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# Genetic Mutations Associated With Cigarette Smoking in Pancreatic Cancer

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## Abstract

**Background—**Cigarette smoking doubles the risk of pancreatic cancer and smoking accounts for 20 to 25% of pancreatic cancers. The recent sequencing of the pancreatic cancer genome provides an unprecedented opportunity to identify mutational patterns associated with smoking.

**Design—**We previously sequenced over 750 million base pairs of DNA from 23,219 transcripts in 24 adenocarcinomas of the pancreas (“Discovery Screen”). In this previous study the 39 genes that were mutated more than once in the Discovery Screen were sequenced in an additional 90 adenocarcinomas of the pancreas (“Validation Screen”). Here we compared the somatic mutations in the cancers obtained from individuals who ever smoked cigarettes (n=64) to the somatic mutations in the cancers obtained from individuals who never smoked cigarettes (n=50).

**Results—**When adjusted for age and gender, analyses of the Discovery Screen revealed significantly more non-synonymous mutations in the carcinomas obtained from ever smokers (mean 53.1 mutations per tumor, SD 27.9) than in the carcinomas obtained from never smokers (mean 38.5, SD 11.1, p=0.04). The difference between smokers and non-smokers was not driven by mutations in known driver genes in pancreatic cancer (*KRAS*, *TP53*, *p16*/*CDKN2A* and *SMAD4*), but instead was predominantly observed in genes mutated at lower frequency. No differences were observed in mutations in carcinomas from the head vs. tail of the gland.

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**Conclusion—**Pancreatic carcinomas from cigarette smokers harbor more mutations than do carcinomas from never smokers. The types and patterns of these mutations provide insight into the mechanisms by which cigarette smoking causes pancreatic cancer.

## INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in the United States [1]. It has been estimated that in the year 2008, approximately 37,680 Americans were diagnosed with pancreatic cancer, and that 34,290 died from this disease [1]. A number of factors have been identified that increase the risk of pancreatic cancer, including advancing age, diets high in meats and fats, diets low in vegetables and folate, diabetes mellitus, obesity, chronic pancreatitis, partial gastrectomy, radiation, a family history of pancreatic cancer, and cigarette smoking [2–6]. Of all of these known risk factors, cigarette smoking remains the leading preventable cause of pancreatic cancer [6,7]. Approximately 20% of cancers of the pancreas are caused by cigarette smoking, and a recent meta-analysis of 82 studies published between 1950 and 2007 on smoking and pancreatic cancer found that current smokers have a 1.74 fold (95% CI 1.61–1.87) increased risk of developing pancreatic cancer [6,7]. Smoking has also been associated with early onset pancreatic cancer and smoking cessation has been shown to reduce pancreatic cancer risk [8–12].

Genetic analyses of other cancers caused by cigarette smoking have revealed increased numbers of mutations in cancer-associated genes as well as specific types of mutations in cancers resected from smokers [13–19]. This link between cigarette smoking and specific genetic changes in a cancer is strongest for lung carcinomas [14,19]. Smoking is associated with an approximate 11-fold increased relative risk of lung cancer, and activating point mutations in the *KRAS* gene are more common in adenocarcinomas of the lung resected from smokers than they are in adenocarcinomas from non-smokers [16,19–21] Most of these mutations are G:C to T:A transversions, a mutation type associated with carcinogens such as polycyclic aromatic hydrocarbons in tobacco smoke [16,22]. Remarkably, these same mutations can be seen in lung cancers obtained from ex-smokers, suggesting that these *KRAS* gene mutations occurred years before the cancers were resected [15]. Similarly, a number of studies have shown that *TP53* gene mutations are more common in lung cancers from smokers than they are in lung cancers from never smokers, and, again, the G:C to T:A transversions predominate with a specificity towards CpG sites [14,17,23–25]. Thus, there is a strong “fingerprint” of tobacco carcinogens in the DNA of lung cancer [24,26].

The recent analysis of the “pancreatic cancer genome,” encompassing the sequencing of 20,661 protein coding genes in a series of 24 pancreatic cancers, provides a unique opportunity to correlate the somatic genetic changes in pancreatic cancer with smoking status [27]. In this previous study over 750 million base pairs of DNA were sequenced in two phases [27]. First, in the “Discovery Screen,” the sequences of the protein-coding exons from 20,661 genes were sequenced in 24 advanced adenocarcinomas of the pancreas. Of the 1562 somatic mutations discovered using this approach, 62.4% were missense, 25.5% were synonymous, 5.0% were small insertions or deletions, 3.8% were nonsense, and 3.3% were in splice sites or within untranslated regions (UTR) [27]. In addition, 198 homozygous deletions and 144 high copy number amplifications were identified in the cancers included in the Discovery Screen using high density oligonucleotide arrays [27]. In the second phase of this study, the “Validation Screen”, 39 genes that were mutated more than once in the Discovery Screen were sequenced in an additional panel of 90 well-characterized adenocarcinomas of the pancreas [27].

Here we correlate these data with patient smoking history as well as with a variety of other clinical factors such as patient age, sex, stage, and location of the cancer within the pancreas.

## MATERIALS AND METHODS

This study was approved by the Johns Hopkins Institutional Review Board.

### Patients

All available records were retrospectively reviewed on the 114 patients (24 in the Discovery Screen and 90 from the Validation Screen). This included a review of the patient's hospital charts, the electronic patient medical records, and the Johns Hopkins Pancreatic Cancer Research Database [28]. Ninety-eight (86%) of the 114 patients included in this study were deceased at the time of the study.

Non-smokers were defined as patients who reported that they had never smoked in their lives. Smokers were defined as patients who reported that they had smoked in their lives. Ex-smokers were defined as smokers who had quit more than one year prior to surgery for their pancreatic cancer. Information was not available on second-hand smoking exposure.

### Statistical analyses

The total numbers of mutations, deletions and amplifications were compared between clinical parameters using a Poisson regression model that adjusted for smoking status and included an over-dispersion term to account for patient-to-patient variation. A similar approach was used to compare the number of mutations between smokers and non-smokers, adjusting for age and gender. Analyses were adjusted for gender because genes specific to the Y chromosome were not sequenced and therefore more alleles were sequenced in the cancers obtained from women then in the cancers obtained from men [27]. The difference in frequency of specific mutation types (base pair changes and insertions/deletions) and the context in which the mutations occurred, were compared between smokers and non-smokers using mixed-effect logistic regression models that adjusted for age and gender. The difference in frequency of mutations and deletions of the known driver genes (i.e. *KRAS*, *TP53*, *SMAD4* and *CDKN2A*/*p16*) between smokers and non-smokers was evaluated with Fisher's Exact test. The number of statistical comparisons was not defined prior to the analyses, therefore the p values presented are not adjusted for the number of comparisons and are included for descriptive purposes only.

## RESULTS

### Patient demographics

A summary of the patient demographics for the Discovery and Validation Screens is provided in Table 1, and the smoking histories of each of the patients included in the original sequencing study are provided in Supplementary Tables S1 and S2[27]. Briefly, the mean age for both smokers and non-smokers was 65 years. The Discovery Screen included 10 males and 14 females, and the Validation Screen 43 males and 47 females[27]. Sixty-four of the 114 patients included were smokers, and 50 were non-smokers. Of the 64 smokers, 38 had reported that they had quit smoking, and 26 of the 38 ex-smokers had quit more than ten years before their diagnosis. The smokers in the Discovery Screen smoked a mean of 43 pack-years, and the smokers in the Validation Screen a mean of 38 pack-years. There were no p-values of 0.05 or less for any of the clinical parameters examined between the smokers and non-smokers (Table 1).

### Mutations in the Discovery Screen

We first examined the mutations identified by sequencing in the Discovery Screen and calculated the total number of mutations per sample for each of the clinical parameters evaluated (Table 2).

There was a trend for more mutations in smokers than in non-smokers. The number of mutations ranged from 40 to 187 per tumor for smokers and from 34 to 72 per tumor for non-smokers. As has been previously reported with lung cancer, the variance of the number of point mutations for smokers was higher than for non-smokers (Variance Ratio estimate = 9.0 (95% C.I. 2.7 – 32.7), $p < 0.001$) [19]. The eleven smokers had a mean of 75.5 intragenic mutations per carcinoma (SD 41.7) and the non-smokers a mean of 56.2 mutations (SD 13.9, p=0.06 when adjusted for age and gender) (Table 3). Thus, approximately 25% of the intragenic mutations in the pancreatic cancers obtained from smokers appear to be smoking-related.

When homozygous deletions and amplifications were also included together with the mutations identified by sequencing, the carcinomas from the eleven smokers had a mean of 90.9 (SD 44.4) genetic alterations per tumor and the carcinomas from the non-smokers a mean of 69.5 (SD 16.4, p=0.08). There were no significant differences observed in the number of amplifications or in the number of deletions in smokers and non-smokers. Though the numbers were small, no significant differences were observed between the ex-smokers and the current smokers with respect to mutation number or type.

No significant differences were observed in the number of mutations for the other clinical variables examined for patients included in the Discovery Screen (Table 2).

## Categories of Mutations in the Discovery Screen (Table 3)

Next we examined the broad categories of alterations observed in the Discovery Screen (Table 3). As noted above, the number of homozygous deletions and amplifications did not differ between the non-smokers and smokers. Our further analyses therefore focused on the mutations identified by sequencing.

When the *KRAS* and *TP53* genes, the two previously reported targets of tobacco-related carcinogens, were excluded from the analyses a larger number of mutations were still identified in the cancers obtained from smokers (mean 73.9, SD 41.9) than in the cancers obtained from non-smokers (mean 54.4, SD 14.1, p=0.06 when adjusted for age and gender). A similar pattern was observed when all four "gene mountains," the *KRAS*, *TP53*, *SMAD4* and *CDKN2A* genes, were excluded from the analyses, with a mean of 73.5 (SD 41.7) mutations in the smokers and 53.9 (SD 14.1) mutations in the non-smokers (p=0.05 when adjusted for age and gender, Table 3) [27]. Finally, we compiled a list of 65 driver genes (Table S3). These 65 driver genes included genes identified in our previous genome-wide sequencing analyses, genes reported as driver genes in the literature, and genes with greater than 10 alterations in the Cosmic database (December 20, 2008; http://www.sanger.ac.uk/genetics/CGP/cosmic/) [27,29–31]. When these driver genes were excluded from the analyses, the difference persisted, with a mean of 73.3 (SD 41.8) mutations in smokers and 53.2 (SD 13.8) mutations in the non-smokers (p=0.05 when adjusted for age and gender). These results suggest that the differences observed in the number of mutations detected by sequencing between smokers and non-smokers are not driven by these major driver genes.

Significantly more non-synonymous mutations were observed in the cancers from smokers (mean 53.1, SD 27.9) than in the cancers from non-smokers (mean 38.5, SD 11.1, p=0.04 when adjusted for age and gender). More synonymous mutations were also observed in the cancers from smokers (mean 18.7, SD 11.3, Table 3) as compared to non-smokers (mean 14.8, SD 5.4, p=0.26), but this difference was not statistically significant.

Transitions were more common in the cancers from smokers (mean 43.7, SD 16.9) than in the cancers from non-smokers (mean 35.3, SD 9, p=0.04 when adjusted for age and gender). There were also more transversions in the cancers from smokers (mean 31.8, SD 25.9) than non-

smokers (mean 20.9, SD 10.3), but this latter difference was not statistically significant (Table 3).

### Types of Mutations in the Discovery Screen (Table 4)

We next examined the specific types of mutations identified in the Discovery Screen (Table 4). Here the mutations were placed into one of thirteen groups: the 12 possible base pair changes (based on the reading strand) and insertions or deletions. Of the 13 possible mutation types, C:G to A:T (Odds Ratio 1.6, 95% CI [1.04,2.46], p=0.03 adjusted age and gender) and T:A to A:T mutations (Odds Ratio 2.32, 95% CI [0.99,5.45], p=0.05 adjusted age and gender) were both more common in the cancers from smokers than in the cancers from non-smokers.

There were no significant differences observed in the context in which the mutations occurred (Table 4). Similar analyses for the Validation Screen are presented in Table S4.

### *TP53* gene mutations and smoking

Point mutations in the *TP53* gene were identified in 82% of the cancers (Table 5). Eighteen of the 24 cancers in the Discovery Screen harbored a *TP53* gene mutation, as did 76 of the 90 cancers in the Validation Screen. The prevalence of *TP53* gene mutations in cancers from smokers did not differ significantly from the prevalence of *TP53* gene mutations in the cancers from non-smokers. Fifty (78%) of the 64 cancers from smokers harbored a *TP53* gene mutation, compared to 44 (88%) of the 50 cancers from non-smokers (p=0.22). In addition, the types and context of the *TP53* gene mutations in smokers and in non-smokers also were similar (Table 6).

### *KRAS* gene mutations and smoking

*KRAS* gene mutations were observed in 113 (99%) of the 114 pancreatic cancers sequenced. With almost universal *KRAS* gene mutations, the number of *KRAS* gene mutations in the cancers from smokers did not differ significantly from the number in cancers from non-smokers. As was true for the *TP53* gene, the types and context of the *KRAS* gene mutations in smokers and in non-smokers were similar (Table 6).

### Other gene mutations and smoking

There were a total of 1562 sequence mutations involving 1315 unique genes in the tumor samples. In addition to the genes presented in Table 5, *TTN* was mutated in 8 carcinomas: 4 smokers and 4 non-smokers. Of the remaining 1310 genes, 1166 were mutated in only 1 tumor sample. The remaining 144 genes were mutated in 2, 3, or 4 tumor samples and were not analyzed for differences by smoking group.

## DISCUSSION

A number of studies have linked cigarette smoking with specific genetic alterations in cancer-associated genes in lung cancer [14,16,17,19,20,23,24] For example, Westra et al. reported significantly more *KRAS* gene mutations in lung adenocarcinomas obtained from current smokers (30%) and former smokers (32%) than in lung adenocarcinomas obtained from never smokers (7%, p=0.015) [15]. Similarly, Le Calvez and colleagues found *TP53* gene mutations in the lung cancers of 47.5% of never smokers, 55.6% of former smokers, and 77.4% of current smokers [14]. More recently, Ding et al. sequenced 623 genes in 188 adenocarcinomas of the lung and found significantly more mutations in the cancers from smokers than in the cancers from never smokers (p=0.02) [19]. All of the cancers obtained from never smokers harbored 5 or fewer mutations, while the cancers obtained from smokers had as many as 49 mutations

[19]. Comparable results have been reported for other cancer types associated with cigarette smoking such as head and neck cancer, and bladder cancer [18,32,33]

Smoking also has been associated with pancreatic cancer through epidemiologic studies and smoking has been linked to specific genetic mutations in pancreatic cancers [6,13,34]. Pancreatic cancers from cigarette smokers have been reported to have more *KRAS* and more *TP53* gene mutations than pancreatic cancers from non-smokers [13,34]. For example, Jiao et al. found that smoking was associated with G:C to A:T mutations in the *KRAS* gene in pancreatic cancer [35]. It should be noted, however, that not all studies have found a link between smoking and specific genetic changes in pancreatic cancer [36,37]. For example, Porta et al. reported on 107 pancreatic cancers and found no relationship between *KRAS* gene mutations and smoking [37].

The sequencing of the pancreatic cancer genome provided a unique opportunity to correlate cigarette smoking and other clinical parameters with specific genetic mutations [27]. We found that although the number of smoking related mutations did not appear to be as high as it was for lung cancer, pancreatic cancers obtained from ever smokers harbored more mutations than cancers obtained from never smokers [19]. As has been previously reported with lung cancer, the variance of the number of point mutations in the pancreatic cancers obtained from smokers was higher than the variance of the number of point mutations in the pancreatic cancers obtained from non-smokers[19]. We estimate that one in four of the mutations in the pancreatic cancers obtained from smokers may be smoking related.

In contrast to several previous reports, however, we did not observe an association between smoking and *KRAS* gene mutations [13]. This likely reflects the selection criteria used to include cases in the sequencing project [27,34,38]. Cancers with variant morphologies, such as medullary carcinoma, were excluded from the project in an effort to increase the uniformity of the cancers sequenced. Medullary carcinomas, as we have reported before, are often microsatellite unstable, they lack *KRAS* gene mutations, and some are caused by germline mutations in a DNA mismatch repair gene [39]. Thus, the selection criteria for the pancreatic cancer genome project tended to exclude the *KRAS* wild-type cases driven by a pathway unrelated to smoking. Simply put, with 99% of the cancers harboring a *KRAS* gene mutation, it would have been virtually impossible to detect an impact of smoking on the *KRAS* gene.

The differences between the number of mutations in smokers and non-smokers were not found in the other genes known to be “driver” genes in pancreatic cancer, such as *TP53*, *CDKN2A* and *SMAD4* [27,40–44]. This observation can be explained by the fact that these mutations are likely required for pancreatic cancer to occur and are highly selected for during the tumorigenic process. Passenger mutations, but not driver mutations, provide a molecular clock that can be used to infer mutation rates [45]. Smokers may develop pancreatic cancer more frequently and at a younger age of onset, but the driver genes that are mutated appear to be the same in the two groups [8]. While this distinction between the passenger mutations and driver mutations has been overlooked in prior literature, it may explain the often unconvincing associations between smoking and driver genetic mutations[37].

We also examined the types of mutations and the context in which these mutations occurred. We did not identify a signature tobacco-related mutation in the smokers. A possible explanation for this heterogeneity is that it reflects the multiple DNA damaging compounds (>100) found in cigarette smoke, and that perhaps the mutagenicity of cigarette smoke is not limited to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (BaP), two well-studied tobacco-derived carcinogens [46]. The data would also be consistent with the hypothesis that the carcinogens in tobacco damage the DNA in the pancreas in a non-specific way, but this latter hypothesis is not consistent with extensive data from the study of lung

cancer, and with the finding of specific tobacco-derived carcinogens in the pancreatic juice of smokers. Other possible explanations include that non-tobacco-related mutagenic risk factors for pancreatic cancer may share mutagenic properties with the tobacco mutagens active in pancreatic tissues, and that the end-organ metabolic products of diverse tobacco carcinogens differ in the lung and the pancreas[19,47].

We examined the number of mutations in the cancers relative to a number of other clinical parameters, such as location within the pancreas (head vs. tail), sex of the patient, age of the patient, tumor grade, margin status, and stage. No statistically significant differences were found.

Limitations of this study should be acknowledged. Because 86% of the patients were deceased at the time of this study, all of the clinical parameters were collected retrospectively by review of the patient's hospital charts, the electronic patient medical records, and the Johns Hopkins Pancreatic Cancer Research Database [28]. While several studies have suggested that self-reporting may under estimate cigarette smoking, the magnitude of this under-reporting is likely small enough to have only a modest impact on our results [48–50].

In conclusion, we found that cigarette smoking is associated with greater numbers of mutations in pancreatic cancer, but that these mutations do not produce a characteristic profile.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Reference List

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96. [PubMed: 18287387]
2. Michaud DS, Giovannucci E, Willett WC, Colditz GA, Stampfer MJ, Fuchs CS. Physical activity, obesity, height, and the risk of pancreatic cancer. JAMA 2001;286:921–9. [PubMed: 11509056]
3. Silverman DT, Schiffman M, Everhart J, et al. Diabetes mellitus, other medical conditions and familial history of cancer as risk factors for pancreatic cancer. Br J Cancer 1999;80:1830–7. [PubMed: 10468306]
4. Ahlgren JD. Epidemiology and risk factors in pancreatic cancer. Semin Oncol 1996;23:241–50. [PubMed: 8623060]
5. Coughlin SS, Calle EE, Patel AV, Thun MJ. Predictors of pancreatic cancer mortality among a large cohort of United States adults. Cancer Causes Control 2000;11:915–23. [PubMed: 11142526]
6. Iodice S, Gandini S, Maisonneuve P, Lowenfels AB. Tobacco and the risk of pancreatic cancer: a review and meta-analysis. Langenbecks Arch Surg. 2008
7. Hassan MM, Bondy ML, Wolff RA, et al. Risk factors for pancreatic cancer: case-control study. Am J Gastroenterol 2007;102:2696–707. [PubMed: 17764494]
8. Raimondi S, Maisonneuve P, Lohr JM, Lowenfels AB. Early onset pancreatic cancer: evidence of a major role for smoking and genetic factors. Cancer Epidemiol Biomarkers Prev 2007;16:1894–7. [PubMed: 17855711]

9. Mulder I, Hoogenveen RT, van Genugten ML, et al. Smoking cessation would substantially reduce the future incidence of pancreatic cancer in the European Union. Eur J Gastroenterol Hepatol 2002;14:1343–53. [PubMed: 12468956]
10. Lin Y, Tamakoshi A, Kawamura T, et al. A prospective cohort study of cigarette smoking and pancreatic cancer in Japan. Cancer Causes Control 2002;13:249–54. [PubMed: 12020106]
11. Fuchs CS, Colditz GA, Stampfer MJ, et al. A prospective study of cigarette smoking and the risk of pancreatic cancer. Arch Intern Med 1996;156:2255–60. [PubMed: 8885826]
12. Boyle P, Maisonneuve P, Bueno dM, et al. Cigarette smoking and pancreas cancer: a case control study of the search programme of the IARC. Int J Cancer 1996;67:63–71. [PubMed: 8690527]
13. Hruban RH, van Mansfeld ADM, Offerhaus GJ, et al. K-*ras* oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization. Am J Pathol 1993;143:545–54. [PubMed: 8342602]
14. Le Calvez F, Mukeria A, Hunt JD, et al. TP53 and KRAS mutation load and types in lung cancers in relation to tobacco smoke: distinct patterns in never, former, and current smokers. Cancer Res 2005;65:5076–83. [PubMed: 15958551]
15. Westra WH, Slebos RJC, Offerhaus GJ, et al. K-ras oncogene activation in lung adenocarcinomas from ex- smokers: Evidence that K-ras mutations are an early an irreversible event in the development of adenocarcinoma of the lung. Cancer 1993;72:1–7. [PubMed: 8508393]
16. Slebos RJ, Hruban RH, Dalesio O, Mooi WJ, Offerhaus GJ, Rodenhuis S. Relationship between K-ras oncogene activation and smoking in adenocarcinoma of the human lung. J Natl Cancer Inst 1991;83:1024–7. [PubMed: 2072410]
17. Westra WH, Offerhaus GJ, Goodman SN, et al. Overexpression of the p53 tumor suppressor gene product in primary lung adenocarcinomas is associated with cigarette smoking. Am J Surg Pathol 1993;17:213–20. [PubMed: 8434702]
18. Brennan JA, Boyle JO, Koch WM, et al. Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck. N Engl J Med 1995;332:712–7. [PubMed: 7854378]
19. Ding L, Getz G, Wheeler DA, et al. Somatic mutations affect key pathways in lung adenocarcinoma. Nature 2008;455:1069–75. [PubMed: 18948947]
20. Ahrendt SA, Decker PA, Alawi EA, et al. Cigarette smoking is strongly associated with mutation of the K-ras gene in patients with primary adenocarcinoma of the lung. Cancer 2001;92:1525–30. [PubMed: 11745231]
21. Gandini S, Botteri E, Iodice S, et al. Tobacco smoking and cancer: a meta-analysis. Int J Cancer 2008;122:155–64. [PubMed: 17893872]
22. Pfeifer GP, Denissenko MF, Olivier M, Tretyakova N, Hecht SS, Hainaut P. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. Oncogene 2002;21:7435–51. [PubMed: 12379884]
23. Ahrendt SA, Chow JT, Yang SC, et al. Alcohol consumption and cigarette smoking increase the frequency of p53 mutations in non-small cell lung cancer. Cancer Res 2000;60:3155–9. [PubMed: 10866304]
24. Shimmyo T, Okada A, Hashimoto T, et al. Etiologic value of p53 mutation spectra and differences with histology in lung cancers. Cancer Sci 2008;99:287–95. [PubMed: 18271927]
25. Denissenko MF, Pao A, Tang M, Pfeifer GP. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53. Science 1996;274:430–2. [PubMed: 8832894]
26. Cox LA Jr, Sanders E. Estimating preventable fractions of disease caused by a specified biological mechanism: PAHs in smoking lung cancers as an example. Risk Anal 2006;26:881–92. [PubMed: 16948683]
27. Jones S, Zhang X, Parsons DW, et al. Core Signaling Pathways in Human Pancreatic Cancers Revealed by Global Genomic Analyses. Science 2008;321:1801–6. [PubMed: 18772397]
28. Herman JM, Swartz MJ, Hsu CC, et al. Analysis of fluorouracil-based adjuvant chemotherapy and radiation after pancreaticoduodenectomy for ductal adenocarcinoma of the pancreas: results of a large, prospectively collected database at the Johns Hopkins Hospital. J Clin Oncol 2008;26:3503–10. [PubMed: 18640931]

29. Leary RJ, Lin JC, Cummins J, et al. Integrated analysis of homozygous deletions, focal amplifications, and sequence alterations in breast and colorectal cancers. Proc Natl Acad Sci U S A 2008;105:16224–9. [PubMed: 18852474]
30. Parsons DW, Jones S, Zhang X, et al. An integrated genomic analysis of human glioblastoma multiforme. Science 2008;321:1807–12. [PubMed: 18772396]
31. Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. Science 2007;318:1108–13. [PubMed: 17932254]
32. Kelsey KT, Hirao T, Hirao S, et al. TP53 alterations and patterns of carcinogen exposure in a U.S. population-based study of bladder cancer. Int J Cancer 2005;117:370–5. [PubMed: 15906354]
33. Ronchetti D, Neglia CB, Cesana BM, et al. Association between p53 gene mutations and tobacco and alcohol exposure in laryngeal squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2004;130:303–6. [PubMed: 15023836]
34. Fryzek JP, Garabrant DH, Schenk M, Kinnard M, Greenson JK, Sarkar FH. The association between selected risk factors for pancreatic cancer and the expression of p53 and K-ras codon 12 mutations. Int J Gastrointest Cancer 2006;37:139–45. [PubMed: 18049799]
35. Jiao L, Zhu J, Hassan MM, Evans DB, Abbruzzese JL, Li D. K-ras mutation and p16 and preproenkephalin promoter hypermethylation in plasma DNA of pancreatic cancer patients: in relation to cigarette smoking. Pancreas 2007;34:55–62. [PubMed: 17198183]
36. Schuller HM, Zhang L, Weddle DL, Castonguay A, Walker K, Miller MS. The cyclooxygenase inhibitor ibuprofen and the FLAP inhibitor MK886 inhibit pancreatic carcinogenesis induced in hamsters by transplacental exposure to ethanol and the tobacco carcinogen NNK. J Cancer Res Clin Oncol 2002;128:525–32. [PubMed: 12384795]
37. Crous-Bou M, Porta M, Lopez T, et al. Lifetime history of tobacco consumption and K-ras mutations in exocrine pancreatic cancer. Pancreas 2007;35:135–41. [PubMed: 17632319]
38. Berger DH, Chang H, Wood M, et al. Mutational activation of K-ras in nonneoplastic exocrine pancreatic lesions in relation to cigarette smoking status. Cancer 1999;85:326–32. [PubMed: 10023699]
39. Wilentz RE, Goggins M, Redston M, et al. Genetic, immunohistochemical, and clinical features of medullary carcinoma of the pancreas: a newly described and characterized entity. Am J Pathol 2000;156:1641–51. [PubMed: 10793075]
40. Caldas C, Hahn SA, da Costa LT, et al. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. Nat Genet 1994;8:27–32. [PubMed: 7726912]
41. Hahn SA, Schutte M, Hoque AT, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 1996;271:350–3. [PubMed: 8553070]
42. Rozenblum E, Schutte M, Goggins M, et al. Tumor-suppressive pathways in pancreatic carcinoma. Cancer Res 1997;57:1731–4. [PubMed: 9135016]
43. Schutte M, Hruban RH, Geradts J, et al. Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. Cancer Res 1997;57:3126–30. [PubMed: 9242437]
44. Hruban RH, Iacobuzio-Donahue CA, Wilentz RE, Goggins M, Kern SE. Molecular pathology of pancreatic cancer. Cancer J 2001;7:251–8. [PubMed: 11561601]
45. Jones S, Chen WD, Parmigiani G, et al. Comparative lesion sequencing provides insights into tumor evolution. Proc Natl Acad Sci U S A 2008;105:4283–8. [PubMed: 18337506]
46. Wittel UA, Hopt UT, Batra SK. Cigarette smoke-induced pancreatic damage-experimental data. Langenbecks Arch Surg 2008;393:581–8. [PubMed: 18193450]
47. Prokopczyk B, Hoffmann D, Bologna M, et al. Identification of tobacco-derived compounds in human pancreatic juice. Chem Res Toxicol 2002;15:677–85. [PubMed: 12018989]
48. Patrick DL, Cheadle A, Thompson DC, Diehr P, Koepsell T, Kinne S. The validity of self-reported smoking: a review and meta-analysis. Am J Public Health 1994;84:1086–93. [PubMed: 8017530]
49. Schofield PE, Hill DJ. How accurate is in-patient smoking status data collected by hospital admissions staff? Aust N Z J Public Health 1999;23:654–6. [PubMed: 10641361]
50. Studts JL, Ghate SR, Gill JL, et al. Validity of self-reported smoking status among participants in a lung cancer screening trial. Cancer Epidemiol Biomarkers Prev 2006;15:1825–8. [PubMed: 17035388]

**Table 1**

Distribution of clinical and smoking characteristics among smokers and non-smokers, separately for the Discovery and Validation screens.

| | Discovery Screen | | Prevalence Screen | |
|---|---|---|---|---|
| | **Non-smokers N = 13** | **Smokers N = 11** | **Non-smokers N = 37** | **Smokers N = 53** |
| Age – Mean (SD) | 64.7 (12.1) | 65.5 (9.7) | 64.9 (11.3) | 65.2 (9.6) |
| Gender – No. (%) | | | | |
| Male | 6 (46.2) | 4 (36.4) | 22 (59.5) | 21 (39.6) |
| Female | 7 (53.8) | 7 (63.6) | 15 (40.5) | 32 (60.4) |
| Race – No. (%) | | | | |
| White | 12 (92.3) | 9 (81.8) | 32 (86.5) | 47 (88.7) |
| Other Race | 1 (7.7) | 2 (18.2) | 5 (13.5) | 6 (11.3) |
| Surgery – No. (%) | | | | |
| Autopsy | 4 (30.8) | 3 (27.3) | 1 (2.7) | 0 (0.0) |
| Whipple | 9 (69.2) | 6 (54.5) | 33 (89.2) | 43 (81.1) |
| Distal Pancreatectomy | 0 (0.0) | 2 (18.2) | 3 (8.1) | 10 (18.9) |
| Location of Tumor – No. (%) | | | | |
| Head | 12 (92.3) | 7 (63.6) | 34 (94.4) | 43 (84.3) |
| Tail | 1 (7.7) | 4 (36.4) | 2 (5.6) | 8 (15.7 |
| Grade – No. (%) | | | | |
| Poor | 8 (61.5) | 7 (70) | 11 (29.7) | 22 (42.3) |
| Moderate/Well | 5 (38.5) | 3 (30) | 26 (70.3) | 30 (57.7) |
| Tumor Size – No. (%) | | | | |
| < 3 cm | 3 (23.1) | 2 (18.2) | 13 (35.1) | 19 (35.8) |
| 3–5 cm | 6 (46.1) | 6 (54.5) | 20 (54.1) | 25 (47.2) |
| > 5 cm | 2 (15.4) | 2 (18.2) | 3 (8.1) | 8 (15.1) |
| Unknown (autopsy cases) | 2 (15.4) | 1 (9.1) | 1 (2.7) | 1 (1.9) |
| Margin – No. (%) | | | | |
| Negative | 6 (46.2) | 5 (45.5) | 23 (62.2) | 37 (69.8) |
| Positive | 4 (30.8) | 3 (27.3) | 13 (35.1) | 16 (30.2) |
| Unknown (autopsy cases) | 3 (23.1) | 3 (27.3) | 1 (2.7) | 0 (0.0) |
| Diabetic – No. (%) | | | | |
| No | 9 (69.2) | 7 (63.6) | 7 (87.5) | 4 (50) |
| Yes | 4 (30.8) | 4 (36.4) | 1 (12.5) | 4 (50) |
| No. of Positive Lymph Nodes – Mean (SD) | 3 (1.7) | 6 (3.6) | 3 (4.6) | 3 (3) |
| No. of Lymph Nodes – Mean (SD) | 22 (12.9) | 18 (5.3) | 15 (8.2) | 15 (8.2) |
| Smoking Status – No. (%) | | | | |
| Current | | 5 (45.5) | | 21 (39.6) |
| Former | | 6 (54.5) | | 32 (60.4) |
| Years Quit – No. (%) | | | | |
| ≤ 10 | | 2 (33.3) | | 10 (31.2) |
| > 10 | | 4 (66.7) | | 22 (68.8) |

| | Discovery Screen | | Prevalence Screen | |
|---|---|---|---|---|
| | **Non-smokers N = 13** | **Smokers N = 11** | **Non-smokers N = 37** | **Smokers N = 53** |
| Pack-Years – Mean (SD) | | 43 (23.3) | | 38 (27.9) |

**Table 2**

Mean (SD) genetic alterations for the Discovery Screen, by clinical parameters. P values for smoking-adjusted differences in the rate of mutations between patient groups. Differences between males and females exclude mutations on chromosome X. Values for age in years are regression coefficients (standard errors) for the average increase in the number of alterations with a yearly increase in age, adjusting for smoking status.

| | N | Mutations | | Deletions | | Amplifications | | Mutations, Deletions & Amplifications | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean (SD) | P | Mean (SD) | P | Mean (SD) | P | Mean (SD) | P |
| Nonsmoker | 13 | 56.2 (13.9) | 0.08 | 7.8 (4.7) | 0.58 | 5.5 (5.4) | 0.73 | 69.5 (16.4) | 0.08 |
| Smoker | 11 | 75.5 (41.7) | | 8.8 (4.3) | | 6.5 (9.1) | | 90.9 (44.4) | |
| Moderate/Well grade | 8 | 58.1 (15.6) | 0.59 | 6.5 (2.7) | 0.23 | 2.6 (4.6) | 0.06 | 67.2 (19) | 0.28 |
| Poor Grade | 15 | 68.9 (37.4) | | 8.7 (4.8) | | 7.4 (7.9) | | 84.9 (39) | |
| Aged < 70 | 16 | 60.8 (19.1) | 0.16 | 8.9 (4.6) | 0.33 | 6.6 (7.3) | 0.59 | 76.2 (24.3) | .37 |
| Aged ≥ 70 | 8 | 73.8 (47.1) | | 7 (4.2) | | 4.9 (7.3) | | 85.6 (48.2) | |
| Black | 3 | 61.3 (15.9) | 0.69 | 7 (2) | 0.54 | 0.3 (0.6) | 0.01 | 68.7 (17.2) | 0.39 |
| White | 21 | 65.6 (32.7) | | 8.4 (4.7) | | 6.8 (7.3) | | 80.9 (35.1) | |
| Female | 10 | 54.3 (13.5) | 0.35 | 6.4 (3.6) | 0.08 | 3 (4.4) | 0.05 | 63.7 (14.5) | 0.10 |
| Male | 14 | 69.3 (37) | | 9.6 (4.7) | | 8.1 (8.1) | | 87 (38.7) | |
| Non-Diabetic | 16 | 68.9 (36) | 0.34 | 8.9 (5.1) | 0.32 | 7.8 (7.9) | 0.04 | 85.6 (37.4) | 0.13 |
| Diabetic | 8 | 57.4 (15.9) | | 7 (2.7) | | 2.4 (3.9) | | 66.8 (19.8) | |
| Tail | 5 | 72.6 (24) | 0.88 | 7.8 (3.1) | 0.68 | 9 (10.9) | 0.39 | 89.4 (34.1) | 0.76 |
| Head | 19 | 63.1 (32.7) | | 8.4 (4.8) | | 5.2 (6) | | 76.7 (33.6) | |
| Age in years | | 0.96 (0.55) | 0.10 | −0.12 (0.08) | 0.16 | −0.06 (0.14) | 0.68 | 0.77 (0.61) | 0.22 |

**Table 3**

Types of mutations by smoking status for the Discovery screen. P values for differences between smokers and non-smokers, adjusting for age and gender.

| | **Non-smokers N = 13** | **Smokers N = 11** | **P** |
|---|---|---|---|
| | **Mean SD** | **Mean SD** | |
| Mutations | 56.2 (13.9) | 75.5 (41.7) | 0.06 |
| Mutations not in *KRAS*, *TP53*, *SMAD4* or *CDKN2A*/*P16* | 53.9 (14.1) | 73.5 (41.7) | 0.05 |
| Mutations not in any driver gene | 53.2 (13.8) | 73.3 (41.8) | 0.05 |
| Synonymous Mutations | 14.8 (5.4) | 18.7 (11.3) | 0.26 |
| Non-synonymous Mutations | 38.5 (11.1) | 53.1 (27.9) | 0.04 |
| Transition Mutations | 35.3 (9) | 43.7 (16.9) | 0.04 |
| Transversion Mutations | 20.9 (10.3) | 31.8 (25.9) | 0.16 |

**Table 4**

Frequency of specific sequence mutations and context for the Discovery Screen, by smoking status. Odds ratios for having each specific type of mutation for smokers v. non-smokers, adjusting for age and gender.

| | Number of mutations in non-smokers | Number of non-smokers with a specific mutation | Number of mutations in smokers | Number of smokers with a specific mutation | Odds Ratio [95% C.I.] | P |
|---|---|---|---|---|---|---|
| ***Sequence Mutations*** | | | | | | |
| A to C | 26 | 10 | 22 | 10 | 0.72 [0.33,1.54] | 0.40 |
| A to G | 32 | 12 | 50 | 11 | 1.38 [0.86,2.21] | 0.18 |
| A to T | 20 | 9 | 29 | 11 | 1.25 [0.69,2.27] | 0.46 |
| C to A | 36 | 13 | 70 | 10 | 1.6 [1.04,2.46] | 0.03 |
| C to G | 34 | 11 | 34 | 10 | 0.76 [0.45,1.26] | 0.28 |
| C to T | 214 | 13 | 197 | 11 | 0.78 [0.57,1.07] | 0.13 |
| G to A | 187 | 13 | 200 | 11 | 1.02 [0.79,1.31] | 0.89 |
| G to C | 39 | 9 | 35 | 10 | 0.8 [0.36,1.76] | 0.57 |
| G to T | 57 | 13 | 83 | 11 | 1.27 [0.88,1.85] | 0.20 |
| T to A | 8 | 6 | 20 | 8 | 2.32 [0.99,5.45] | 0.05 |
| T to C | 26 | 11 | 34 | 8 | 1.15 [0.64,2.06] | 0.63 |
| T to G | 15 | 8 | 16 | 7 | 0.85 [0.41,1.79] | 0.68 |
| Insertion or Deletion | 37 | 13 | 41 | 11 | 1.01 [0.63,1.63] | 0.96 |
| ***Context*** | | | | | | |
| A | 78 | 13 | 101 | 11 | 1.07 [0.78,1.48] | 0.66 |
| C | 90 | 13 | 104 | 11 | 0.91 [0.67,1.25] | 0.57 |
| C*pG | 152 | 13 | 140 | 11 | 0.82 [0.6,1.12] | 0.21 |
| CpG* | 128 | 13 | 143 | 11 | 1.16 [0.8,1.69] | 0.43 |
| G | 92 | 13 | 119 | 11 | 1.11 [0.82,1.5] | 0.49 |
| G*pA | 63 | 12 | 56 | 11 | 0.79 [0.41,1.53] | 0.49 |
| T | 49 | 12 | 70 | 11 | 1.25 [0.81,1.92] | 0.31 |
| TpC* | 42 | 12 | 57 | 10 | 1.16 [0.76,1.77] | 0.49 |
| ***All Sequence Mutations*** | ***731*** | ***13*** | ***831*** | ***11*** | ***1.26 [0.74,2.15]*** | ***0.40*** |

**Table 5**

Frequency distribution of the number of patients with none or at least one sequencing mutation in *KRAS*, *SMAD4*, *CDKN2A*/*P16* or *TP53*. (NS= non-smoker, S= smoker)

| | Discovery Screen | | | | | Validation Screen | | | | | Combined Samples | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Non-smokers | | Smokers | | P | Non-smokers | | Smokers | | P | Non-smokers | | Smokers | | P |
| | N | % | N | % | | N | % | N | % | | N | % | N | % | |
| ***KRAS*** | | | | | | | | | | | | | | | |
| Wild-type | 0 | (0) | 0 | (0) | - | 0 | (0) | 1 | (2) | - | 0 | (0) | 1 | (2) | 1.00 |
| Mutated | 13 | (100) | 11 | (100) | | 37 | (100) | 52 | (98) | | 50 | (100) | 63 | (98) | |
| ***SMAD4*** | | | | | | | | | | | | | | | |
| Wild-type | 8 | (62) | 8 | (73) | 0.68 | 28 | (76) | 39 | (74) | 1.00 | 36 | (72) | 47 | (73) | 1.00 |
| Mutated | 5 | (38) | 3 | (27) | | 9 | (24) | 14 | (26) | | 14 | (28) | 17 | (27) | |
| ***CDKN2A/p16*** | | | | | | | | | | | | | | | |
| Wild-type | 12 | (92) | 10 | (91) | 1.00 | 30 | (81) | 38 | (72) | 0.33 | 42 | (84) | 48 | (75) | 0.26 |
| Mutated | 1 | (8) | 1 | (9) | | 7 | (19) | 15 | (28) | | 8 | (16) | 16 | (25) | |
| ***TP53*** | | | | | | | | | | | | | | | |
| Wild-type | 2 | (15) | 4 | (36) | 0.36 | 4 | (11) | 10 | (19) | 0.38 | 6 | (12) | 14 | (22) | 0.22 |
| Mutated | 11 | (85) | 7 | (64) | | 33 | (89) | 43 | (81) | | 44 | (88) | 50 | (78) | |

**Table 6**

Frequency distribution of the number of sequencing mutations in *KRAS*, *SMAD4*, *TP53*, *CDKN2A/p16* and all other genes, by mutation type and context, comparing non-smokers and smokers, adjusting for age and gender. Some patients had more than one mutation on an individual gene, and total represents the number of mutations across all patients within smoking category (NS= non-smoker, S= smoker).

| | ***KRAS*** | | | ***SMAD4*** | | | ***TP53*** | | | ***CDKN2A/p16*** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **NS** | **S** | **P** | **NS** | **S** | **P** | **NS** | **S** | **P** | **NS** | **S** | **P** |
| *Sequence Mutations* | | | | | | | | | | | | |
| A to C | 1 | 2 | 0.99 | 1 | 0 | >0.99 | 0 | 1 | >0.99 | 0 | 1 | >0.99 |
| A to G | 0 | 1 | >0.99 | 0 | 2 | >0.99 | 4 | 3 | 0.89 | 0 | 0 | - |
| A to T | 0 | 0 | - | 0 | 0 | - | 2 | 1 | 0.99 | 0 | 1 | >0.99 |
| C to A | 0 | 0 | - | 0 | 0 | - | 1 | 2 | 0.99 | 0 | 1 | >0.99 |
| C to G | 0 | 0 | - | 1 | 0 | >0.99 | 1 | 2 | 0.99 | 1 | 2 | 0.85 |
| C to T | 0 | 0 | - | 6 | 3 | 0.17 | 9 | 10 | >0.99 | 4 | 6 | 0.32 |
| G to A | 26 | 34 | 0.9 | 0 | 3 | >0.99 | 9 | 12 | 0.58 | 0 | 2 | >0.99 |
| G to C | 6 | 10 | 0.99 | 0 | 1 | >0.99 | 1 | 0 | >0.99 | 0 | 1 | >0.99 |
| G to T | 17 | 17 | 0.37 | 2 | 1 | 0.91 | 5 | 4 | 0.85 | 1 | 1 | 0.99 |
| InDel | 0 | 0 | - | 4 | 7 | 0.39 | 11 | 8 | 0.36 | 2 | 4 | 0.77 |
| T to A | 0 | 0 | - | 0 | 0 | >0.99 | 1 | 2 | 0.99 | 0 | 0 | - |
| T to C | 0 | 0 | - | 1 | 0 | >0.99 | 2 | 3 | 0.99 | 0 | 0 | - |
| T to G | 0 | 0 | - | 0 | 0 | - | 1 | 2 | 0.99 | 0 | 1 | >0.99 |
| ***Context*** | | | | | | | | | | | | |
| A | 1 | 3 | 0.99 | 1 | 2 | 0.99 | 6 | 5 | 0.86 | 0 | 2 | >0.99 |
| C | 0 | 0 | - | 1 | 0 | >0.99 | 2 | 2 | >0.99 | 2 | 5 | >0.99 |
| C*pG | 0 | 0 | - | 4 | 1 | 0.14 | 8 | 11 | 0.99 | 3 | 4 | 0.34 |
| CpG* | 0 | 0 | - | 0 | 0 | - | 5 | 8 | 0.97 | 0 | 0 | - |
| G | 49 | 61 | 0.99 | 1 | 4 | 0.98 | 4 | 7 | 0.99 | 0 | 3 | >0.99 |
| G*pA | 0 | 0 | - | 1 | 1 | 0.99 | 6 | 1 | 0.67 | 1 | 1 | 0.99 |
| T | 0 | 0 | - | 1 | 0 | >0.99 | 4 | 7 | 0.99 | 0 | 1 | >0.99 |
| TpC* | 0 | 0 | - | 2 | 2 | >0.99 | 1 | 1 | >0.99 | 0 | 0 | |
| ***Total Mutations*** | ***50*** | ***64*** | ***>0.99*** | ***15*** | ***17*** | ***0.12*** | ***47*** | ***50*** | ***>0.99*** | ***8*** | ***20*** | ***0.02*** |