Smoking correlates with increased cytoskeletal protein-related coding region mutations in the lung and head and neck datasets of the cancer genome atlas

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Abstract
Cancer from smoking tobacco is considered dependent on mutagens, but significant molecular aspects of smoking-specific, cancer development remain unknown. We defined sets of coding regions for oncoproteins, tumor suppressor proteins, and cytoskeletal-related proteins that were compared between nonsmokers and smokers, for mutation occurrences, in the lung adenocarcinoma (LUAD), head and neck squamous carcinoma (HNSC), bladder carcinoma (BLCA), and pancreatic adenocarcinoma (PAAD) datasets from the cancer genome atlas (TCGA). We uncovered significant differences in overall mutation rates, and in mutation rates in cytoskeletal protein-related coding regions (CPCRs, including extracellular matrix protein coding regions), between nonsmokers and smokers in LUAD and HNSC (P < 0.001), raising the question of whether the CPCR mutation differences lead to different clinical courses for nonsmoker and smoker cancers. Another important question inspired by these results is, whether high smoker cancer mutation rates would facilitate genotoxicity or neoantigen-based therapies. No significant, mutation-based differences were found in the BLCA or PAAD datasets, between nonsmokers and smokers. However, a significant difference was uncovered for the average number of overall cancer mutations, in LUAD, for persons who stopped smoking more than 15 years ago, compared with more recent smokers (P < 0.032).

Introduction
Tobacco use has long been associated with increased incidence of lung (Kasala et al. 2015), head and neck (Hayes et al. 2015), pancreatic (Maisonneuve and Lowenfels 2015) and bladder cancer (Cumberbatch et al. 2016), but the mechanisms of cancer initiation and progression dependent on tobacco use are not comprehensively understood. In particular, mutations caused by smoke-related mutagens are considered an important cause (Kim et al. 1993), but other factors, such as smoking dependent, reduced CpG island methylation, could also play a role (Klingbeil et al. 2014; Philibert et al. 2014). We grouped nonsmokers and smokers using the TCGA clinical files and compared their mutation occurrences. The analysis of the data below indicates that recognized cancer driver mutations in lung adenocarcinoma distinguish nonsmokers from smokers but not in head and neck, bladder or pancreatic cancer. In bladder cancer, in particular, our analysis indicates that lower methylation (and thereby reduced suppression) of oncogenes in smokers may be the distinguishing factor between nonsmoker- and smoker-related disease.

Cytoskeletal proteins are emerging as a distinct class of cancer driver proteins, particularly in view of their likely
capacity for function as dominant-negative drivers (Fawcett et al. 2015; Parry et al. 2015; Parry and Blanck 2016), in that mutations in any one of many places in the coding region of a filamentous protein would have the impact of disrupting polymer formation, as happens with any number of mutations in the coding regions for the collagen subunits that form collagen molecules and cartilage. In addition, cytoskeletal-related protein coding regions (CPCRs), including extracellular matrix (ECM) protein coding regions, occupy a relatively large genomic space. For example, the CPCR set that is among the top 25 most mutated coding regions among 10 cancer datasets represents about 15% of all of the human coding regions (Parry and Blanck 2016). Thus, this CPCR set has great potential as a biomarker of mutagenesis. This is particularly true keeping in mind the many reagents and processes available to assess cytoskeletal and ECM integrity and the fact that a disorganized cytoskeleton has been long associated with tumorigeneis (Verderame et al. 1980). Thus, this report includes an assessment of mutation rates for this previously established, cancer-relevant CPCR set.

**Methods**

Clinical and somatic mutation data (BI_Illumina) were downloaded for the lung adenocarcinoma (LUAD), head and neck squamous carcinoma (HNSC), bladder carcinoma (BLCA), and PAAD datasets from the TCGA data portal (http://cancergenome.nih.gov/), following NIH approval (request #27073-3 for project #6300). The clinical patient file was used to sort barcodes into lifelong nonsmokers and smokers for each cancer dataset. Tumor sample barcodes in the somatic mutation file were truncated to contain only the following characters, TCGA-###. A cancer file, for example, “SOM, LUAD results”, was created for each cancer set. The truncated tumor sample barcodes along with the nonsmoker and smoker barcodes were then copied into the total mutations sheet of each cancer file. A COUNTIF function was used to determine the total number of mutations for each barcode in both the nonsmoking and smoking categories. Barcodes in nonsmoker and smoker categories, respectively, that did not appear in the mutation file were eliminated. The final lists of nonsmoker and smoker barcodes representing mutations were determined. The nonsmoking and smoking barcodes were then compared based on their total mutation frequencies.

Three sets of coding regions, for analysis of mutation occurrence between nonsmoker and smoker categories, were established: (1) cytoskeletal protein-related coding regions (CPCRs) (Fawcett et al. 2015; Parry and Blanck 2016; Parry et al. 2015); (2) oncoprotein (Fawcett et al. 2015; Parry et al. 2015); (3) tumor suppressor proteins (Fawcett et al. 2015; Parry et al. 2015). The CPCRs were previously determine in ref. (Parry et al. 2015), based on their occurrence in the top 25 most commonly mutated coding regions among the five TCGA datasets studied in ref. (Parry et al. 2015) (BLCA, COAD, LUAD, GBM, STAD). Three additional CPCRs were added to the set indicated in the preceding sentence, representing commonly mutated CPCRs in the SKCM dataset. The HUGO symbols for the coding regions for all sets are in Table 1.

Within each cancer file (e.g., “SOM, LUAD results”), a sheet for total mutations, including only the original barcodes truncated as indicated above and two sheets for each coding region set, for example, “CPCR Mut.” and “CPCR Results,” were created. The coding region mutation sheet includes information from the comprehensive mutation file, such as HUGO symbol, truncated tumor sample barcode, and mutation type (amino acid altering or silent). The list of nonsmoker and smoker barcodes was compared to the comprehensive mutation data using a COUNTIF function (in the coding region mutation sheet) to determine the number of mutations per barcode. The coding region mutations of nonsmokers and smokers were compared in the “results” sheet.

### Table 1. HUGO symbols for the CPCR, oncoprotein, and tumor suppressor protein gene sets (detailed in the SOM file labeled, “SOM Table 1, source file”).

| Gene set              | Cytoskeletal | Oncoprotein | Tumor suppressor |
|-----------------------|--------------|-------------|------------------|
| ANK2                  | MUC4         | ACVR1       | AKAP12           |
| APC                   | NEB          | ALK         | AXIN1            |
| COL11A1               | NEFH         | ARAF        | BMP2             |
| DNAH10                | NF1          | BRAF        | BMP1R3           |
| DNAH11                | PCDH15       | CTNNB1      | BMPRA2           |
| DNAH3                 | PCDHAC2      | EGFR        | BRCWA1           |
| DNAH5                 | PCDHG5       | FGFR2       | BRCWA2           |
| DNAH7                 | PCL1         | FLT3        | BRMS1            |
| DNAH8                 | PKHD1        | FRK         | CASZ1            |
| DSCAM                 | PLEC         | HRAS        | CDKN2A           |
| DST                   | RELN         | JAK2        | CHD5             |
| FAT3                  | SPTA1        | KRAS        | CHEK2            |
| FAT4                  | SPTAN1       | MTO1        | CTCF             |
| FBN2                  | SSPO         | NRS1        | DCL1             |
| FGL                   | SYNE1        | PRKACA      | DOK2             |
| GPR98                 | SYNE2        | RAFL        | FLCN             |
| MUC16                 | TTN          | FOXP3       | SP100            |
| MUC17                 | XIRP2        | GPR68       | TFP2             |
|                       |              | ING1        | TMFR5511A        |
|                       |              | ING4        | TXNIP            |
|                       |              | INPP4B      | VHL              |
|                       |              | KISS1       | WWOX             |

CPCR, cytoskeletal protein-related coding regions.
From the LUAD clinical patient file, available data on the years started and stopped smoking were collected ("SOM Table 2, source file"). Groups of (1) less than or equal to, (2) greater than 15 years from the time of smoking cessation, (3) less than or equal to, and (4) greater than 30 years of total years of smoking were established and then compared based on the number of total mutations per barcode.

The number of deleterious amino acid changes was determined using PROVEAN. The chromosome number, start position, reference allele, and tumor sequence allele for the CPCRs and tumor suppressor datasets for nonsmokers and smokers in each cancer were copied and pasted into PROVEAN under the Human Genome Variants protocol. The removal of duplicates from the “#ROW_NO.” column in the PROVEAN output was used to determine the number of deleterious amino acid changes for the CPCRs and tumor suppressor datasets. The total was then divided by the sample size to determine the average number of deleterious amino acid changes for nonsmokers and smokers in each cancer set. (An example with additional detail is provided in the SOM file labeled, "SOM Example Deleterious AA, LUAD CPCRs").

**Results**

The TCGA datasets of LUAD, HNSC, BLCA, and PAAD were compared based on mutation frequencies between lifelong nonsmokers and smokers. The total number of mutations per barcode was calculated, and the average number of mutations per barcode was determined for nonsmokers and smokers for each cancer type (Fig. 1). The overall mutation rates for LUAD and HNSC, distinguishing nonsmokers and smokers, were found to be statistically significant, however, no such distinction could be established for the BLCA and PAAD datasets.

Next, three cancer driver gene subsets (Fawcett et al. 2015; Parry et al. 2015) were established for analysis: (1) CPCRs; (2) oncprotein; (3) tumor suppressor proteins (Table 1). Mutation frequencies were evaluated for nonsmokers and smokers for each gene set for the four cancer types. Smokers had a significantly higher average mutation rate for all three coding region sets for LUAD (Fig. 2); and smokers had a significantly higher number of mutations for the CPCRs gene set in HNSC (Fig. 3), reflecting the HNSC results for the overall mutation rates and the distinction between nonsmokers and smokers (Fig. 1). Mutations rates of the remaining gene sets for HNSC (oncoprotein and tumor suppressor protein) failed to distinguish nonsmokers and smokers in the indicated analyses. None of the gene set mutation rates distinguished nonsmokers from smokers for the BLCA and PAAD datasets.

To further emphasize the distinction in mutation rates, between nonsmokers and smokers, for LUAD and HNSC, the 25 barcodes with the highest mutation frequency and the 25 barcodes with the lowest mutation frequency were compared based on their inclusion in the nonsmoker or smoker groups (detailed in the SOM files labeled, “SOM, LUAD results” and “SOM, HNSC results”). In both cases, the high-frequency mutation groups were dominated by smoker barcodes (LUAD $P < 2E^{-12}$, HNSC $P < 6E^{-8}$) and the low-frequency mutation groups were about evenly distributed between nonsmokers and smokers.

| Table 2. Comparison of available data from the LUAD dataset based on the year started and stopped smoking (detailed in the SOM file labeled, “SOM Table 2, source file”). |
|---------------------------------------------------------------|
| Number of years since stopped smoking (≤15 yrs vs. >15 yrs) | Total number of years smoked (≤30 yrs vs. >30 yrs) |
|---------------------------------------------------------------|
| **P-value for ≤15 yrs versus >15 yrs since stopped smoking** | **P-value for ≤30 yrs versus >30 yrs of smoking** |
| Avg number of mutations/barcode for ≤15 yrs | 604.06 |
| Avg number of mutations/barcode for >15 yrs | 482.55 |
| Avg number of mutations/barcode for ≤15 yrs | 493.11 |

LUAD, lung adenocarcinoma.
Public health data have strongly indicated, for decades, that smoking cessation is accompanied by a reduced risk of cancer and the results continue to be confirmed with recent work (Wynder and Hoffmann 1976; Wynder and Stellman 1977; Ogihara et al. 2016). However, possible molecular explanations for this phenomenon have been limited (Wang et al. 1999), usually to specific genes. We found that groups of (1) less than or equal to, and (2) greater than 15 years since smoking cessation had a significant difference in the number of mutations per barcode. Barcode groups of (3) less than or equal to, and (4) greater than 30 years of total years smoked had no difference (Table 2), indicating that the significant difference in the number of mutations between the smoking and smoking-cessation groups was not simply due to fewer years smoked among the smoking-cessation group. To further address this (trivial) possibility, we examined the distribution of the total number of years smoked in all of the above-indicated sets, and it is clear that the smoking and smoking cessation groups have a much larger overlap of years smoked than do the two groups based on total number of years smoked (Fig. 4). This strongly indicates that the significant difference in the number of mutations between the smoking and smoking-cessation groups is not due to a difference in the number of mutations accumulating on a per-year basis. If that were the case, the difference in the number of mutations distinguishing the “over 30 years” and under 30 years groups would likely be significant, that is, parallel to the average number of years smoked. (In this latter case, the average number of years smoked is dramatically different based on the distributions of years smoked (Fig. 4B), but the number of mutations in the cancer samples is not (Table 2).)

To address whether the higher smoker mutation rates could have an impact on cellular function, the number of deleterious amino acid changes for the CPCR and tumor suppressor datasets were also considered (Table 3). The LUAD dataset had the largest average number of deleterious amino acid changes per barcode, as well as, the greatest difference between nonsmokers and smokers in both the CPCR and tumor suppressor datasets, consistent with the above distinctions between nonsmokers and smokers based on mutations alone.

Data indicated that both BLCA and PAAD development are associated with smoking. Likewise, HNSC has been linked to smoking. Yet, in none of these cases were we able to observe oncoprotein or tumor suppressor protein mutation increases among the smokers. There are numerous possible explanations for this, not the least of which is that the difference between nonsmokers and smokers is related to incidence of occurrence of a “single hit” basis for the cancers and not due to any “amplification” requirement that could be traceable to an increased number of mutations. Nevertheless, the increased number of mutations in cancer driver coding regions in the LUAD dataset raises the question of whether nonsmokers...
Figure 3. (A) Normal distribution of total mutations per barcode in LUAD for nonsmokers versus smokers. ($P < 1.0e^{-17}$) (detailed in the SOM file labeled, “SOM, LUAD results”). (B) Normal distribution of total mutations per barcode in HNSC for nonsmokers versus smokers. ($P < 0.0006$) (detailed in the SOM file labeled, “SOM, HNSC results”). (C) Normal distribution of CPCR mutations per barcode in LUAD for nonsmokers versus smokers. ($P < 1.3e^{-20}$) (detailed in the SOM file labeled, “SOM, LUAD results”). (D) Normal distribution of CPCR mutations per barcode in HNSC for nonsmokers versus smokers. ($P < 1.5e^{-7}$) (detailed in the SOM file labeled, “SOM, HNSC results”). (E) Normal distribution of CPCR mutations per barcode in BLCA for nonsmokers versus smokers. ($P > 0.05$ not significant) (detailed in the SOM file labeled, “SOM, BLCA results”). (F) Normal distribution of CPCR mutations per barcode in PAAD for nonsmokers versus smokers. ($P > 0.05$ not significant) (detailed in the SOM file labeled, “SOM, PAAD results”). CPCR, cytoskeletal protein-related coding regions; HNSC, head and neck squamous carcinoma; LUAD, lung adenocarcinoma; PAAD, pancreatic adenocarcinoma.
could be distinguished from smokers via another molecular attribute related to cancer development.

Thus, we considered the possibility that nonsmokers and smokers could be distinguished on the basis of reduced methylation of oncoprotein coding regions, presumably leading to a relative upregulation of oncoprotein expression. Indeed, there have been reports linking smoking chemicals to interference in CpG island methylation (Klingbeil et al. 2014; Philibert et al. 2014; Bjaanaes et al. 2016). Thus, we compared the methylation averages for the top five available barcodes with the highest mutation burdens for nonsmokers and smokers in each cancer set.

Figure 4. (A) Normal distributions for the total years smoked between the ≤15 years since quitting and >15 years since quitting smoking groups (detailed in the SOM file labeled, “SOM Table 2, source file”). (B) Normal distributions of total years smoked between the ≥30 years and <30 years of total years smoking (detailed in the SOM file labeled, “SOM Table 2, source file”). BLCA, bladder carcinoma; HNSC, head and neck squamous carcinoma.
likely representative of a significant stochastic process of and smoker cancers. Both of the preceding results are in lung and head and neck cancers, between nonsmoker cation of a distinction in the number of CPCR mutations ing. Furthermore, the data above represent the first indi-

## Discussion

Specific cancer mutations have represented distinctions between nonsmoker and smoker cancers in previous studies (Albrecht and Theron 1988; Kondo et al. 1992), but this is the first direct, confirmation of an increased overall mutation rate in lung cancer that is attributed to smoking. Furthermore, the data above represent the first indication of a distinction in the number of CPCR mutations in lung and head and neck cancers, between nonsmoker and smoker cancers. Both of the preceding results are likely representative of a significant stochastic process of smoking-dependent mutagenesis, keeping in mind that CPCR coding regions are relatively large and thus represent large mutagen targets.

The above work also indicates that the significant difference in CPCR mutations, between nonsmokers and smokers, leads to more deleterious amino acid substitutions among the smoker CPCR set. Because CPCR mutations are likely to have a dominant-negative impact, consistent with their role in the formation of, and potential corrup-

### Table 3. Average number of deleterious mutations per barcode in the CPCR and tumor suppressor data sets for all four cancer sets (detailed in the SOM file labeled, "SOM Table 3, source file" and additional information for the method is detailed in the SOM file labeled, "SOM Example Deleterious AA, LUAD CPCR").

|                | LUAD          | HNSC          | BLCA          | PAAD          |
|----------------|---------------|---------------|---------------|---------------|
|                | Smokers | Nonsmokers | Smokers | Nonsmokers | Smokers | Nonsmokers | Smokers | Nonsmokers |
| Avg # of deleterious CPCR mut/barcode | 4.43 | 0.86 | 2.28 | 0.90 | 2.15 | 1.83 | 0.19 | 0.19 |
| Avg # of deleterious tumor suppressor mut/barcode | 0.52 | 0.15 | 0.45 | 0.36 | 0.37 | 0.50 | 0.09 | 0.06 |

BLCA, bladder carcinoma; CPCR, cytoskeletal protein-related coding regions; HNSC, head and neck squamous carcinoma; LUAD, lung adenocarcinoma; PAAD, pancreatic adenocarcinoma.
several related questions. Certain studies have indicated that higher cancer mutation rates indicate greater sensitivity to mutagenic drugs. This result could be due to reduced DNA repair functions or due to “weakened” cells unable to tolerate additional genotoxicity. Are there distinctions between nonsmoker and smoker responses to mutagenic chemotherapy? In addition, the above data would indicate that clinical differences dependent on oncogene or tumor suppressor gene mutations would not be apparent for nonsmoker and smoker cancers.

There are many possible explanations for cancer development besides mutations, including copy number variation, partial deletions, and epigenetic processes. In particular, certain studies have associated DNA methylation, or lack of methylation with cancers arising from tobacco use (Philibert et al. 2014; Bjaanaes et al. 2016). Data presented here do indicate an alternative explanation for a lack of mutation-based distinction of nonsmoker and smoker cancers: demethylation of oncogenes in BLCA. These data are consistent with the possibility that nonsmoker and smoker pancreatic cancers are not distinguishable on the basis of mutation frequency.

Finally, the above data indicate that lung cancer arising after cessation of smoking represented reduced levels of overall mutations, the first such indication, although specific gene mutation profiles are known to be different in cancers arising years after smoking cessation (Bernardini et al. 2001; Ha and Califano 2002). The reduced number of mutations in smoking-cessation cancers raises interesting questions about the change in the mutation profiles of the lung cells over the years since the use of tobacco. For example, are the heavily mutated cells indeed weakened and therefore lost to genotoxicity? Is there a natural rate of turnover in the cells that are sources of lung cancer, such that smoking-cessation cancers are essentially the result of the same processes that lead to low mutation burdened, nonsmoker lung cancers?

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### Conflict of Interest

None declared.

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Table 4. Methylation beta averages for the top five available barcodes with the highest mutation burdens for smokers and nonsmokers in all four cancer sets for the overall genome methylation and the oncoprotein dataset (detailed in the SOM file labeled, “SOM Table 4, source file”).

| Cancer Type | Barcode | Total Beta P-value | Oncoprotein Beta P-value |
|-------------|---------|--------------------|--------------------------|
| LUAD Smoker | TCGA-44-2656 | 0.1202 | 0.0827 |
| TCGA-44-2659 | 0.1202 | 0.0827 |
| TCGA-44-2668 | 0.1202 | 0.0827 |
| TCGA-44-5644 | 0.1202 | 0.0827 |
| TCGA-44-7670 | 0.1202 | 0.0827 |
| Nonsmoker | TCGA-44-2665 | 0.5145 | 0.2851 |
| TCGA-44-2659 | 0.5145 | 0.2851 |
| TCGA-50-5066 | 0.5145 | 0.2851 |
| TCGA-86-8279 | 0.5145 | 0.2851 |
| TCGA-86-8585 | 0.5145 | 0.2851 |
| TCGA-86-8672 | 0.5145 | 0.2851 |
| HNSC Smoker | TCGA-CN-4723 | 0.5145 | 0.2851 |
| TCGA-CR-7402 | 0.5145 | 0.2851 |
| TCGA-CV-6961 | 0.5145 | 0.2851 |
| TCGA-CV-7245 | 0.5145 | 0.2851 |
| TCGA-D6-6516 | 0.5145 | 0.2851 |
| Nonsmoker | TCGA-BA-5152 | 0.5145 | 0.2851 |
| TCGA-88-4223 | 0.5145 | 0.2851 |
| TCGA-CR-6472 | 0.5145 | 0.2851 |
| TCGA-CR-6481 | 0.5145 | 0.2851 |
| TCGA-CV-7252 | 0.5145 | 0.2851 |
| BLCA Smoker | TCGA-BT-A2LB | 0.0081 | 0.0100 |
| TCGA-DK-A6AW | 0.0081 | 0.0100 |
| TCGA-DK-A6Y6 | 0.0081 | 0.0100 |
| TCGA-MA-A51V | 0.0081 | 0.0100 |
| TCGA-SY-A9GS | 0.0081 | 0.0100 |
| Nonsmoker | TCGA-DK-A3WW | 0.0081 | 0.0100 |
| TCGA-G2-A2EO | 0.0081 | 0.0100 |
| TCGA-K4-A6YZ | 0.0081 | 0.0100 |
| TCGA-MA-A51V | 0.0081 | 0.0100 |
| TCGA-SY-A9GS | 0.0081 | 0.0100 |
| PAAD Smoker | TCGA-3A-A919 | 0.0476 | 0.0622 |
| TCGA-H6-8124 | 0.0476 | 0.0622 |
| TCGA-LB-A75X | 0.0476 | 0.0622 |
| TCGA-OE-A75W | 0.0476 | 0.0622 |
| TCGA-PZ-ASRE | 0.0476 | 0.0622 |
| Nonsmoker | TCGA-2L-AAQ1 | 0.0476 | 0.0622 |
| TCGA-3A-A910 | 0.0476 | 0.0622 |
| TCGA-FB-AAPU | 0.0476 | 0.0622 |
| TCGA-HV-A7OP | 0.0476 | 0.0622 |
| TCGA-IB-7652 | 0.0476 | 0.0622 |

BLCA, bladder carcinoma; HNSC, head and neck squamous carcinoma; LUAD, lung adenocarcinoma; PAAD, pancreatic adenocarcinoma.
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