls ($p < 0.05$). Gene symbol and Genbank number are shown for all 30 genes

Figure 3.

Comparison of microarray (dotted line) and qRT-PCR (solid line) analyses of differentially expressed genes involved PI3 Kinase related kinase (*SMG1*), metabolism (*ALDH7A1*), metallothionein (*MT1F*), and DNA binding (*HIST3H3*) by qRT-PCR of RNA from Ecto1 E6/E7 cells treated with increasing doses of NNK. Microarray analyses of gene expression was determined from control and from NNK-treated cells in duplicate (n=2); qRT-PCR analyses were performed 4 times for each PCR reaction and the values were expressed as mean $\pm$ standard deviation.

Figure 4. Histone 3 and phosphorylated Histone 3(Ser10) protein expression in cell lysates of Ecto1/E6E7cervical cells treated with increasing doses of NNK. Analyses were performed in duplicates from control and from NNK-treated cells. β -actin was used as a loading control.

Table 1
Effects of NNK on gene expression in human ectocervical cells.^a

Function	Name	Description	NNK treatment (μM) Fold change					
			Up-regulated			Down-regulated		
			1	10	100	1	10	100
Immune response	<i>LTB4R</i>	G-protein signaling, Host defense, Inflammation				0.94	0.64	0.52
	<i>SMG1</i>	Inositol/phosphatidylinositol kinase activity, RNA surveillance, genotoxic stress response	0.92	1.32	1.96			
Metabolism	<i>SELO</i>	Selenoprotein O	1.73	1.96	2.42			
	<i>ALDH7A1</i>	Aldehyde dehydrogenase 7 family, member A1	0.96	1.33	2.04			
	<i>ASL</i>	Arginine biosynthesis, urea cycle	1.02	1.29	1.96			
	<i>SOAT2</i>	Sterol O-acyltransferase2 Cholesterol metabolism				0.85	0.63	0.47
	<i>RHCG</i>	Integral to plasma membrane ammonium transport				0.92	0.61	0.53
	<i>PGD</i>	Phosphogluconatedehydrogenase mRNA degradation				1.01	0.87	0.47
	<i>SLC9A9</i>	Antiporter activity Regulation of pH, sodium ion transport				0.87	0.67	0.51
	<i>MTIF</i>	Maintain metal ion homeostasis	3.76	3.17	5.03			
	<i>CHD1L</i>	Chromodomain helicase DNA binding protein 1-like	1.27	1.31	2.15			
	<i>FLJ45246</i>	DNA binding; nucleic acid binding; zinc ion binding	1.70	1.98	2.13			
DNA repair	<i>HIST3H3</i>	Chromosome organization and biogenesis nucleosome assembly	0.99	1.65	2.19			
	<i>RAD23B</i>	Nucleotide-excision repair				0.85	0.48	0.30
Protein biosynthesis	<i>UBA52</i>	Protein biosynthesis	1.03	1.78	2.58			
TGF-β	<i>TSC22</i>	Transforming growth factor beta- stimulated protein				1.10	0.73	0.50
Neuro-peptide signaling	<i>HCRT2</i>	neuropeptide signaling pathway	1.22	1.45	2.24			
Unknown function	<i>C21orf45</i>	Transcribed sequences	1.04	1.61	2.19			
	<i>UBE2R2</i>	Transcribed sequences	1.26	1.64	1.95			
	<i>COMMD1</i>	Transcribed sequences	1.65	2.03	2.62			
	<i>SMOCl</i>	Transcribed sequences	0.86	1.58	1.95			
	<i>NPCI</i>	Transcribed sequences	1.57	1.54	2.66			

^aRNA was isolated from cells treated with different doses of NNK for 12 weeks in duplicate and microarray analysis was performed using the Codelink Human Whole Genome Bioarray system. Genes with expression ≥ 2.0 or ≤ 0.5 in cells treated with 1, 10 and 100 μM NNK are reported.