Lung Cancer 71 (2011) 11–18

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

The function of IGF-IR in NNK-mediated lung tumorigenesis

Megan D. Siwicky, James J. Petrik, Roger A. Moorehead *

Department of Biomedical Sciences, Ontario Veterinary College, 50 Stone Road East, University of Guelph, Guelph, ON, Canada N1G2W1

ARTICLE INFO

Article history:
Received 10 December 2009
Received in revised form 19 February 2010
Accepted 21 March 2010

Keywords:
Lung cancer
IGF-IR
NNK
Akt
CREB
Erk1/2

ABSTRACT

The type I insulin-like growth factor receptor (IGF-IR) is associated with many different types of cancer and it has been found to be involved in many aspects of the malignant phenotype, such as mitogenesis, survival, transformation and metastasis. This receptor has been observed to be overexpressed in the majority of lung cancer cell lines and human lung tumor biopsies. Two doxycycline-inducible transgenic mouse models in which the human IGF-IR was overexpressed in either the Clara cells or the type II alveolar cells of the lung were used in this study to examine the interaction between the nicotine derivative, nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and the IGF-IR. NNK was injected into both of the transgenic mouse models that overexpress human IGF-IR in the lung tissue in order to determine whether IGF-IR overexpression would affect NNK-induced tumorigenesis. No significant differences in the overall tumor burden were found between mice overexpressing the IGF-IR transgene that were treated with NNK and those that were not, however NNK-treated mice expressing high levels of IGF-IR transgene developed larger tumors than mice expressing high levels of IGF-IR transgene that did not receive NNK injections. In addition, endogenous murine IGF-IR was found to be expressed at high levels in the tumors that developed in the wild type, NNK injected mice suggesting that NNK induces lung tumors through inducing endogenous IGF-IR expression.

1. Introduction

Lung cancer is one of the few cancers in which there is a known causal factor for the majority of the cases. Tobacco smoke is known to cause up to 90% of lung cancer cases, with active smokers having a 10–15% chance of developing lung cancer within their lifetime [1,2]. Tobacco smoke contains more than sixty carcinogens, with the most potent being the polycyclic hydrocarbons and the nitrosamines [3]. One of the most carcinogenic nitrosamines, a nicotine derivative known as nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), has been found to induce lung tumor formation in all species tested [4]. NNK presents itself as a procarcinogen, which is metabolized and activated by enzymes in the lung and liver [5]. The active metabolites that form largely from the carbonyl reduction of NNK are electrophilic and mutagenic. These metabolites attack and mutate specific genes such as the well known oncogenes K-ras, p53 and the EGFR [3,6–8] or the CYP genes that encode for the CYP450 enzymes that are necessary for the metabolism of these carcinogens [9,10]. Mutations in critical genes such as these can then lead to tumor formation and carcinogenesis.

In addition to the DNA mutagenesis activated by the metabolism of NNK, this carcinogen has also been found to activate specific signaling pathways at the cellular level, through the binding of NNK to the α_7 nicotinic acetylcholine receptor, or the β_1-adrenergic and β_2-adrenergic receptors [11–15]. For example, NNK has been found to activate the PI3K pathway through the phosphorylation of Akt, which in turn increases cellular proliferation and cellular survival [13]. This carcinogen has also been found to upregulate cellular proliferation through the activation of the MAPK/ERK pathway in several different studies [11,12,15].

While NNK has been shown to mutate or activate some well known oncogenes such as K-ras, p53 or the EGFR, its involvement with a specific oncogene known as the type I insulin-like growth factor receptor (IGF-IR) has not been studied thus far. The IGF-IR is a tyrosine kinase receptor that belongs to a family of peptides which consists of two ligands (IGF-I and IGF-II), three receptors (IGF-I receptor, IGF-II receptor, and the insulin receptor) and six binding proteins (IGFBP1-6). Ligand activation of the IGF-IR has been found to be implicated in carcinogenesis. This receptor is often overexpressed in many common cancers, such as breast, colon and lung cancer. For example, in lung cancer the IGF-IR has been found to be overexpressed in most NSCLC and SCLC cell lines, and in almost all lung tumor tissue extracts [16–19]. Overexpression and activation of the IGF-IR signaling pathways has been found to be associated with many aspects of the malignant phenotype such as mitogenesis, survival, transformation and metastasis [20–22]. The IGF-IR

* Corresponding author. Tel.: +1 519 824 4120x54950.
E-mail address: rmoorehe@uoguelph.ca (R.A. Moorehead).

0169-5002/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.lungcan.2010.03.012

has been found to promote these malignant processes by activating some of the same pathways that NNK activates such as the PI3K pathway and the MAPK/ERK pathway [21]. In addition, characterization of two doxycycline-inducible transgenic mouse models generated in our lab revealed that overexpression of full length human IGF-IR cDNA in either lung type II alveolar cells (SPC-IGFIR) or Clara cells (CCSP-IGFIR) resulted in lung tumor development [23].

Considering that lung cancer is largely caused by tobacco smoke carcinogens such as NNK, and that the majority of lung cancer tumor tissue extracts express high levels of IGF-IR, our lab was interested in investigating the association between these two molecules. While we did not find that IGF-IR overexpression was capable of significantly exacerbating the NNK-induced tumorigenesis, we did discover that a considerable amount of endogenous murine IGF-IR was upregulated in the tumor tissue of wild type mice that were injected with NNK. These results suggest that NNK is capable of upregulating endogenous IGF-IR and altered IGF-IR expression may promote NNK-mediated lung tumorigenesis.

2. Materials and methods

2.1. Mice

Transgenic mice containing either the surfactant protein C (SPC)-reverse tetracycline transactivator (rtTA) or the Clara cell secretory protein (CCSP)-rtTA were mated with mice containing the tetracycline response element (TRE)-IGF-IR transgene as previously described [23]. This mating strategy generated double transgenic mice referred to as SPC-IGFIR and CCSP-IGFIR. Both the CCSP-IGFIR and SPC-IGFIR transgenic lines were injected i.p. with NNK (Toronto Research Chemicals Inc., North York, ON, Canada) at a concentration of 10 μmol (2 mg dissolved in 0.2 mL of 0.9% sterile saline) per mouse one time per week for a period of 6 weeks. The first injection was given at 21 days of age. The NNK concentration and schedule was based on a study performed by Abdel-Aziz et al. [24]. SPC-IGFIR and CCSP-IGFIR transgenic mice which did not receive NNK injections were also evaluated. All SPC-IGFIR and CCSP-IGFIR mice were fed rodent chow containing 2 g/kg of doxycycline (Bio-Serv, Frenchtown, NJ, USA) ad libitum starting at 21 days of age in order to induce human IGF-IR transgene expression. Wild type or single transgenic mice (which do not overexpress the IGF-IR transgene) were also used as controls, with some mice receiving NNK injections (2 mg NNK/0.2 mL 0.9% saline) and others receiving 0.2 mL of 0.9% sterile saline given at the same frequency and duration as the SPC-IGFIR and CCSP-IGFIR transgenic mice. Mice in all treatment and control groups were sacrificed and the lung tissue was collected at 5 months after the commencement of doxycycline or NNK administration (i.e. at 6 months of age). Mice were maintained at the Central Animal Facility, University of Guelph, following the Canadian Council on Animal Care guidelines.

2.2. H&E and Immunohistochemistry

H&E and immunohistochemistry was performed using previously described methods [23,25]. Primary antibodies were used at the following dilutions, anti-Akt1 1:100, anti-Akt2 1:50, anti-phospho-Erk1/Erk2 (Thr202/Tyr204) 1:25, anti-CREB 1:100, anti-human and mouse IGF-IR 1:100 (Cell Signaling Technology, Beverly, MA), anti-human IGF-IR 1:100 (R&D Systems Inc., Minneapolis, MN), anti-TTF-1 1:500, anti-CCSP and anti-SPC 1:1000 (Abcam, Cambridge, MA). Quantitative analysis was performed by using Aperio ImageScope software (Aperio Technologies, Inc., Vista, CA, USA).

2.3. Western blotting

Western blotting and protein detection were performed as previously described [23,25]. Western blotting was carried out using isolated tumors when available, and lung tissue from doxycycline treated transgenic mice when the tumors were not large enough for isolation. Each lane of the western blot represents tissue from a different animal. All primary antibodies were used at a 1:1000 dilution, anti-human IGF-IR (R&D Systems Inc., Minneapolis, MN), anti-phospho-Akt (Ser473), anti-Akt1, anti-Akt2, anti-phospho-Erk1/Erk2 (Thr202/Tyr204), anti-phospho-CREB (Ser133), anti-human and mouse IGF-IR, and anti-β-actin (Cell Signaling Technology, Beverly, MA). Western blots were captured using a Fluorchem8800 gel documentation system and band intensities were quantified using AlphaEase software (Alpha Innotech, San Leandro, CA).

2.4. Statistics

The Student's *t*-test was used to compare two means and an ANOVA followed by a Tukey's HSD test was used to compare multiple means. Differences were considered to be significant at $p < 0.05$.

3. Results

3.1. NNK treatment increases lung tumor size but not tumor burden

Tumor burden was assessed by counting the number of internal tumors per lobe in two separate sections of paraffin fixed lung tissue for each mouse. For the SPC-IGFIR mice, the tumor burden was slightly larger for the NNK injected SPC-IGFIR mice ($n = 5$) compared to the SPC-IGFIR control mice ($n = 7$), however this difference was not statistically significant (Fig. 1A). The same pattern was found for the CCSP-IGFIR mice, where the NNK injected CCSP-IGFIR mice ($n = 5$) had a greater but non-significant tumor burden in comparison to the CCSP-IGFIR control mice ($n = 5$) (Fig. 1A). The tumor burden in all of the transgenic mice was greater than that of the NNK injected wild type or single transgenic mice. The tumor burden was also assessed by manually counting the surface tumors on each lung lobe of each mouse at the time of necropsy. The same pattern was found when evaluating the tumor burden using this method, however no surface tumors were visible in the NNK injected wild type or single transgenic mice ($n = 4$) (Fig. 1B).

While the overall tumor burden was not significantly greater in the NNK injected transgenic mice compared to the transgenic control mice, it was found that only the NNK injected transgenic mice developed large nodular tumors. The largest tumor observed in the SPC-IGFIR or CCSP-IGFIR transgenic mice (not treated with NNK) was 687 μm^2. Four of the five NNK injected SPC-IGFIR mice developed tumors greater than 687 μm^2, while one of the five NNK injected CCSP-IGFIR mice developed tumors greater than 687 μm^2 (Table 1).

Table 1
Tumor size in NNK injected and control SPC-IGFIR and CCSP-IGFIR mice.

Genotype	Number of mice with tumors > 687 μm^2
SPC-IGFIR + NNK	4/5
CCSP-IGFIR + NNK	1/5
SPC-IGFIR	0/7
CCSP-IGFIR	0/5
NNK	0/4