An Immune-Inflammation Gene Expression Signature in Prostate Tumors of Smokers

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Abstract

Smokers develop metastatic prostate cancer more frequently than nonsmokers, suggesting that a tobacco-derived factor is driving metastatic progression. To identify smoking-induced alterations in human prostate cancer, we analyzed gene and protein expression patterns in tumors collected from current, past, and never smokers. By this route, we elucidated a distinct pattern of molecular alterations characterized by an immune and inflammation signature in tumors from current smokers that were either attenuated or absent in past and never smokers. Specifically, this signature included elevated immunoglobulin expression by tumor-infiltrating B cells, NF-κB activation, and increased chemokine expression. In an alternate approach to characterize smoking-induced oncogenic alterations, we also explored the effects of nicotine in human prostate cancer cells and prostate cancer–prone TRAMP mice. These investigations showed that nicotine increased glutamine consumption and invasiveness of cancer cells in vitro and accelerated metastatic progression in tumor-bearing TRAMP mice. Overall, our findings suggest that nicotine is sufficient to induce a phenotype resembling the epidemiology of smoking-associated prostate cancer progression, illuminating a novel candidate driver underlying metastatic prostate cancer in current smokers. Cancer Res; 76(5); 1055–65. © 2015 AACR.

Introduction

Prostate cancer is a leading cause of cancer mortality among men (1). Few environmental factors have been consistently asso-

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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do: 10.1158/0008-5472.CAN-14-3630
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paraffin-embedded (FFPE) tumor specimens were collected at UMD. Tissue collection was approved by the institutional review boards at the participating institutions. Written informed consent was obtained from all donors. CPCTR has been described previously (11). Smoking information at the time of surgery (current, past, never) was obtained from medical records and cancer registry entries for CPCTR and JHU. For patients from UMD, this information was abstracted from an epidemiologic questionnaire. The human immortalized prostate epithelial cell line, RWPE-1, and human prostate cancer cell lines (22Rv1, PC-3, LNCaP, DU145) were obtained from the ATCC between 2006 and 2010. Authentication of these cell lines was performed in December, 2013, using a short tandem repeat analysis with GenePrint10 (9 loci and 2010. Authentication of these cell lines was performed in December, 2013, using a short tandem repeat analysis with GenePrint10 (9 loci + amelogenin for sex determination). For details on tissue collection and assessment of smoking status, see Supplementary Methods.

RNA extraction from frozen bulk tissue and cell lines

Tissue macrodissection and isolation of total RNA from tissues and cell lines was performed according to standard methods described in Supplementary Methods.

Affymetrix microarrays

RNA labeling and hybridization were performed according to Affymetrix standard protocols, as described previously (12). Labeled cRNA was hybridized either to Affymetrix GeneChip HG-U133A 2.0 or mouse 1.0 ST arrays. In accordance with Minimum Information About a Microarray Experiment guidelines, we deposited the CEL files for the microarray data and additional patient information into the Gene Expression Omnibus (GEO) repository [http://www.ncbi.nlm.nih.gov/geo/]. The GEO submission accession number for the 47 bulk tissue tumors, which were initially analyzed, is GSE6956. GSE68138 contains the gene expression data for the additional 20 bulk tissue tumors (JHU samples) and the laser capture microdissected tumor samples (n = 10), and for prostate tumors from TRAMP mice ± nicotine treatment (n = 10), and cell lines (22Rv1 and LNCaP cells) ± nicotine treatment (n = 12). For more information, see Supplementary Methods.

RNA isolation from microdissected prostate tumors

Enriched tumor epithelium was obtained from 5 current and 5 never smokers with laser capture microdissection (LCM) of frozen tissue samples. These tumors were also analyzed as bulk tissues. A total of 5,000 to 15,000 cells per tumor were collected. RNA was isolated using the PicoPure protocol (Arcturus). mRNA was amplified with two linear amplification steps by in vitro transcription using the MEGAscript T7 Kit (Ambion) followed by labeling using the BioArray HighYield RNA Transcription Labeling Kit T3 from Enzo Life Sciences. Labeled cRNA was hybridized onto arrays.

Data normalization and statistical analysis of gene expression data

All chips were normalized using the Robust Multi-array Average procedure (13). Because two sets of array data were analyzed for human prostate tumors, we controlled for a batch effect using the Partek Genomics Suite (www.partek.com) or the Bioconductor limma R package (www.bioconductor.org). To generate lists of differently expressed genes, the resulting datasets were subjected to the significance analysis of microarray procedure (14) or linear modeling features implemented in limma. Supplementary Tables S7–S11 describe differentially expressed genes in LCM tumor epithelium comparing current (n = 5) versus never smokers (n = 5; S7–S8), nicotine-treated (n = 3) versus untreated (n = 3) 22Rv1 and LNCaP cells (S9–S10), and prostate tumors from nicotine-treated (n = 5) versus untreated (n = 5) TRAMP mice (S11), respectively. For more information, see Supplementary Methods.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed as described previously (15). For details, see Supplementary Methods.

Additional methods

For more information on methods not described (qPCR of gene expression; in situ hybridization for immunoglobulin κ and λ light chain expression in prostate tumors and IHC; proliferation, motility, and invasion assays of nicotine-treated cells; integrin cell surface expression and extracellular matrix protein-binding assays; Western blot analysis of nicotine-treated cells; and measurement of IL8 in human plasma samples), see Supplementary Methods.

Glutamine consumption in nicotine-treated prostate cancer cells

22Rv1 and LNCaP cells were plated in T150 flasks, serum starved, and treated with 100 nmol/L nicotine. One milliliter of media was collected and cell pellets were prepared. Dried extracts of these samples were resuspended in injection solvent composed of water:methanol (50:50) and subjected to LC/MS. Details can be found in Supplementary Methods.

Nicotin treatment of prostate cancer–prone TRAMP mice and evaluation of lung metastasis

Male TRAMP mice were bred at the Assisted Reproduction Laboratory, Frederick National Laboratory for Cancer Research (Frederick, MD), using in vitro fertilization (B6×FVB F1). At 8 to 9 weeks of age, they received either tap water or a solution of either 100 or 250 µg/ml of nicotine in tap water, which is similar to a previous described protocol (16). The three groups consisted of 20 to 25 animals each. At the selected concentration, nicotine generates nicotine plasma concentrations comparable with those of active smokers and causes some weight loss (Supplementary Fig. S1). All mice were euthanized after 80 days or when they became moribund because of prostate cancer. To assess the effects of nicotine on prostate cancer development and metastasis, the prostate glands and lungs were collected and were formalin-fixed for histologic examination by a boarded veterinary pathologist. All described animal procedures were reviewed and approved by the NCI-Frederick Institutional Biosafety Committee (IBC registration #06-060 and 11-041). NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. More details can be found in Supplementary Methods.

Statistical analysis

Statistical analyses were performed using STATA (Stata Corp) or GraphPad Prism 6 (GraphPad Software). All statistical tests were
two sided and an association was considered statistically significant with \( P < 0.05 \). The Spearman rank correlation (e.g., for continuous B-cell numbers in never, past, current smokers) or the Fisher exact tests (e.g., for nuclear \( p\)-NF-kB stratified into present or absent in never, past, current smokers) were used to calculate \( P_{\text{corrd}} \). The Wilcoxon rank-sum test was used as a nonparametric statistical test to compare two independent groups. Comparisons among more than two independent groups were performed with the ANOVA and Kruskal–Wallace tests.

### Results

A smoking-associated gene expression signature in prostate tumors

We evaluated gene expression characteristics from tumors comparing current with past and never smokers. Patients are described in Supplementary Table S1: current, past and never smokers did not differ significantly by age, race/ethnicity, or clinicopathology. Initially, we analyzed the gene expression profiles of 47 tumors from 9 current, 21 past, and 17 never smokers using Affymetrix GeneChip microarrays. This analysis revealed an immune signature in tumors from current smokers. The most upregulated transcripts among current smokers represented immunoglobulins (Supplementary Table S2). When we performed a hierarchical cluster analysis, immunoglobulin expression separated tumors into two clusters (Fig. 1). Tumors from current smokers were significantly overrepresented in cluster 2, which consisted of tumors with upregulated immunoglobulin expression. Furthermore, we applied a linear regression model to examine whether the differences in immunoglobulin expression by smoking status are confounded by race/ethnicity and found that these differences were independent of race/ethnicity.

To further investigate the immunoglobulin signature, we conducted \( \text{in situ} \) hybridization (ISH) for signature validation, and to localize expression. ISH for both \( \kappa \) and \( \lambda \) light chain mRNA expression was performed on additional 22 FFPE tumors (6 current, 7 past, 9 never smokers). This approach revealed an elevated number of immunoglobulin-expressing B lymphocytes in tumors of current smokers compared with past and never smokers (Fig. 2). The lymphocytes infiltrated the tumor stroma (Fig. 2A and B and Supplementary Fig. S2). Average number of \( \lambda \) light chain–positive B lymphocytes per \( 250 \times 250 \) field increased from 3.6 (range: 0–24) among never smokers to 5 (range: 0–16) among past smokers to 23 (range: 1–86) among current smokers (Spearman rank correlation, \( r = 0.51; P = 0.02 \); Fig. 2C and D).

Because our initial analysis described only few genes other than immunoglobulins as being altered in tumors from current smokers, gene expression profiles from additional patients (JHU dataset: 7 current, 7 past, 6 never smokers) were combined with the discovery dataset to allow identification of more genes that are differentially expressed between current and never/past smokers. For the combined dataset (67 tumors), we generated two gene lists using Significance Analysis of Microarrays: one for differentially expressed genes between current (\( n = 16 \)) and never smokers (\( n = 23 \)), and one for differentially expressed genes between current (\( n = 16 \)) and never/past smokers (\( n = 51 \)). The comparison of tumors from current and never smokers yielded 98 transcripts that represented 73 differentially expressed genes at a false-discovery rate \( \leq 30\% \) (Supplementary Table S3). The second comparison, current versus never/past smokers, yielded 70 transcripts representing 40 differently expressed genes (Supplementary Table S4). Notably, many of the differentially expressed genes in the two lists have known immune-regulatory functions, and their expression was increased in current smokers [e.g., immunoglobulins, indoleamine-2,3-dioxygenase (IDO1), and chemokines]. Quantitative (q)RT-PCR analysis confirmed overexpression of immunoglobulins (IGH, IGL, IGK, IGL1, IDO1, and several chemokines (CCL5, CXCL10, CXCL11) among current smokers in an analysis of 57 tumors (15 current, 18 never, 24 past smokers) from the microarray study (Supplementary Fig. S3) whereas CXCL8 (IL8) did not validate. Expression of these immune genes tended to be low or absent in tumors from never or past smokers. Lastly, we generated additional gene lists for classification using Bioconductor limma R (Supplementary Fig. S4). Differentially expressed genes were then assessed using the linear modeling features implemented in limma. \( P \) values \( < 0.05 \) were used to generate two gene lists for current versus never and current versus never/smokers. Probesets with same differential expression in both gene lists (\( n = 601 \), Supplementary Table S5) were selected for classification. As shown in Fig. 3, the gene expression pattern defined by these probesets separated the 67 tumors into two clusters with greatly different gene expression. Upregulation of genes in immune-related pathways was the main characteristic of tumors from current smokers that differentiated them from others.

Nuclear accumulation of NF-kB in tumors and increased IL8 in blood of current smokers

Next, we tested whether increased stress signaling through NF-kB may occur in tumors from smokers because B-cell activation has been linked to NF-kB signaling (17). We used IHC to determine nuclear localization of phosphorylated NF-kB, \( p\delta 5 \) subunit (Ser536), in the tumor epithelium as described previously (18), to assess NF-kB activation (Fig. 4A and B). Analyzing 69 tumors, we found nuclear NF-kB \( p\delta 536 \) in 5/26 tumors from never smokers (19%), 12/24 tumors from past smokers (50%), and 11/19 tumors from current smokers (58%; \( P_{\text{wald}} = 0.014 \); Fisher exact test). Thus, accumulation of phosphorylated NF-kB correlated with smoking status. Because our microarray analysis initially indicated that IL8 is upregulated in tumors from current smokers, we also examined plasma levels of IL8 in 97 prostate cancer patients and 89 controls to assess whether circulating IL8 is increased in patients who are current smokers. The analysis showed that IL8 levels were significantly elevated in plasma from current smokers with prostate cancer (Fig. 4C), but not among current smokers without the disease (Fig. 4D).

**GSEA**

We used GSEA to identify common features between the smoking-related gene signature in prostate tumors, nicotine-induced gene signatures in human prostate cancer cells, and archived signatures in the Molecular Signatures Database (MSigDB) (http://www.broad.mit.edu/gsea/msigdb; ref. 15). We aimed to identify candidate mechanisms by which smoking may induce gene expression alterations in the cancerous prostate, and to better define possible roles of nicotine.

Four signatures were subjected to GSEA. The first contained genes that were differentially expressed between current and never smokers in bulk tumors. The second signature was derived...
from the same contrast, but used microdissected tumor epithelium from 5 current and 5 never smokers as the source for the mRNA. The last two signatures were generated from LNCaP and 22Rv1 prostate cancer cells treated with 100 nmol/L nicotine (vs. untreated), which is within the physiologic concentration range for nicotine in current smokers (~10–500 nmol/L blood; refs. 19, 20). Results are summarized in Supplementary Fig. S5. In short, a hepatocyte growth factor (HGF)-induced gene signature in monocytes (21) and a glutamine starvation signature (22) were the two MSIGDB-archived gene signatures with significant associations among all four gene lists. The data suggest that nicotine may influence cell metabolism, leading to
Increased glutamine consumption, and also exerts functions that may mimic HGF in human prostate tumors. Glutamine deprivation can occur when glutamine is excessively metabolized, and it has been shown that glutamine deprivation leads to NF-κB activation (23). To validate this GSEA prediction, we examined glutamine consumption of nicotine-treated prostate cancer cells. As shown in Fig. 5A and B, nicotine increased glutamine consumption in these cells, resulting in glutamine deprivation in culture medium.

Nicotine activates the Akt pathway and induces a prometastatic phenotype

Nicotine may have prometastatic properties in prostate cancer patients. We tested this hypothesis but first examined whether nicotinic acetylcholine receptors (nAChR) are expressed in the cancerous prostate. qRT-PCR showed that various nAChR subunits are expressed in prostate tumors and cancer cell lines (Supplementary Fig. S6). Notably, the nAChR α7 subunit, which has been linked to PI3K-Akt pathway activation and other oncogenic effects (24), was significantly upregulated in tumors. Next, we investigated whether nicotine activates oncogenic Akt signaling in human prostate cancer cell lines and in RWPE-1 immortalized prostate epithelial cells. Treatment of 22Rv1 prostate cancer cells with 10 nmol/L and 100 nmol/L nicotine led to phosphorylation of Akt (the key activating step for Akt) and its downstream targets, for example, GSK3β and human MDM2 (Fig. 5C–E). Akt pathway activation was also observed in other cell lines (Supplementary Fig. S7). Both mecamylamine, an inhibitor of nAChR signaling, and the PI3 kinase inhibitor, LY294002, blocked nicotine-induced Akt phosphorylation (Fig. 5D and Supplementary Fig. S7B). Next, we assessed whether nicotine can induce a metastatic phenotype in prostate cancer cells and evaluated migration and Matrigel invasion in response to nicotine. Nicotine enhanced Matrigel invasion of both 22Rv1 and PC-3 cells (Fig. 6A and B), which further increased when nicotine and HGF were added together (Fig. 6C). However, only nicotine-induced, but not HGF-induced, invasion was inhibited by mecamylamine (Fig. 6D). Nicotine also enhanced migration of 22Rv1 cells but did not increase it in PC-3 cells (Supplementary Fig. S8). Our findings suggest that nicotine may selectively enhance invasive properties of prostate cancer cells. This hypothesis was further supported by the observation that nicotine affected cell surface integrin expression and extracellular matrix binding, as nicotine increased binding of 22Rv1 cells to the bone-associated filaments, collagen type I and IV (Supplementary Fig. S9), which is associated with metastasis-promoting integrin signaling (25, 26).

Nicotine accelerates the onset of metastasis in TRAMP mice

Our experiments showed that nicotine induces metastasis-related phenotypes in cell culture. For further corroboration, we evaluated the effect of nicotine on metastasis in TRAMP mice, which develop aggressive prostate tumors at 100% penetrance and start to develop visible pulmonary metastatic lesions at 24 weeks of age (27). We treated animals with 100 or 250 μg nicotine/mL in the drinking water (n = 20–25) and assessed primary tumor growth and metastasis to the lung after 80 days...
of treatment. Our treatment regimens yielded an average 34 nmol/L (5.5 ± 5.2 ng nicotine/mL, n = 6) or 105 nmol/L (17.1 ± 9.0 ng/mL, n = 6) plasma nicotine concentrations, respectively, whereas nicotine was undetectable in untreated animals (P < 0.01). Nicotine did not increase the size of the cancerous prostate (Table 1), consistent with our observation that 100 nmol/L nicotine either did not or only modestly enhance proliferation of RWPE-1, 22Rv1, or PC-3 cells (Supplementary Figs. S10 and S11). In addition, we did not observe significant histologic differences in the cancerous prostate.
between the treatment groups. However, the nicotine treatment produced metastatic lesions in the lung that were not present in control animals (Table 1), indicating it accelerates the onset of metastasis in this model. A total of 13 out of 45 nicotine-treated animals (29%) were positive for lung metastasis, compared with none of the 20 controls (\(P = 0.006\), Fisher exact test). To gain insights on the nicotine-induced molecular alterations, we analyzed gene expression in the cancerous prostate of untreated and 250 \(\mu\)g/ml nicotine-treated TRAMP mice (\(n = 5\) each group). The analysis showed that nicotine-treated tumors have increased expression of genes regulating synaptic signal transduction (Supplementary Table S6). An Ingenuity pathway
analysis also suggested an association of the differentially expressed genes with G2–M DNA damage checkpoint regulation \((P = 1.1 \times 10^{-6})\), mitotic roles of Polo-like kinases \((P = 6.6 \times 10^{-6})\), and the complement system \((P = 4.7 \times 10^{-5})\), and their strongest disease association was with cancer \((P = 3.4 \times 10^{-13})\). Lastly, we did not observe an immune signature with B-cell infiltration in these tumors.

**Discussion**

In this study, we describe an immune and inflammation signature in prostate tumors from current smokers. We further discovered that nicotine increases invasiveness of human prostate cancer cells and accelerates the onset of metastases in tumor-bearing TRAMP mice. These observations point to previously unrecognized mechanisms by which smoking may enhance prostate cancer progression. While mechanistically novel, they are in agreement with epidemiologic studies and a recent publication describing inflammation in prostate tumors of current smokers (28). Also, a systematic review of the relationship between smokeless tobacco and cancer revealed that prostate cancer is one of the few cancers associated with the use of smokeless tobacco (29), which is a key source of nicotine and nicotine-derived nitrosamines, which both activate nAChRs (9, 10, 30).

Our analysis of prostate tumors indicated an increased presence of immunoglobulin-expressing B cells in tumors of current smokers whereas nicotine did not increase their numbers in tumors of TRAMP mice. B-cell numbers are commonly increased in human prostate tumors, but this increase was not found to correlate with standard markers of disease.

**Table 1. Lung metastasis in nicotine-treated TRAMP mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tap water</th>
<th>Nicotine 100 μg/mL</th>
<th>Nicotine 250 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td># of animals</td>
<td>20</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Lung (number examined)</td>
<td>20</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Lung metastasis</td>
<td>(0%)</td>
<td>6 (28%)</td>
<td>7 (31%)^a</td>
</tr>
<tr>
<td>Adenocarcinoma with metastasis to the lung</td>
<td>(0%)</td>
<td>1 (5%)</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Neuroendocrine carcinoma with metastasis to the lung</td>
<td>(0%)</td>
<td>5 (23%)</td>
<td>5 (22%)</td>
</tr>
<tr>
<td>Lymph node (# examined)</td>
<td>3</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>(0%)</td>
<td>2 (29%)^b</td>
<td>1 (33%)^b</td>
</tr>
<tr>
<td>Urogenital tract weight (mg) without seminal vesicles^c</td>
<td>592 ± 231</td>
<td>513 ± 195</td>
<td>532 ± 462</td>
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^aP = 0.046; Fisher exact test for trend.
^bNeuroendocrine histology.
^cNot significantly different between groups in both the animal weight-adjusted and unadjusted analysis (ANOVA test).
aggressiveness [31]. Although their presence in primary tumors may not immediately have a progression-enhancing effect, their increase in current smokers could be deleterious at the transition to a castration-resistant prostate cancer (CRPC). It was shown that B cells accelerate this transition in a CRPC mouse model while B-cell depletion delayed CRPC development [32]. The critical role of tumor infiltrating B cells was attributed to lymphotoxin-β secretion enhancing inflammation in the animal study. We examined lymphotoxin-β plasma levels in prostate cancer patients but could not detect elevated lymphotoxin-β in current smokers (Supplemental Fig. S12). Perhaps, increased lymphotoxin-β is restricted to the tumor microenvironment. Alternatively, B cells may require stimuli for lymphotoxin-β release that specifically arise in the environment of CRPC.

Several studies reported that B cells enhance cancer development. In a mouse model of skin carcinogenesis, cancer progression driven by chronic inflammation was shown to be B cell–dependent [33]. Here, deposition of circulating immune complexes into the tumor parenchyma led to the release of proangiogenic and prometastatic molecules [34]. Similarly, we observed NF-kB activation and increased expression of chemokines in prostate tumors of current smokers with high B-cell counts, indicative of a proinflammatory tumor microenvironment. One of the chemokines, CCL5, has been linked to disease progression of multiple cancers including prostate cancer [35]. Likewise, increased NF-kB signaling predicts prostate cancer progression in prostate [36, 32]. Hence, our finding that prostate tumors of current smokers tend to have an immune response consistent with their increased metastatic potential.

Nicotine has oncogenic properties that meet the criteria of a prometastatic factor [10]. Because we could not directly examine the effects of nicotine in cancer patients, we investigated them in cell culture and TRAMP mice and observed that nicotine increases glutamine consumption of cancer cells and enhances invasion and metastasis. Increased glutamine consumption is a hallmark of cancer and predicts poor survival in breast cancer [37]. In TRAMP mice, primary tumors from nicotine-treated animals showed increased expression of genes regulating synaptic signal transduction, whereas nicotine-driven Akt pathway activation was prominent in cell culture. Akt signaling enhances prostate cancer progression [38, 39]. The cancer-promoting effects of nicotine have also been evaluated in animal models of lung cancer. Although one study reported that nicotine promotes tumor growth and metastasis [40], two other studies could not find a nicotine effect on tumor growth [41, 42]. Thus, the effect of nicotine on tumor growth is controversial and model-dependent. Moreover, metastasis-related rather than tumor growth–related phenotypes may develop in nicotine-treated animals. We believe that the use of TRAMP mice was justified because this model captures a neuroendocrine differentiation that is also observed in CRPC [43]. Nicotine may promote progression of cancers with neuroendocrine features such as a subset of castration-resistant tumors and metastatic prostate cancers, or the aggressive small-cell lung cancer [44]. In addition, autonomic nerve development has recently been shown to contribute to prostate cancer progression [45]. Note-worthy, in this context, is our finding that nicotine increased expression of genes in synaptic signal transduction, thus potentially increasing nerve development and signaling.

Finally, we found that circulating IL8 levels are increased in current smokers with prostate cancer. IL8 expression can be induced by nicotine in human neutrophils [46], and its expression correlates with metastasis in prostate cancer [47, 48]. Although nicotine may induce IL8 directly, upregulated IL8 in smokers may also arise from other mechanisms, for example, activation of monocytes by various smoking-related xenobiotics. Nonetheless, the finding of increased IL8 in current smokers with prostate cancer reveals another candidate mechanism by which tobacco use following a prostate cancer diagnosis could enhance metastasis.

In summary, our study uncovered several mechanisms by which smoking may increase metastasis in prostate cancer patients. However, our study has few limitations; for example, we used TRAMP mice to show that nicotine accelerates metastasis in vivo. These animals develop mainly neuroendocrine tumors that are different from typical adenocarcinomas. Nevertheless, our findings point to the need of additional mechanistic and population-based studies, to define the relative contribution of nicotine to prostate cancer metastasis in current smokers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.A. Glynn, M. Yi, J. Luo, K.E. Stagliano, J.W. Gillespie, R.S. Hudson, J.L. Shoe, D.C. Haines, S.V. Jordan, J.F. Borin, M.J. Naslund, R.B. Alexander, C.A. Loffredo, N. Putluri, A. Steekum, A.A. Hurwitz


Writing, review, and/or revision of the manuscript: R.L. Prueitt, T.A. Wallace, S.A. Glynn, M. Yi, J. Luo, K.E. Stagliano, J.W. Gillespie, A. Terunuma, D.C. Haines, M. Han, D.N. Martin, C.A. Loffredo, N. Putluri, A. Steekum, A.A. Hurwitz, S. Ambs

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.L. Prueitt, T.A. Wallace, S.A. Glynn, M. Yi, J. Luo, T.H. Dorney, K.E. Stagliano, D.C. Haines, D.H. Lee

Study supervision: C.A. Loffredo, A.A. Hurwitz, S. Ambs

Acknowledgments

The authors thank CPCTR for providing tissue specimens and supporting data and also thank personnel at the University of Maryland and the Baltimore Veterans Administration Hospital for their contributions with the recruitment of subjects.

Grant Support

This research was supported by the Intramural Research Program of the NIH, NCI, Center for Cancer Research, and was also funded with federal funds from the NCI under Contract No. HHSN261200800001E. In addition, grants to A. Steekum and N. Putluri supported this work [NB15SW112-1-0130 from DOD, DMS 1161759 from NSF, NIH 1PO1 CA167234, CPRIT Metabolomics Core-RP120092 Alkek CMD Grants]. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 10, 2014; revised October 13, 2015; accepted December 7, 2015; published OnlineFirst December 30, 2015.
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