C. elegans and mutants with chronic nicotine exposure as a novel model of cancer phenotype

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ABSTRACT
We previously investigated MET and its oncogenic mutants relevant to lung cancer in C. elegans. The inactive orthologues of the receptor tyrosine kinase Eph and MET, namely vab-1 and RB2088 respectively, the temperature sensitive constitutively active form of KRAS, SD551 (let-60; G3889) and the inactive c-CBL equivalent mutants in sli-1 (PS2728, PS1258, and MT13032) when subjected to chronic exposure of nicotine resulted in a significant loss in egg-laying capacity and fertility. While the vab-1 mutant revealed increased circular motion in response to nicotine, the other mutant strains failed to show any effect. Overall locomotion speed increased with increasing nicotine concentration in all tested mutant strains except in the vab-1 mutants. Moreover, chronic nicotine exposure, in general, upregulated kinases and phosphatases. Taken together, these studies provide evidence in support of C. elegans as initial in vivo model to study nicotine and its effects on oncogenic mutations identified in humans.

Introduction
Lung cancer is a devastating disease that afflicts more than 200,000 people per year in United States alone and the associated number of deaths is more than any other type of cancer. Cigarette smoking is the major underlying etiological factor. Although cigarette smoke contains more than 60 carcinogens, nicotine, a major component of cigarette smoke, is a carcinogen, and the principal addictive agent. The nicotine-derived nitrosamines are nevertheless carcinogenic. Moreover, nicotine is known to promote cancer cell proliferation, angiogenesis and epithelial-mesenchymal transition thereby aiding tumor growth and metastasis. The conduits for nicotine mediated signaling are the ubiquitously expressed acetylcholine receptors (nACHR) whose downstream signaling targets are also common to pathways mediated by receptor tyrosine kinases (RTK).

The simple soil nematode, C. elegans, is gaining acceptance in vivo model for the rapid screening MET gain-of-function mutations in a variety of cancers. In this regard, we and others have shown that the RTKs such as EGFR, MET, RON and Eph are not only overexpressed but frequently undergo gain-of-function mutations in a variety of cancers. Our continued effort to identify potential lung cancer therapeutic targets led us to the discovery of increased occurrence of gain-of-function mutations in RTKs such as MET, EphB4, key intracellular signaling molecules such as RAS GTPase, the adapter focal adhesion molecule Paxillin and the PAX transcription factors 5 and 8. Their downstream target such as RAS is also known to undergo oncogenic mutation. On the other hand, mutations in c-CBL, the E3 ubiquitin ligase that negatively regulates RTKs, characterized in lung cancer, turned out to promote cell growth and proliferation and enhance cell motility. The c-CBL wild type (wt), however, suppressed the above effects. Moreover, we have previously demonstrated that C. elegans can be used as an in vivo model for the rapid screening MET gain-of-function
mutants discovered in human lung cancer. Transgenic worms that expressed either MET R988C or MET T1010I suffered from significantly low fecundity and abnormal vulval development characterized by hyperplasia compared to those worms that expressed wt MET. Interestingly, the above effects were exacerbated by nicotine treatment.36

Our search of the C. elegans data base revealed the existence of strains that harbored non-functional mutants of MET (RB2088), Eph (vab-1(e2)III), RAS (SD551 derived from let-60) and c-CBL (PS2728, PS1258, MT13032 derived from sli-1). Since the RTKs MET and Eph, and the RAS GTPase are known to be over expressed in several cancers and support cell proliferation and growth, we hypothesized that non-functional mutants of these molecules will adversely affect the survival and related functions in the worms. In comparison, the wt c-CBL negatively regulates RTKs, and worms harboring non-functional sli-1 are therefore expected to have abnormal growth and motility. Based on our previous work, we also postulated that chronic exposure to nicotine would further aggravate the various functional behaviors in the above mutant strains.36

The current studies are aimed at understanding the effect of chronic exposure to nicotine on the phenotype, survival, fertility, egg-laying capacity, locomotion and gene expression profiling on select C. elegans mutants relevant to cancer. The mutant worms used were vab-1 (inactive, Eph), RB2088 (inactive, MET), SD551 (temperature sensitive strain expressing constitutively active form of KRAS), and 3 sli-1 mutants PS2728, PS1258, and MT13032 (inactive, c-CBL). Here we report the changes in the intrinsic behavioral and functional aspects of the above mutants compared to wt N2 control animals. Also, we carried out gene expression profiling studies. Finally, we also present here the effects of chronic nicotine treatment on the above mutants that support our contention that C. elegans is a suitable in vivo model to screen functionality of cancer mutations.

Methods

Nematode strains and culturing

The Bristol N2 strain was used as a wt standard in all the experiments. Non-functional mutant strains used in this study were vab-1 (inactive, Eph), RB2088 (inactive, MET), SD551 (temperature sensitive strain expressing constitutively active form of KRAS), and 3 sli-1 mutants PS2728, PS1258, and MT13032 (inactive, c-CBL). Here we report the changes in the intrinsic behavioral and functional aspects of the above mutants compared to wt N2 control animals. Also, we carried out gene expression profiling studies. Finally, we also present here the effects of chronic nicotine treatment on the above mutants that support our contention that C. elegans is a suitable in vivo model to screen functionality of cancer mutations.

Table 1. Various strains of C. elegans used in the study explaining their genotypes and phenotypes.

| Strain ID | C.elegans Ortholog | H.Sapiens Ortholog | Genotype | Variation | Source | Phenotype | Notes |
|-----------|---------------------|--------------------|----------|-----------|--------|-----------|-------|
| N2        | —                   | —                  | —        | —         | CGC    | WT Bristol Variant | Exons 6–11 missing (most of kinase domain) |<.70% penetrance | |
| SD551     | let-60              | Ras family         | let-60(ga89) IV | unknown   | CGC    | varied, primarily Muv | SynMuv | |
| CB2       | vab-1               | Eph family         | vab-1(e2) II  | 2735G>A    | CGC    | notched head         | SynMuv | |
| RB2088    | F11E6.8             | Met family         | F11E6.8(ok2754) IV | 906-bp deletion | CGC    | SynMuv | |
| MT13032   | sli-1               | Cbl family         | sli-1(n5358) X | 914C>T (S305L) | CGC    | SynMuv | |
| PS2728    | sli-1               | Cbl family         | sli-1(y143) X | 454C>T (Q152*) | CGC    | SynMuv | |

Reagents

Nicotine (cat. #N0267), 5-Fluoro-2’-deoxyuridine, (FUDR) (cat. # F0503), Ampicillin (cat.# A1593) were obtained from Sigma. Trizol was obtained from Life Technologies (cat. #13596–06).

Kaplan-meyer survival assays on NGM agar

NGM agar plates were prepared with Amp/FUDR to which nicotine was added at various concentrations (control, 50 μM and 500 μM) according to the protocol (http://www.jove.com/index/details.stp?ID=1152). For each group, 2 plates were used and on each plate 20 – 25 age synchronized L4 worms were transferred and the worms were grown at 20°C. Every two days the worms were transferred to a new NGM agar plate. The worms were counted every 12 h for 2 weeks. Survival curves were plotted using the survival package for the R Statistical Software.38-40

Egg-laying assay

Worms were grown from synchronized egg populations on nicotine and control plates to early L4 stage and 4 worms were then transferred to a single well in a 24-well tissue culture plate with identical NGM agar composition.41,42 Newly laid eggs were counted after 6.0 h from all the wells. The rate of egg-laying was calculated as eggs/worm. For each strain the assay was repeated 3 to 4 times.

Fertility assay

Eggs were isolated from gravid adult hermaphrodite worms by alkaline hypochlorite treatment and then grown on the NGM agar plates with and without nicotine till early L4 stage. Single L4-stage worms grown at 20°C were transferred to fresh plates every day until they stopped laying eggs. All progeny plates were incubated at 20°C for 2 days, and the number of progeny developed was counted for every plate.42 The assay was repeated for each strain at least 3 to 4 times.

Liquid culturing of C. elegans for phenotypic study

The worms were grown in 24-well tissue culture plate according to the method of Fitzgerald.43 In brief the liquid culture was prepared with sterilized S basal buffer (5.85g NaCl, 1g K2HP04, 6 g KH2PO4 and water to 1 L) supplemented with OP50 E. Coli bacteria, cholesterol, FUDR (25μM) to suppress reproduction along with nicotine. Synchronized L4 worms were obtained from the Caenorhabditis Genetic Center at the University of Minnesota.
were added at a density of 12 ± 6 worms per well. Liquid culture was changed daily by carefully aspirating the liquid. The plates containing worms were kept on orbital shaker at room temperature. The experiment was carried out for 10 d. The worms were monitored daily and photographed for any phenotypic changes using inverted microscope (Olympus IX71, Center valley, PA, USA).

**Locomotion analysis**

Locomotion behavior was analyzed using an automated worm tracking system as previously described. In brief, eggs were isolated from adult worms by alkaline hypochlorite treatment and grown on NGM agar plates with and without nicotine to obtain synchronized L4 population. For analysis, 20 L4 worms were transferred to 35 mm NGM agar bacteria free plate with an identical agar composition to the original culture plate. The experimental worms were then transferred to fresh plates one hour before the analysis so as to get them acclimatized. For each condition, we used 5 such plates. The temperature was maintained at 20–21°C with relative humidity of 30–40%. Behavior was recorded for 10 minutes at 2 frames per second using a CCD camera (Prosilica GC2450, Allied Vision Technologies, Stadtroda, Germany). Movies were analyzed using a modified version of existing MATLAB (MathWorks, Natick, MA) scripts. After background subtraction, individual animals were detected based on a pixel intensity threshold and particle size and their centroid coordinates determined for each frame. Speed for each worm was computed as mean centroid displacement (mm) per second excluding regions where the animal made sharp-angle turns.

**Gene expression analysis**

Eggs were isolated using hypochlorite treatment and grown on control and nicotine containing plates till early L4 stage. A total 5, 10 cm NGM agar plates, with and without nicotine, were used for each group. Once the worms became adults, total RNA was extracted from all the strains using Trizol. RNA extraction was done from 3 independent experiments. Global gene expression analysis was determined by microarray analysis of the extracted RNA as described below.

Gene expression microarray experiments were conducted with biological replication in all samples. Sample processing order was randomized. RNA quality was assessed by Bioanalyzer (minimum RIN = 7.5). cRNA was produced using the Agilent Low-Input Linear amplification and labeling kit. Array hybridizations (Agilent C. elegans (V2) Gene Expression Microarray, 4x44K) were performed at the University of Texas at Austin.

Figure 1. Effect of Nicotine on survival of *C. elegans*. Survival plots of *C. elegans* when exposed to different concentrations of nicotine are shown. The experiment was repeated twice and the results were comparable. The data was subjected to Kaplan Meier survival analysis using Graphpad Prism Software 4.02V.
of Chicago and Argonne National Labs high throughput genome analysis core facility, according the manufacturer’s instructions. The Agilent FE software was used to extract feature intensities and to flag saturated, non-uniform and outlier features. Probe intensity was adjusted by subtracting background intensity using the minimum method and quantile normalized between arrays. Outlier arrays were eliminated based on total number of flagged probes, intra-array variance, inter-array variance, biological replicate variance and spike-in linearity. Multiprobe probe sets were hierarchically clustered. Using one minus the Pearson correlation coefficients as a distance matrix, clusters were divided into groups by cutting clusters at a dendrogram height of 0.5 (roughly producing clusters with internal correlation coefficients 0.5). All downstream analyses were performed independently on each resulting cluster and all single probe probesets. For each probe set, surrogate variable analysis (SVA) was performed on the matrix of expression measurements, after controlling for the effects of genotype and nicotine exposure. For each probe set, we then constructed a linear fixed effects model $y = m + (\text{genotype} \times \text{nicotine}) + \epsilon$, where $y$ is the log2 transformed probe intensity, $m$ is the expected probe intensity, genotype is a factor representing the effect of genotype, nicotine is a factor representing the effect of nicotine and probe the effect of the oligonucleotide probe and $\epsilon$ is the residual error. The $\times$ represents the genotype and nicotine effects are fully crossed, to identify interactions between genotype and nicotine. The

Figure 2. Effect of Nicotine on egg-laying capacity and fertility of *C. elegans*. a. Egg-laying responses to chronic exposure of nicotine were assayed on NGM agar. Each condition had 24 worms and the assay was repeated 4 times. Rate at which eggs were laid was calculated as eggs/worm and percent relative egg-laying compared to untreated N2 worms is shown. b. To quantify the number of progeny produced by hermaphrodites, synchronized L4 worms were collected and at least 4 worms were allowed to lay eggs on individual plates. Animals were examined until no progeny were produced within a 24-h period and percent relative fertility compared to untreated N2 worms is shown.
significance of covariate effects was assessed by estimating false discovery rates, using Storey’s q-value method.\textsuperscript{48} Differentially expressed genes, defined by FDR cutoff (see above) were entered into DAVID (the database for annotation, visualization and integrated discovery) bioinformatics resources.\textsuperscript{49,50} All genes on the array were used as a background.

**Statistical analysis**

Cox proportional hazards model was used to assess the effect of nicotine and strains on survival. For egg laying and fertility assays, we estimated the effect of nicotine and variant using mixed effects linear model with treatment and variant type and their interaction terms as covariates. A random effect for batch

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**Figure 3.** Phenotypic changes in *C. elegans* after chronic treatment with nicotine. Phenotypes of SD551 mutant. 10X and 20X magnification images of SD551 a. control showing normal vulva phenotype and b. with nicotine showing multiple vulva phenotype. Phenotypes of PS2728 and MT13032 (Sli-1) mutants. 10X and 20X magnification images of PS2728 c. control showing normal body size and d. with nicotine showing shrinkage in the body size (MT13032 not shown).
was used to account for the fact that some batches tend to be more productive than others.

Locomotion data was analyzed using speed as outcome and treatment as a categorical covariate. Regular, log-transformed and robust regression approaches were used to assess sensitivity of results to outliers and distributional assumptions. Analysis of locomotion was done according to a 2 sample z test which is a standard hypothesis test comparing 2 means. The absolute value of the z score had to be greater than 1.96 to corresponds to the p = 0.05 significance level that was used. Otherwise, both means were considered to be the same.

Results

Chronic nicotine treatment decreases C. elegans survival

Survival assays were carried out over a period of 2 weeks on worms grown in NGM agar plates that were exposed to nicotine (50 \( \mu \)M and 500 \( \mu \)M). As shown in Fig. 1, chronic exposure to nicotine, especially at higher concentration, had significant impact on survival of all strains of worms except for the 2 \( sli-1 \) mutants (MT13032 and PS1258). The \( vab-1 \) (e2)II and SD551 strains suffered the maximum loss in survival compared to the wt worms and other mutant strains. Treatment with nicotine reduced the lifespan of worms for all strains (p = .0034). Survival also depends on strain, with N2 being the longest living group (p = .0004). We did not find significant difference in nicotine treatment effect on survival across different strains. Since nicotine is known to induce behavioral changes in worms, we next determined the effects of nicotine treatment on egg-laying capacity, fertility and locomotion.

Nicotine suppresses egg-laying capacity and fertility of C. elegans

The average number of eggs laid by L4 worms during a 6 hour period after 10 d of nicotine treatment is presented in Fig. 2A. Except for RB2088 and SD551, the egg-laying capacity of all other strains was somewhat lower as compared to N2 worms. Nicotine treatment had a suppressive effect on the egg-laying capacity of all strains, including that of control worms, reducing egg production by 9% at 50 \( \mu \)M nicotine and 27% at 500 \( \mu \)M nicotine. As shown in Fig. 2A, the rate of nicotine-induced decrease in egg-laying capacity was affected significantly more in the \( sli-1 \) mutant strain PS2728 (p = .0091) and suggestively more affected in the MET mutant equivalent strain RB2088 (p = .0643).

We next determined the total number of progeny generated in a worm’s life span for each of the strains. Fig. 2B shows percent relative fertility compared to the N2 control. Overall, the fertility of most of the untreated mutant strains was lower compared to N2 worms by about 20% except for SD551. With increasing concentrations of nicotine, the percentage fertility of each strain decreased. At the highest concentration of nicotine, differences between the untreated strains was completely abrogated. N2 and SD551 were the most affected strains, showing maximum reduction in fertility upon nicotine exposure.

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**Table 2. % of C. elegans phenotypes observed with chronic nicotine treatment.**

| Strain   | Control | 500 \( \mu \)M Nicotine | 1000 \( \mu \)M Nicotine |
|----------|---------|---------------------------|--------------------------|
| PS2728   | 2.22 ± 2.22 | 10.74 ± 3.53          | 11.85 ± 2.67            |
| MT13032  | 0.00 ± 0.00  | 7.04 ± 1.61             | 9.26 ± 3.76             |
| SD551    | 1.11 ± 1.11  | 11.11 ± 5.13            | 12.59 ± 4.51            |

Synchronized L4 worms of all the strains were exposed to nicotine in liquid culture for 10 d and their phenotypes were observed. Values are given as Average ± SEM. There is a significant difference between the % phenotypes of control, 500 \( \mu \)M and 1000 \( \mu \)M nicotine within each strain (p < .05).

Nicotine induces morphological changes in C. elegans

We next wanted to know whether the chronic exposure of nicotine has any effect on the morphology of these mutants. The RAS mutant SD551 revealed multivulva phenotype that was exacerbated upon chronic exposure to nicotine (Fig. 3a and b). Meanwhile, the \( sli-1 \) mutant strains, PS2728 and MT13032, had a dramatic decrease in body size as compared to untreated control worms (Fig. 3c and d). Percentage of SD551, PS2728 and MT13032 worms with phenotypic changes in the presence and absence of nicotine are summarized in Table 2. Treatment with nicotine significantly increased morphological changes in each of the mutant strains compared to control (p < .05). However, exposure to nicotine did not induce any morphological changes in N2 (data not shown).

Chronic exposure to nicotine enhanced locomotion in C. elegans

In the absence of nicotine, there is a significant decrease in speed of all mutant worms compared to the wt N2 worms (Fig. 4a). Our data shows that N2 worms had maximum average speed while SD551 had minimum average speed in comparison to other mutants. Notably, chronic exposure to nicotine significantly enhanced the locomotion speeds in wt and all mutants except \( vab-1 \) (Fig. 4a). The relative distribution of speeds is shown for all the worms and the average speeds before and after nicotine treatment in the various groups of worms tested are summarized in Table 3. We also found that \( vab-1 \) mutant showed increased circular motion compared to the wt N2 (Fig. 4b, supplementary videos 1, 2, 3, 4).

Chronic exposure to nicotine and gene expression analysis in C. elegans

The gene expression experiments focused on the Eph receptor mutation (\( vab-1 \)) and the RAS mutation (SD551) with nicotine concentrations of 0 and 500 \( \mu \)M. The linear models used in these experiments provide differentially expressed genes for each genotype, for nicotine alone, and for the interaction between nicotine and each genotype. Mutation of \( vab-1 \) dramatically altered gene expression patterns, with 4496 genes differentially expressed (FDR < 0.01). SD551 mutations also significantly altered gene expression, with 1276 genes significantly differentially expressed (FDR < 0.01). Nicotine had a smaller effect, affecting the expression of 986 genes (FDR < 0.05). This can also be seen in the principal components analysis; the first 2 principal components (Fig. 5a) largely separate the samples based on genotype (although the second PCA does...
Figure 4. (A). Effect of chronic exposure of nicotine on the locomotion velocity of different *C. elegans* strains. Synchronized worms were grown on plates with and without nicotine. 20-30 L4 worms were transferred to bacteria free plates with nicotine and incubated for 20 min before recording tracks. Worms were tracked for 600 seconds. The corresponding normalized histograms of N2, SD551, *vab-1*, RB2088, MT13032, PS1258 and PS2728 speeds with (a, c, e, g, i, k, m) and without nicotine (b, d, f, h, j, l, n) are respectively shown. Average velocity (mm/s) of each mutant is shown in Table 3. (B). Effect of chronic exposure of nicotine on worm path. Worms (N2 wt, and *vab-1*) were tracked for 10 minutes. The tracks of 20 worms with and without nicotine are shown.
also show separation based on nicotine exposure). Interestingly, however, the normal genotype exposed to 500 μM nicotine clusters with the RAS mutant worms (Fig. 5, 5b), suggesting that, at least with regards to global gene expression, exposure of normal worms to nicotine phenocopies mutation of RAS (Fig. 5c). PCAs 2–4 (Fig. 5b, c) separate samples based on nicotine exposure.

To understand the biological process affected by mutation of Eph, RAS and exposure to nicotine, we performed pathway analysis on differentially expressed genes. Mutation of Eph significantly altered several important processes, including cell cycle, embryonic development and larval development (Supplementary Fig. 1). Mutation of RAS altered a huge number of F-box containing proteins (Supplementary Fig. 2). C. elegans had an expansion of F-box proteins during evolution and the function of many of these proteins is poorly understood. RAS also significantly affected the expression of several serine-threonine kinases, as well as structural genes and genes involved in locomotion and response to stimulus. Nicotine exposure by itself significantly affected important pathways, such as post-embryonic and larval development and signal transduction (Fig. 5d).

The interaction of Eph and nicotine led to altered expression of numerous F-box proteins as well. However, it also led to differential expression of several interesting classes of genes, including positive regulation of growth rate (Fig. 5e). A heat map of the linear model effect size of the 280 genes (Supplementary Table 1) in positive regulation of growth rate shows that mutation of Eph and, to a lesser extent, exposure to nicotine, drives gene expression in the same direction. Interestingly, however, the interaction between Eph and nicotine strongly drives gene expression in the opposite direction. As specific examples, expression of Paxillin and EGL-15 (i.e. FGFR homolog) are strongly increased by mutation of Eph, and their expression is downregulated in EphA mutants exposed to nicotine. Alternatively, PAR-6, a gene involved in epithelial cell polarity, is downregulated by Eph and upregulated by the combination of Eph and nicotine. This is in contrast to several serine-threonine kinases that are significantly differentially expressed in both Eph and RAS mutants (Fig. 6a–f). Most kinases are strongly upregulated by mutations and by nicotine, and even more strongly upregulated by the interaction between genotype and nicotine.

Given the phenotypes above, we next asked whether expression of genes known to have similar phenotypes when mutated was altered by mutation of RAS or Eph, or addition of nicotine. For example mutation of Eph altered expressed of 54 UNCoordinated genes, and the interaction between Eph and nicotine altered expression of 18 UNCoordinated genes. Expression of Egg-Laying (egl) defective genes were also altered by mutation of Eph (18 genes) and RAS (11 genes). Interestingly, although mutations of RAS generated a multi-vulva phenotype that was exacerbated by exposure to nicotine, only 1 of the 38 genes known to have a SynMuv phenotype demonstrated significantly altered expression by RAS mutation or exposure to nicotine.

**Discussion**

Lung cancer is the second most common cancer and the leading cause of death among all cancers. The overall prognosis for lung cancer has remained poor with a survival rate of mere 16% for the first 5 y With the advent of targeted molecular therapies this is expected to improve. In lieu of rapid development of resistance, it is imperative that we constantly add to the arsenal by developing novel targeted therapeutics. In this context, we have previously demonstrated the use of *C. elegans* as a simple model to study oncogenic mutants discovered in human lung cancer.\(^3\)\(^6\) *C. elegans* engineered to over-express the MET mutant revealed an abnormal vulval phenotype with hyperplasia. Interestingly, exposure to nicotine significantly aggravated the above phenotype suggesting that *C. elegans* can be used as an *in vivo* model for rapid screening of c-MET mutants as well as drugs. Here we carried out systematic studies to evaluate the effects of chronic exposure to nicotine on wt and mutant worms such as *vab-1* (Eph), SD551 (KRAS), RB2088 (MET) and 3 *sli-1* (c-CBL) mutants, namely PS2728, PS1258 and MT13032. In general, the mutants suffered loss in survival, egg-laying capacity and fertility that was aggravated by exposure to nicotine. Locomotion studies revealed that with the increase in concentration of nicotine, there was a significant increase in locomotion speed in all strains except for *vab-1*. Wt N2 showed the maximum speed while SD551 had the least. We also found *vab-1* mutant worms had increased circular path motion in that was enhanced with chronic nicotine exposure. The expression of these genes was already elevated in SD551 that was further increased in response to nicotine. Our findings further strengthen the role of nicotine as a promoter of cancer.

*C. elegans* has served as an excellent model to investigate essential signaling pathways and behavioral responses due to its simple, non-redundant genetic makeup and ease of culture. Its behavioral responses to nicotine such as acute response, tolerance, withdrawal and sensitization mirror those that seen in mammals and require specific nAChRs that are highly conserved. For instance, lack of TRPC (transient receptor potential canonical) channels in *C. elegans* results in defective behavioral response to nicotine that can be rescued using human TRPC gene.\(^1\)\(^4\) Previously, we showed that *C. elegans* can serve as a model for mechanistic studies related to lung cancer. Worms forced to express a constitutively active MET mutant frequently associated with lung cancer suffered from significantly

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**Table 3. Effect of chronic exposure of nicotine on locomotion speed (mm/s) of different mutants.**

| Strain | Control Nicotine 500 μM |
|--------|--------------------------|
| N2     | 0.13134 ± 0.00018        |
| SD551  | 0.07222 ± 0.00028        |
| vab-1  | 0.10525 ± 0.00016        |
| RB2088 | 0.09740 ± 0.00036        |
| MT13032| 0.07693 ± 0.00015        |
| PS1258 | 0.10011 ± 0.00029        |
| PS2728 | 0.10188 ± 0.00028        |

With exposure to nicotine, locomotion speed of all the strains has increased except for *vab-1*. N2 has maximum speed while SD551 has the least. Values are given as Average ± SEM.

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With the advent of targeted molecular therapies this is expected to improve. In lieu of rapid development of resistance, it is imperative that we constantly add to the arsenal by developing novel targeted therapeutics. In this context, we have previously demonstrated the use of *C. elegans* as a simple model to study oncogenic mutants discovered in human lung cancer.\(^3\)\(^6\) *C. elegans* engineered to over-express the MET mutant revealed an abnormal vulval phenotype with hyperplasia. Interestingly, exposure to nicotine significantly aggravated the above phenotype suggesting that *C. elegans* can be used as an *in vivo* model for rapid screening of c-MET mutants as well as drugs. Here we carried out systematic studies to evaluate the effects of chronic exposure to nicotine on wt and mutant worms such as *vab-1* (Eph), SD551 (KRAS), RB2088 (MET) and 3 *sli-1* (c-CBL) mutants, namely PS2728, PS1258 and MT13032. In general, the mutants suffered loss in survival, egg-laying capacity and fertility that was aggravated by exposure to nicotine. Locomotion studies revealed that with the increase in concentration of nicotine, there was a significant increase in locomotion speed in all strains except for *vab-1*. Wt N2 showed the maximum speed while SD551 had the least. We also found *vab-1* mutant worms had increased circular path motion in that was enhanced with chronic nicotine exposure. The expression of these genes was already elevated in SD551 that was further increased in response to nicotine. Our findings further strengthen the role of nicotine as a promoter of cancer.

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increased incidence of multi-vulval phenotype and associated hyperplasia that was exacerbated upon chronic exposure to nicotine. Here, we have extended the studies to investigate the behavioral and genetic changes in response to chronic nicotine treatment using select mutants of *C. elegans* that are relevant to human cancers.

Figure 5. Effect of Nicotine on Gene Expression analysis of N2, vab-1 and SD551 mutants of *C. elegans*. Panels a-c: Principle Components Analysis plots of the first 3 PCAs. The letter designates the genotype of the strain (v = vab-1, s = SD551, n = N2) and the color designates nicotine exposure (blue = 500 x nicotine, red = 0 x nicotine). Panels d-f: -log10 p-value (fisher’s exact test) for enrichment of pathways and process in differentially expressed genes. Panel d shows nicotine alone, Panel e show the interaction between nicotine and vab-1, and Panel f shows the interaction between nicotine and SD551.
The strong association between smoking and lung cancer is well known and has been the subject of intense investigation for more than 3 decades. The major addictive principle of cigarettes is the nicotine in the tobacco. Although, nicotine is not a carcinogen, it does play a significant role in promoting tumor growth and metastasis, and therefore can be termed a 'cocarcinogen'. It promotes tumor growth through enhanced proliferation, cell motility and invasion, epithelial-mesenchymal transition, angiogenesis and by triggering signaling pathways that are associated with autocrine loops linked to tumor growth.\textsuperscript{51,52} Significant association between smoking and the development of pancreatic and lung cancers has been reported and moreover, the prognosis is poor in smokers suffering from above cancers.\textsuperscript{53,54} Cigarette smoke and the increased risk of pulmonary metastasis of breast cancer has been known for some time and was confirmed in a murine model.\textsuperscript{55,56} The link between nicotine and metastasis can be appreciated by the fact that it promotes epithelial mesenchymal transition, a process that is fundamental to cancer invasion.\textsuperscript{57} Interestingly, a dose dependent increase in the proliferation and invasion of breast, lung and pancreatic cancer cells using matrigel in response to nicotine was noted.\textsuperscript{5} It was also established by the same group that the effect was mediated through nAChRs.

Current experiments utilized relatively higher concentrations of nicotine that ranged from 50 to 500 \( \mu \text{M} \). Since, little is known about the uptake mechanism of nicotine by the nematode \textit{C. elegans}, it is rather difficult to determine the actual effective concentration of nicotine achieved in the worm. The
range of concentrations used in the present study are comparable to those used by previously.58

We have utilized in this study several C. elegans mutants that have either non-functional or constitutive forms of key signaling molecules known to play a role in tumorigenesis. Others and we have previously shown that MET mutations, RAS mutations, CBL mutations can occur in the context of smoking. We have recently shown a critical role for EphB4 receptor tyrosine kinase in esophageal cancer and lung cancer.59 C. elegans has one equivalent receptor vab-1. We used here the kinase inactive C. elegans mutant vab-1 (G912E) to investigate the chronic effects of nicotine.60 RAS is an important oncogene that is mutated in a number of tumors, such as pancreatic, colon and lung cancer. There are a number of KRAS mutations in lung cancers, especially on codon 12, 13 and 61. KRAS appears to be an oncogenic driver in lung cancer. Oncogenic mutations in the RAS gene are present in approximately 30% of all human cancers. KRAS mutations occur in more than 90% of pancreatic and colon cancers and about 20–30% in non-small-cell lung cancer. It is important to note that non-small lung cancer patients with KRAS mutations are also unresponsive to EGFR or ALK targeted therapies. In general, the prognosis is poor with KRAS mutations.1,2,61,62 We therefore investigated its equivalent in C. elegans the mutant SD551 (G912E), a gain of function temperature sensitive mutant that is highly active at 20°C but remains non-functional at 15°C.53

We were one of the first to identify the RTK MET as an important target in lung and other cancers and our mechanistic, translational and clinical studies conducted over the past 20 y have now yielded several small molecule chemotherapeutic drugs that are now in various clinical trials.63 Also in this study, we utilized the non-functional MET receptor kinase ortholog RB2088 (F11E6.8) that has a 900 base pairs deletion in the kinase domain. Various RTKs that contribute to tumorigenesis are negatively regulated by c-CBL, an E3 ubiquitin ligase. We recently reported that c-CBL frequently suffers loss-of-function mutations in lung cancer and plays a key role in tumorigenesis.55 Here, we also utilized 3 (c-CBL ortholog) mutants: PS2728, PS1258 and MT13032, that are non-functional.

The mutants in general revealed lower egg-laying capacity and fertility compared to wt worms that were further worsened by chronic nicotine treatment. This is in agreement with the fact that cigarette smoking is associated with lower fecundity. The fact that cigarette smoking is associated with lower fecundity would now be useful to determine if this model can be used to test novel inhibitory strategies for various mutants and environmental exposure.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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