CHRNA3/CHRNA5 and Lung Tumorigenesis

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Abstract

CHRNA3/CHRNA5 and lung tumorigenesis

Past genome-wide association studies (GWAS) looked for genes contributing to lung cancer. Of the genes identified, two key genes, CHRNA3 and CHRNA5, both located on Chromosome 15q25, encoding for the alpha 3 (α3) and alpha 5 (α5) subunits of nicotinic acetylcholine receptors (nAChR). As smoking is a significant risk factors in lung cancer, these genes were examined closely and single nucleotide polymorphisms (SNPs) in each of these genes were linked to elevated lung cancer risk in GWAS studies, as well as playing a role in the quantity smoked, nicotine dependence, the intensity of smoking and internal dosage exposure to nitrosamines. However, these studies found conflicting results regarding whether the link between the CHRNA3, CHRNA5 genes and lung cancer was direct or if it was indirectly associated by differences in smoking behavior. To expand on these studies, this research will use mouse models (CHRNA5 wild type, heterozygous, and knockout; CHRNA3 wild type and heterozygous, knockout is embryonic lethal) to determine the relationship of the CHRNA3 and CHRNA5 genes and the development of lung tumors (tumor multiplicity), tumor volume, and tumor burden following exposure to the carcinogenic metabolite of nicotine, 4-(methylnitrosamine)1-(3-pyridyl)-1-butanone (NNK). All mice will receive the same dosage of NNK via an Alzet micro-osmotic pump for seven days because we believed that CHRNA3 and CHRNA5 are the receptors for NNK. The removal of nicotine (epidemiology suggests that nicotine is not a carcinogen) and dose differences from the study will allow for us to look at cancer risk associated with the genes. There were no significant difference in the tumor multiplicities and tumor burdens between the genotypes for both CHRNA3 and CHRNA5. Individual tumor volumes were not

significantly different between CHRNA3 wild-type and heterozygous mice but there was a significant increase in individual tumor volumes in CHRNA5 knockout mice in comparison to their wild-type and heterozygous littermates. Because of the tumor multiplicities and burdens being nearly equivalent for all the genotypes of the mice, there is no evidence that the NNK uses α3 and α5 nAChRs as receptors and thus the link between the SNPs in the CHRNA3 and CHRNA5 genes are not directly related to lung cancer.

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List of Abbreviations

COPD – chronic obstructive pulmonary disease

GWAS – genome-wide association study

MMIHS – megacystis-microcolon-intestinal hypoperistalsis syndrome

nAChR – nicotinic acetylcholine receptor

NNK – 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

NNN – N'-nitrosonornicotine

NSCLC – non-small cell lung cancer

SCLC – small cell lung cancer

SNP – single nucleotide polymorphism

Introduction:

Smoking is known as the major causative agent for lung cancer and lung cancer is attributed to the highest number of cancer deaths per year in the United States in both men and women (Siegel et al., 2013). The 5 year survival rate for lung cancer is a dismal 17%. Lung cancer is normally classified as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The development of lung cancer can be caused by genetic changes. In 2008, genome-wide association studies (GWAS) looked for genes contributing to lung cancer (Amos et al., 2008; Hung et al., 2008; Thorgeirsson et al., 2008). The genes CHRNA3 and CHRNA5 were highlighted in each of these studies as well as GWAS studies for nicotine dependence and chronic obstructive pulmonary disease (COPD) (NL Saccone et al., 2009; JZ Liu et al., 2010; Pillai et al., 2009). The risk variant of the CHRNA5 allele at rs16969968 caused the single nucleotide polymorphism (SNP) in exon 5 that leads to a D398N mutation in the second intracellular loop (Saccone et al, 2010). A study by Le Marchand and colleagues found that smokers that carried the SNP are actually exposed to higher dosages of carcinogens in tobacco smoke than those without the SNP (Le Marchand et al., 2008). The SNP may be inherited or be caused by DNA adducts from carcinogens (Improgo et al., 2010).

Both genes are located on Chromosome 15q25 and encode for the alpha 3 and alpha 5 subunits of nicotinic acetylcholine receptors (nAChR) and are also clustered with the CHRNB4 gene that encodes for the β4 subunit. This close cluster of genes is in high linkage disequilibrium (LD) and the tightness of this cluster can be observed in Figure 1 (taken from Improgo et al, 2010). This figure also shows that CHRNA5 is transcribed in

the opposite direction of CHRNA3 and CHRNB4. The subunits these genes encode make up heteromeric ligand-gated ion channels. In mice, these genes are encoded for on Chromosome 9 (Eng et al., 1991).

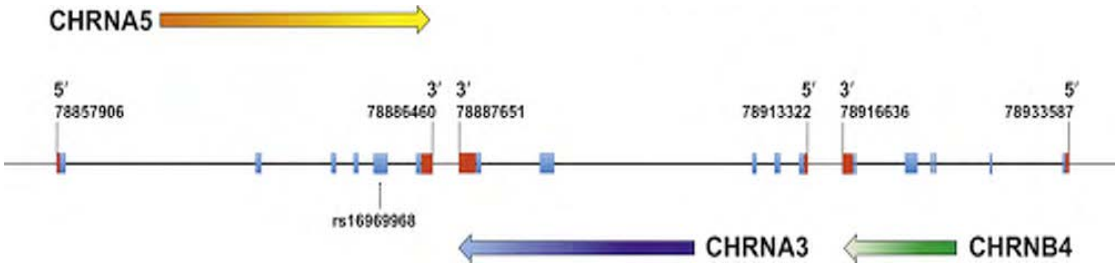

Figure 1. The CHRNA5/CHRNA3/CHRNB4 cluster on Chromosome 15q25, the directions in which the genes are transcribed and the risk allele rs16969968 in CHRNA5 is indicated.

The main addictive agent in tobacco products, nicotine, has been found by epidemiological studies to not be carcinogenic (Hecht, 2003). It has been suggested through studies that nicotine may influence cancer through cell proliferation, tumor invasion, angiogenesis and the inhibition of apoptosis (Wei et al., 2011). The nicotine metabolites, NNK and N'-nitrosonornicotine (NNN) are known carcinogens that are prevalent in cigarette smoke (Hecht and Hoffman, 1988). These carcinogens are suspected to cause the formation of DNA adducts, leading to the mutations that cause cancer (Weitberg and Corvese, 1993). NNK and NNN have been associated with nAChRs and pathways promoting tumorigenesis (Nishioka et al., 2010). The homomeric α7 nAChR is known to bind NNK with the greatest affinity and NNN binds epibatidine-sensitive nAChRs with high affinity (Catassi et al., 2008).

The α3 subunit encoded by the CHRNA3 gene is expressed in the predominantly neuronal tissues of the autonomic ganglia and in the brain. The knockout of CHRNA3 causes mice that, if they survive to birth, have bladder enlargement and infections, seeping urination, urinary stones and pupils that are dilated and do not contract as a reaction to light (Xu et al., 1999). Although there were no noteworthy brain or tissue defects aside from the bladder, none of the mice that were born survived past 8 weeks and are not viable for experimentation. In humans, there is a rare genetic disorder known as megacystis-microcolon-intestinal hypoperistalsis syndrome (MMIHS) that is similar to the phenotypes observed in the CHRNA3 knockout mice. These patients do not express α3 mRNA (Richardson et al., 2001).

CHRNA5 encodes the α5 subunit is primarily expressed in the peripheral nervous system as well as the brain and the gastrointestinal tract (Improgo et al, 2010). Knockout of this gene leads to viable mice. The risk allele rs16969968 has been of great interest since the GWA studies were published. In humans, nicotine dependence risk for heterozygotes is increased 1.3-fold whereas homozygotes for the risk allele have a 2-fold increased risk (Stevens et al., 2008).

As smoking is a significant risk factors in lung cancer, these genes were examined closely and single nucleotide polymorphisms in each of these genes were linked to elevated lung cancer risk in GWA studies, as well as playing a role in the quantity smoked, nicotine dependence, the intensity of smoking and internal dosage exposure to nitrosamines. These studies found conflicting results regarding whether the link between the CHRNA3, CHRNA5 genes and lung cancer was direct or if it was indirectly associated by differences in smoking behavior. To expand on these studies, this research will use mouse models (CHRNA5 wild type, heterozygous, and knockout; CHRNA3 wild type and heterozygous, knockout is embryonic lethal) to determine the relationship of the CHRNA3 and CHRNA5 genes and lung cancer based on the development of lung tumors (tumor multiplicity), tumor volume, and tumor burden. All mice will receive the same dosage of the nicotine metabolite and potent carcinogen NNK via an Alzet micro-osmotic pump for seven days. The hypothesis is that CHRNA3 and CHRNA5 are the receptors for NNK. Because epidemiology studies suggest nicotine is not a carcinogen, the removal of nicotine and dose differences from the study will allow for us to look at direct cancer risk associated with the genes.

Materials & Methods

All experiments were done under an approved NCI animal study protocol where mice were housed in Plexiglas cages and given autoclaved NIH 31 diet and water ad libidum. CHRNA mice from Jackson Laboratories (Bar Harbor, ME, USA). Lung tumor susceptibility was introduced into CHRNA3 +/- and CHRNA 5 +/- mice through mating with A/J mice and selection of mice harboring two copies of the A/J K-ras sensitive allele, which has been shown to be the major determinant of lung tumor susceptibility in this strain. For this study, breeder pairs of +/- mice from each strain were used to generate mice for treatment. Mice were genotyped by PCR on tail DNA. Mice were backcrossed for eight generations. As a mixed genetic background was used, littermates consisting of +/+, +/- and -/- mice for each strain were used. The numbers of each genotype used are indicated in Table 1. The carcinogen NNK was used to induce lung tumorigenesis and NNK treatment was begun in the mice at six weeks of age. The NNK was obtained from Toronto Research Chemicals (Toronto, Ontario, Canada).

Table 1. Number of mice of each genotype used in the experiment.

Genotype	Number of Mice
CHRNA3 +/+	11
CHRNA3 +/-	14
CHRNA3 -/-	0 – embryonic lethal
CHRNA5 +/+	8
CHRNA5 +/-	14
CHRNA5 -/-	8

NNK was prepared at a concentration of 60 mg/ml in 50% dimethyl sulfoxide (DMSO), with a total dose of 6mg, approximating 300 mg/kg for a 20 g mouse. Previous studies from this lab have included 100 mg/kg doses for once a week for three consecutive weeks. The NNK preparation was prepared in a sterile 1.5 ml tube. Each Alzet micro-osmotic pump, model 1007D (Alzet Osmotic Pumps, Cupertino, CA, USA), was 15mm long and 6mm in diameter, holding a total of 0.4 g, a total displacement of 0.5 ml and delivered 0.5 ul/hr for 7 days. The pump was used in this study as to not flood the receptors of interest with carcinogen all at once, but rather over a period of time.

Sterile 1.5 ml tubes were weighed empty, masses recorded then reweighed with the pump and flow moderator. Alzet pumps and flow moderators were removed from their packaging in a sterile laminar flow hood and using sterile forceps. The filling tube included in the packaging was attached to a 1 ml syringe, NNK was drawn up, air bubbles removed, the syringe held upright and the plunger used to push air out of the syringe until liquid was at the tip of the filling tube. With the Alzet pump being held upright, the filling tube was inserted directly into the pump so it reached the bottom and 100 ul of NNK solution dispensed into the reservoir. The filling tube would then be removed, excess solution wiped off and the flow moderator inserted until the cap was flush with the top of the pump. Overflow was wiped off and the pump placed back into the 1.5 ml tube. The tube was reweighed to determine fill volume and the weight of the volume should be at least 900 mg with no bubbles present. A volume of 1ml of sterile normal saline (0.9% NaCl) was added to each tube and incubated overnight at 37°C.

Fifteen minutes prior to surgery, the rumps of mice were shaved and were injected with 100 ul of 0.25% Marcaine. Mice were anesthetized using 3% isofluorane and maintained on a nose cone throughout surgery. Skin was disinfected using betadine soaked cotton tipped applicator and followed by 70% ethanol. After air drying, a 1 cm horizontal incision was made and a subcutaneous pocket created by gentle opening of a sterile hemostat. The pump was inserted flow moderator side first and the wound was closed with staples/wound clips. Mice were monitored until ambulatory. Pumps were removed one week later via an incision at the end of the pump furthest from the initial incision and closed using wound clips (Figure 2). The pumps were removed with sterile forceps using the same analgesia, anesthesia, surgical preparation and technique. Wound clips were removed one week after surgery.

Figure 2. Mouse with Alzet pump prior to anesthesia for pump removal surgery.

Sixteen weeks after NNK treatment, mice were sacrificed via cervical dislocation. Lungs were harvested and inflated with 10% neutral formalin. Lung lobes were separated and visible surface lung tumors were counted and measured using a dissecting microscope. Only surface lung tumors exist at age of sacrifice. Prism software (GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis. For analysis, an unpaired t-test was used.

Results:

The role of the CHRNA3 and CHRNA5 genes on lung tumorigenesis was investigated through the use of carcinogen-driven and genetic mouse models. The C57BL/6 mice are tumor resistant and thus were crossed with A/J mice to confer lung tumor susceptibility (Belinsky et al., 1989). Number of tumors per mouse, tumor burden and individual lung tumor volumes were examined in each CHRNA3 and CHRNA5 mice genotypes. Macrophages were present in the lungs of all genotypes of mice used in this study. There were no trends observed that would differentiate the genotypes utilized.

The CHRNA3 +/- mice did not develop more or fewer tumors than their CHRNA3 +/+ littermates with the heterozygous mice with 20.21 ± 2.28 tumors and wild-type mice with 17.18 ± 3.57 tumors (Figure 3A). The tumor burdens of the mice of the two genotypes were also relatively the same with wild-type having a burden 5.45 ± 1.47 mm^3 and heterozygous with 6.06 ± 0.98 mm^3 (Figure 3B). The individual tumors observed in the mice were of approximately the same sizes with the average wild-type mouse tumor being 0.3169 ± 0.0337 mm^3 and the average heterozygous mouse tumor of 0.3106 ± 0.0217 mm^3 (Figure 3C). Although the CHRNA3 +/- mice did have slightly more tumors, a marginal increase in tumor burden and a minor rise in individual tumor sizes, none of these categories were statistically significant.

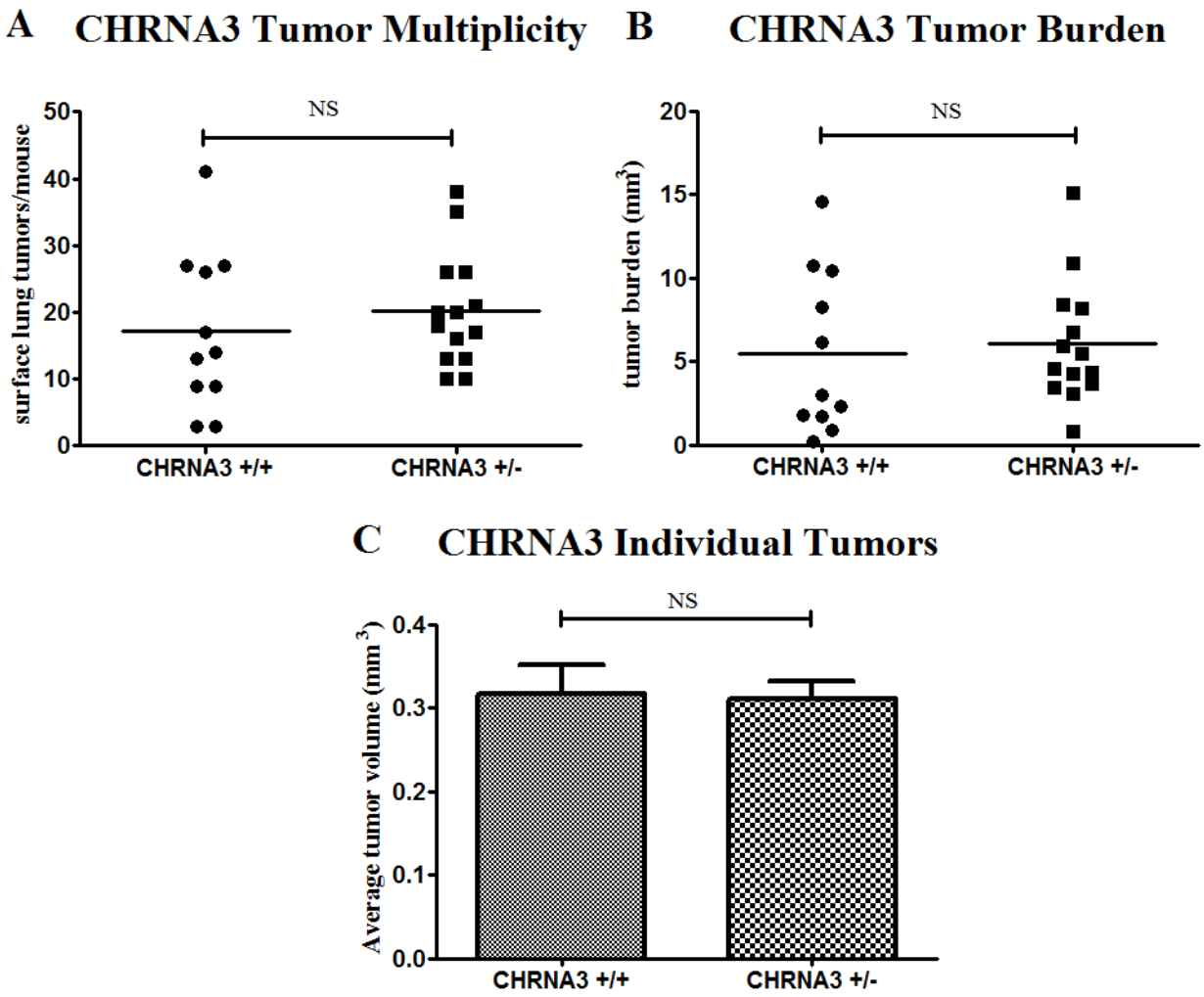

Figure 3. CHRNA3 Tumor Data. A. Tumor multiplicity was similar between the CHRNA3 wild-type and heterozygous mice despite genotype differences. B. Tumor Burden was nearly equivalent for both the wild-type and heterozygous CHRNA3 mice. C. Individual Tumor Volumes were comparable between the CHRNA3 wild-type and heterozygous genotypes.

The CHRNA5 wild-type mice had more tumors at 20.75 ± 2.76 tumors than their heterozygous littermates, who had more tumors at 18.36 ± 2.86 tumors than their knockout littermates with 14.88 ± 2.02 tumors (Figure 4A). However, there was no statistical significance to the minute differences in tumor multiplicity amongst the genotypes. The wild-type and heterozygous CHRNA5 mice had nearly equivalent tumor burdens with wild-type at 4.993 ± 1.45 mm^3 and heterozygous with 4.784 ± 0.87 mm^3 (Figure 4B). The CHRNA5 knockout mice have a slightly increased tumor burden at 6.102 ± 1.49 mm^3, but this was not statistically significant. Individual tumor volumes were comparable in the CHRNA5 wild-type and heterozygous mice and this was not statistically significant. The individual surface lung tumor volumes of the CHRNA5 knockout mice was 0.4102 ± 0.049 mm^3 were significantly larger than those of their wild-type littermates at 0.2406 ± 0.028 mm^3 and the heterozygous littermates at 0.2606 ± 0.026 mm^3 (Figure 5). The CHRNA5 knockout mice had lower tumor multiplicity and higher tumor burden which can be explained by the larger individual tumor volumes present on their lungs. However, if one compares individual tumor sizes between the CHRNA3 wild-type mice and the CHRNA5 knockout mice, the difference is not statistically significant.

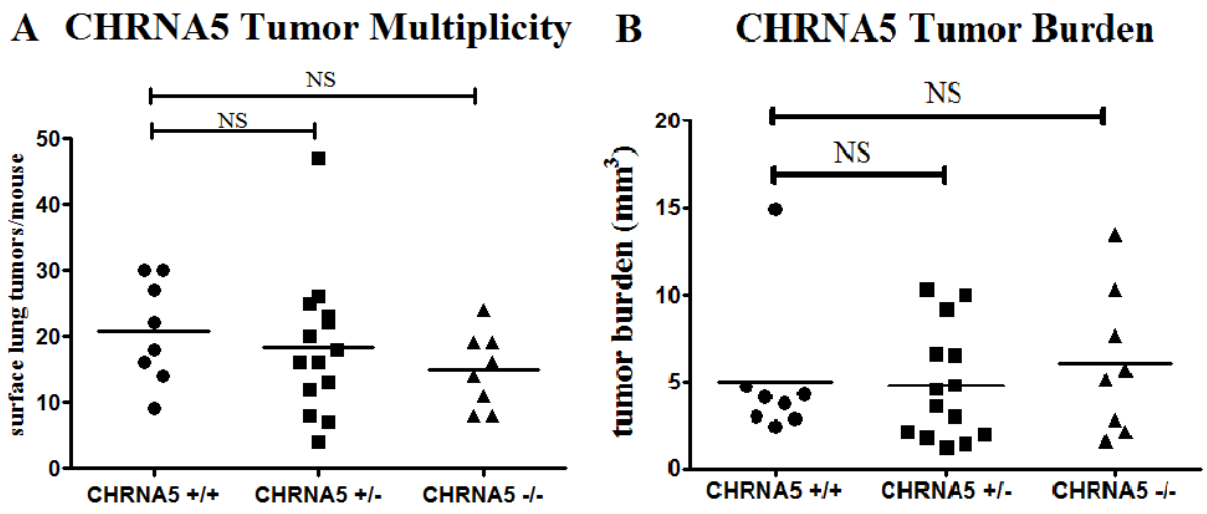

Figure 4. CHRNA 5 tumor data. A. Tumor multiplicity in the CHRNA5 genotypes. Although the knockout mice have fewer tumors than the wild-type mice, difference between the two groups is not significant. B. Tumor Burden in the CHRNA5 mice. Although the CHRNA5 knockout mice have a greater tumor burden than their wild-type and heterozygous littermates, this difference are not significant.

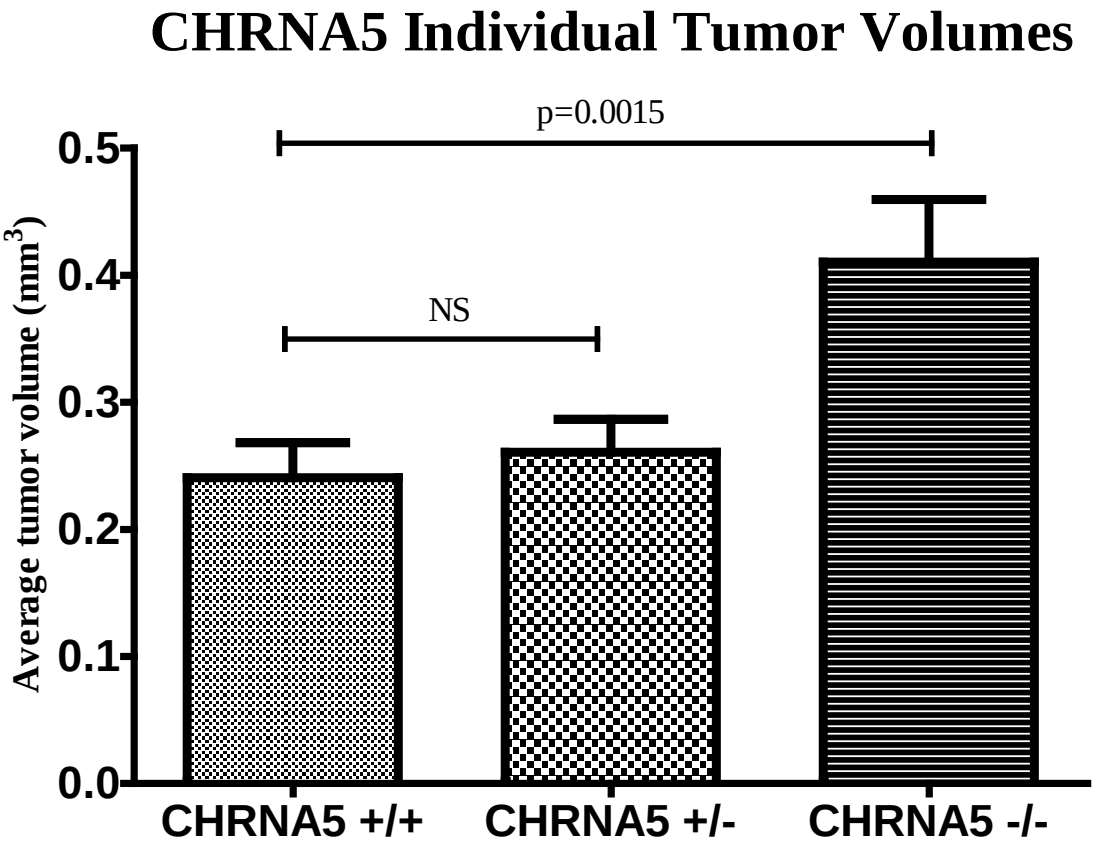

Figure 5. CHRNA5 Individual Tumor Volumes. The CHRNA5 wild-type and heterozygous mice had similar volumes of individual tumors whereas their knockout littermates had individual tumors that were nearly two-fold larger.

Discussion:

The CHRNA3 mice did not have any significant differences in tumor multiplicity, burden or individual tumor volumes (Figure 3). This suggests that α3 subunit containing nAChRs are not involved in NNK binding. If these nAChRs are not involved in the binding of NNK, their role in lung cancer is not direct. The possibility of the SNP in this gene being important through another avenue may still exist, however there is no direct relationship between CHRNA3 and lung cancer. Since the comparison is between the CHRNA3 wild-type and CHRNA3 heterozygous mice, it is possible that the CHRNA3 heterozygous mice actually have CHRNA3 protein at a level similar to that in CHRNA3 wild-type mice, and thus there is no difference in the tumor data

There were no significant differences between the CHRNA5 mouse genotypes concerning tumor multiplicity and tumor burden, although the CHRNA5 knockout mice had slightly fewer tumors and slightly higher tumor burden than their wild-type and heterozygous littermates (Figure 4). The CHRNA5 knockout mice however have significantly larger individual tumor volumes than the wild-types and heterozygotes (Figure 5). Although the α5 containing nAChRs are not likely to be the receptor of NNK that is directly involved in lung cancer development, the potential role for this receptor to be involved in tumor cell proliferation is not as likely when you consider the similarities between the CHRNA3 wild-type mice and CHRNA5 knockout mice individual tumor sizes and the linkage disequilibrium between the genes. Since CHRNA3 knockout genotype is embryo lethal and CHRNA5 knockout mice are viable, CHRNA3 and CHRNA5 have different biological functions or at least the function of CHRNA5 may be

substituted by another protein. Therefore, faster tumor growth rate in CHRNA5 knockout mice suggests a CHRNA5 effect, either directly or indirectly, on cell proliferation. Knockout mouse embryo fibroblast (MEF) cell experiments could be used in determining whether or not this gene is involved in cell proliferation and tumor size.

The removal of smoking quantity in this experiment caused for the mice to get equivalent dosages of carcinogen. The similar tumor multiplicities and burdens observed in the different genotypes of mice leads us to the conclusion that the CHRNA3 and CHRNA5 genes are not directly linked to lung cancer development following exposure to NNK. In a 2011 study by Chen et al, it was the CHRNA3 gene and risk allele rs1051730 that was found to be associated with larger tumor size at diagnosis and it did not appear that they looked at CHRNA5 and risk allele rs16969968. It is CHRNA7, not CHRNA5 or CHRNA3, that has been most associated with cancer, including links to tumor growth and survival, cell survival and resistance to apoptosis, tumor angiogenesis, epithelial to mesenchymal transition and tumor metastasis (Thunnissen, 2009; Singh, Pillai & Chellappan, 2011). Also, the extremely high affinity for NNK over nicotine makes CHRNA7 a very viable target and suspect for lung cancer susceptibility.

There is a likelihood that CHRNA5 and CHRNA3 variants are associated with nicotine-related habits but not the diseases caused by the habits. The risk alleles rs16969968 and rs1051730 were found to play a role in nicotine dependence (X Chen et al., 2009). The CHRNA3 risk allele rs1051730 was found in regular and heavy smokers with greater commonality than in non-smokers and was believed to be directly related to smoking in adulthood (Ducci et al., 2011). The CHRNA5 SNP was also looked at with

respects to nicotine addiction strength, lung cancer and COPD. Nicotine addiction strength was found to be related to carriers of the risk allele but they did not find an increased risk of lung cancer or COPD (Wojas-Krawczyk et al., 2012). CHRNA5 knockout mice have previously been shown to have a decreased sensitivity to effects of nicotine, needing a greater dosage to produce the same effects as in their wild-type littermates (Salas et al., 2003; KJ Jackson et al., 2010; CD Fowler et al., 2011). The addictive effects of the CHRNA5/CHRNA3/CHRNB4 cluster for nicotine is possibly mediated through the brain as suggested by studies that show increased functional activity of α3β4-nAChRs in response to nicotine (Gallego et al., 2012). A more recent study suggests that the α5 subunit may mediate smoking behavior as carriers of one or two copies of the risk allele rs16969968 had increased puff volume and the amount of smoke inhaled is a likely intermediary between the genetic variant and lung cancer (MacQueen et al., 2013). The role of CHRNA5 and CHRNA3 directly on lung cancer does not exist. These genes do likely play a role in nicotine addiction and dependence. Existence of a mediating factor of these genes on cell proliferation and tumor size may still exist.

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