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## Reciprocal effects of NNK and SLURP-1 on oncogene expression in target epithelial cells

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### Abstract

**Aims**—To elucidate how the nicotinic acetylcholine receptors expressed on bronchial and oral epithelial cells targeted by the tobacco nitrosamine (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) (NNK) facilitate carcinogenic transformation.

**Main methods**—Since NNK-dependent transformation can be abolished by the nicotinic secreted mammalian Ly-6/urokinase plasminogen activator receptor related protein-1 (SLURP-1), we compared effects of NNK and recombinant (r)SLURP-1 on the expression of genes related to tumorigenesis in human immortalized bronchial and oral epithelial cell lines BEP-2D and Het-1A, respectively.

**Key findings**—NNK stimulated expression of oncogenic genes, including MYB and PIK3CA in BEP2D, ETS1, NRAS and SRC in Het-1A, and AKT1, KIT and RB1 in both cell types, which could be abolished in the presence of rSLURP-1. Other cancer-related genes whose upregulation by NNK was abolishable by rSLURP-1 were the growth factors EGF in BEP2D cells and HGF in Het-1A cells, and the transcription factors CDKN2A and STAT3 (Het-1A only). NNK also upregulated the anti-apoptotic BCL2 (Het-1A) and downregulated the pro-apoptotic TNF (Het-1A), BAX and CASP8 (BEP2D), all of which could be abolished, in part, by rSLURP-1. NNK decreased expression of the CTNNB1 gene encoding the intercellular adhesion molecule  $\beta$ -catenin (BEP2D), as well as tumor suppressors CDKN3 and FOXD3 in BEP2D cells, SERPINB5 in Het-1A cells, and RUNX3 in both cell types. These pro-oncogenic effects of NNK were also abolished by rSLURP-1.

**Significance**—The obtained results identified target genes for both NNK and SLURP-1 and shed light on the molecular mechanism of their reciprocal effects on tumorigenic transformation of bronchial and oral epithelial cells.

### Keywords

lung cancer; head and neck cancer; NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone); SLURP-1 (secreted mammalian Ly-6/urokinase plasminogen activator receptor related protein-1); gene expression; BEP2D cells; Het-1A cells

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## Introduction

Tobacco nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are involved in cancers of the lung, oral cavity, esophagus, and pancreas (Hecht et al., 1993). The cells exposed to *N*-nitrosamines continue to divide, and display focal growth and morphologic changes suggestive of early stages of cell transformation (Murrat et al., 1993). NNK can displace the endogenous ligand acetylcholine from the nicotinic class of acetylcholine receptors (nAChRs) expressed on the plasma membrane of both bronchial and oral epithelial cells (Arredondo et al., 2006a, Arredondo et al., 2006b). Thus, in addition to a well-formulated role of genotoxic damage in the etiology of tobacco-related lung cancer, *N*-nitrosamines may contribute directly to tumorigenesis through the receptor-mediated mechanisms.

A novel paradigm of cell regulation *via* nAChRs has been discovered in studies of the cholinergic protein termed SLURP-1 (secreted mammalian **L**y-6/**u**rokinase plasminogen activator **r**eceptor related **p**rotein-1) (Chimient et al., 2003). The amino acid composition of SLURP-1 is homologous to that of single domain frog cytotoxin and snake venom neurotoxins, such as  $\alpha$ -bungarotoxin. SLURP-1 was detected in aerodigestive epithelia, lymphocytes, uterus, stomach, cornea, blood, saliva, sweat and urine (reviewed in Grando, 2008). Radioligand binding inhibition studies showed that SLURP-1 preferentially ligates  $\alpha$ 7 nAChR, and alters expression of cell cycle regulators, differentiation markers, and activate caspases (Arredondo et al., 2005). SLURP-1 competes with nicotine and NNK at the nAChR ligand-binding site and regulates vital function of normal and malignant cells (Grando, 2008). We have recently demonstrated that NNK-dependent transformation of immortalized human bronchial and oral epithelial cells BEP2D and Het-1A, respectively, can be abolished by recombinant (r)SLURP-1 (Chernyavskiy et al., 2008).

In this study, we compared the effects of NNK and rSLURP-1 on the expression of genes related to tumorigenesis in BEP2D and Het-1A cells.

## Materials and Methods

### Treatment of Cells with rSLURP-1

The full length rSLURP-1 was manufactured at Virusys Corporation (Sykesville, MD), as detailed by us before (Chernyavskiy et al., 2008). The BEP2D cells — an established clonal population of HPV-18-immortalized human bronchial epithelial cells—was a generous gift from Dr. Harris (NCI, NIH). The Het-1A cell line — an established clonal population of SV40-immortalized human esophageal epithelial cells — was purchased from ATCC (Manassas, VA). The BEP2D cells and Het-1A cells were grown in EpiLife® Medium containing 60  $\mu$ M calcium and Human Corneal Growth Supplement (Invitrogen, Carlsbad, CA) in a humidified incubator at 5% CO<sub>2</sub> and 37°C. At 80% confluence, experimental cells were exposed for 24 h to either 1  $\mu$ M NNK alone or in combination with 1  $\mu$ g/ml of rSLURP-1, and control cells were left untreated.

### PCR Array and Data Analysis

Total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA). The First-Strand Kit, which contains a mixture of oligo(dT) primer and random hexamers (SABiosciences, Frederick, MD), was utilized for cDNA synthesis, using 400 ng of total RNA from experimental and control (untreated) cells. The RT<sup>2</sup>-Profiler PCR Array for human Oncogenes & Tumor Suppressor genes (PAHS-502E, SABiosciences) that profiles expression of 84 key genes involved in oncogenesis was carried out according to manufacturer's instructions using ABI Prism 7900 HT (Applied Biosystems, Carlsbad, CA).

Subsequent to amplification, characterization of products was performed by melting-curve analysis. SYBR Green fluorescence was detected in each well during the annealing step of each cycle. Analyses of the raw data were done through the SuperArray Data Analysis Web Portal (SABiosciences). Three independent experiments, each starting with fresh cells and the isolation of fresh RNA, were conducted. Each independent experiment was performed in triplicates. The  $C_t$  values for each sample corresponded to the point at which the fluorescence crosses the threshold. Results were expressed as fold change compared to intact control. The  $p$  values were calculated based on a Student's t-test of the replicate  $2^{-\Delta C_t}$  values for the gene in each treatment group. To determine fold change in gene expression, the normalized gene expression in the experimental sample was divided by the normalized expression of the same gene in the control sample, as follows:

$$\frac{2^{-\Delta C_t(\text{expt})}}{2^{-\Delta C_t(\text{ctrl})}} = 2^{\Delta\Delta C_t} \quad \text{where } \Delta\Delta C_t \text{ is equal to } \Delta C_t(\text{expt}) - \Delta C_t(\text{ctrl})$$

## Results

In each cell type, NNK stimulated expression of a number of oncogenic genes, such as MYB and PIK3CA in BEP2D cells (Fig. 1), ETS1, NRAS and SRC in Het-1A cells (Fig. 2), and AKT1, KIT and RB1 in both cell types, which could be abolished in the presence of rSLURP-1. Other cancer-related genes whose upregulation by NNK was abolishable by rSLURP-1 were the growth factors EGF in BEP2D cells and HGF in Het-1A cells, and the transcription factors CDKN2A and STAT3 in Het-1A cells. NNK also upregulated the anti-apoptotic BCL2 (Het-1A only) and downregulated the pro-apoptotic TNF (Het-1A), BAX and CASP8 (BEP2D) genes, all of which could be abolished, in part, in the presence of rSLURP-1. NNK decreased expression of the CTNNA1 gene encoding the intercellular adhesion molecule  $\beta$ -catenin (BEP2D), as well as tumor suppressors CDKN3 and FOXD3 in BEP2D cells, SERPINB5 in Het-1A cells, and RUNX3 in both cell types. These pro-oncogenic effects of NNK were also abolished by rSLURP.

## Discussion

Results of the screening experiments reported herein identify target genes for both NNK and SLURP-1 and shed light on the molecular mechanism of their reciprocal regulation of neoplastic transformation of bronchial oral and epithelial cells reported elsewhere (Chernyavskiy et al., 2008). By analogy with other cell types (Faumontet et al., 2009), immortalization might have affected gene expression profiles in BEP2D and Het-1A cells. This possibility, however, would not produce a confounding effect on the results of our study, because the cells treated with NNK  $\pm$  rSLURP-1 were compared to untreated cells, taken as control. NNK might cause some increase of RUNX3 in both cells lines probably by mediating an indirect protective effect, whereas SLURP-1 increased its expression substantially, probably unmasking an endogenous mechanism.

The nAChR-mediated mechanism of the initiation and progression of NNK-induced cancers has been demonstrated by our previous observations that NNK competes with nicotinic radioligands for binding to BEP2D and Het-1A cells (Arredondo et al., 2006a, Arredondo et al., 2006b). SLURP-1 is structurally similar to snake venom toxin  $\alpha$ -bungarotoxin, which acts as  $\alpha 7$  nAChR antagonist (Chimient et al., 2003). Since both NNK and SLURP-1 can ligate  $\alpha 7$  nAChR (Grando, 2008), rSLURP-1 might antagonize NNK effects on the expression of oncogenes by displacing it from the  $\alpha 7$  nAChR ligand-binding site.

The specific role of nAChRs in lung cancer pathophysiology has been substantiated by the results of Wide Genome Association studies showing that single nucleotide polymorphisms of nAChR subunits affect carcinogenicity of nicotine and related compounds in lung cancer, in addition to being related to addiction to tobacco smoking (Amos et al., 2008, Kaur-Knudsen et al., 2011). The nAChRs are selectively overexpressed in a variety of cancers, e.g.,  $\alpha 7$  in lung cancer and  $\alpha 9$  in breast cancer, and inhibition of nAChR protein levels attenuates nicotine- and nitrosamine-induced cell proliferation in tumor models (reviewed in (Singhet et al., 2011, Wuet al., 2011)). Most recently, it has been reported that naturally occurring variants of human  $\alpha 9$  nAChR differentially affect bronchial cell proliferation and transformation (Chikova and Grando, 2011). Therefore, it is currently believed that nAChRs in lung cells act as central mediators in the activation of cancer signaling pathways (Schuller, 2009), and epithelial nAChRs are considered as novel drug targets for prevention and treatment of cancer (Paleariet al., 2009, Russoet al., 2006).

## Conclusions

rSLURP-1 and NNK produce reciprocal effect on gene expression in human bronchial and oral epithelial cells that may mediate their pro- and anti-oncogenic effects, respectively. The pro-oncogenic effects of NNK can be abolished, in part, by rSLURP-1. The cellular nAChRs that mediate biologic effects of NNK and SLURP-1,  $\alpha 7$  and  $\alpha 9$  in particular, may provide a novel molecular target to prevent, reverse, or retard progression of lung and oral cancers by nicotinic ligands. Future studies should ultimately establish the nAChR subtype(s) mediating the reciprocal effects of NNK and SLURP-1, and identify the downstream signaling pathways linking the nAChR subtypes to the tumorigenic transformation of epithelial cells leading to lung and oral cancers.

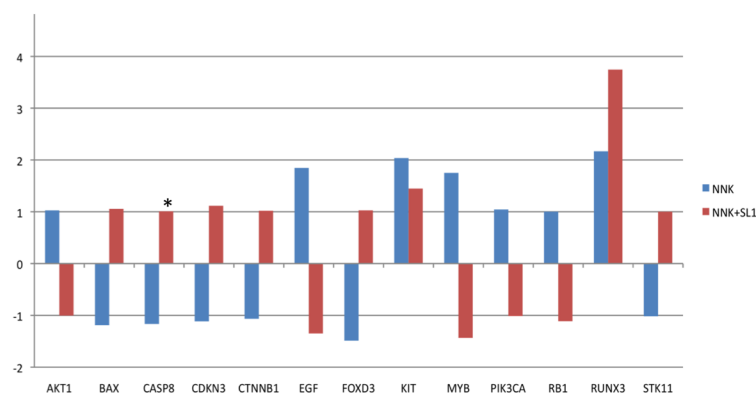
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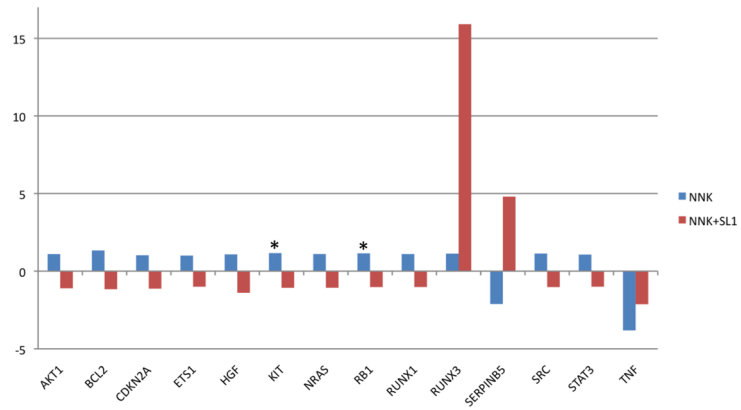
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Symbol	Full Name	NNK	NNK+SLUPR-1
		Fold change	Fold change
AKT1	v-akt murine thymoma viral oncogene homolog 1	1.027	-1.0032
BAX	BCL2-associated X protein	-1.1901	1.0567
CASP8	caspase 8, apoptosis-related cysteine peptidase	-1.1667	1.0091
CDKN3	cyclin-dependent kinase inhibitor 3 (tumor suppressor)	-1.1161	1.116
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	-1.0666	1.0201
EGF	epidermal growth factor	1.8462	-1.3486
FOXD3	forkhead box D3 (tumor suppressor)	-1.4881	1.0273
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	2.0381	1.4482
MYB	v-myb myeloblastosis viral oncogene homolog (avian)	1.7515	-1.4349
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	1.0432	-1.014
RB1	retinoblastoma 1	1.003	-1.1129
RUNX3	runt-related transcription factor 3 (tumor suppressor)	2.1681	3.7466
STK11	serine/threonine kinase 11	-1.0186	1.0013

**Figure 1. Alterations of the expression of oncogenesis-related genes in BEP2D cells**

The cells were treated with either 1  $\mu$ M NNK alone or in combination with 1  $\mu$ g/ml of rSLURP-1 (SL1) and then used in the RT<sup>2</sup>-Profiler PCR Array for human Oncogenes & Tumor Suppressor genes as detailed in Materials and Methods. The vertical axis on the graph shows the fold change values compared to intact control. Fold change values greater than one indicate an up-regulation, and fold-change values less than one indicate a down-regulation of gene expression. Asterisk represents significant ( $p < 0.05$ ) difference between two treatment conditions.



Symbol	Full Name	NNK	NNK+SLURP-1
		Fold change	Fold change
AKT1	v-akt murine thymoma viral oncogene homolog 1	1.1014	-1.1039
BCL2	B-cell CLL/lymphoma 2	1.331	-1.1644
CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	1.0253	-1.1241
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	1.0042	-1.0017
HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	1.0778	-1.3931
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	1.1621	-1.0707
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	1.1064	-1.0681
RB1	retinoblastoma 1	1.1496	-1.0275
RUNX1	runt-related transcription factor 1	1.1026	-1.024
RUNX3	runt-related transcription factor 3 (tumor suppressor)	1.1325	15.911
SERPINB5	serpin peptidase inhibitor, clade B (ovalbumin), member 5 (tumor suppressor)	-2.1148	4.7994
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	1.1369	-1.0271
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	1.0672	-1.0014
TNF	tumor necrosis factor	-3.814	-2.1319

**Figure 2. Alterations of the expression of oncogenesis-related genes in Het-1A cells**  
 The cells were treated, and the gene expression changes were measured and displayed as detailed in the capture to Fig. 1.