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Feasibility of using an epigenetic marker of risk for lung cancer, methylation of p16, to promote smoking cessation among US veterans

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ABSTRACT

Introduction: Providing smokers feedback using epigenetic markers of lung cancer risk has yet to be tested as a strategy to motivate smoking cessation. Epigenetic modification of Rb-p16 (*p16*) due to tobacco exposure is associated with increased risk of developing lung cancer. This study examined the acceptance of testing for methylated *p16* and the understanding of test results in smokers at risk for development of lung cancer.

Methods: Thirty-five current smokers with airways obstruction viewed an educational presentation regarding *p16* function followed by testing for the presence of methylated *p16* in sputum. Participants were offered smoking cessation assistance and asked to complete surveys at the time of enrolment regarding their understanding of the educational material, perception of risk associated with smoking and desire to quit. Participants were notified of their test result and follow-up surveys were administered 2 and 10 weeks after notification of their test result.

Results: Twenty per cent of participants had methylated *p16*. Participants showed high degree of understanding of educational materials regarding the function and risk associated with *p16* methylation. Sixty-seven per cent and 57% of participants with low-risk and high-risk test results, respectively, reported that the information was more likely to motivate them to quit smoking. Smoking cessation rates were similar between methylated and non-methylated participants.

Conclusions: Testing for an epigenetic marker of lung cancer risk is accepted and understood by active smokers. A low-risk test result does not decrease motivation to stop smoking.

Trial registration number: NCT01038492.

KEY MESSAGES

- Limited data exists on the efficacy of biomarkers to promote smoking cessation.
- Evidence of epigenetic changes related to tobacco exposure that increase risk of developing lung cancer are accepted and understood by heavy smokers
- Low-risk test results do not decrease motivation to stop smoking.

INTRODUCTION

Lung cancer remains the leading cause of cancer death in the USA, with 224 210 new cases diagnosed, and 159 260 deaths estimated for 2014.[1] Among those diagnosed with lung cancer, 79–90% are current or ever cigarette smokers,[2] and despite the strong associations between smoking and lung cancer, 16% of adults in the USA continue to smoke based on 2011 estimates.[3] The most important and cost-effective strategy for the prevention of death due to lung cancer is smoking avoidance and cessation. Yet rates of cessation remain as low as 4–7% in the general population.[4]

Enhancing perceived smoking-related risk via use of biomarker testing may help motivate smokers to quit. Strategies to motivate and achieve cessation that have used biomarkers of exposure (eg, carbon monoxide feedback) and harm (eg, pulmonary function testing and identification of atherosclerotic plaque using ultrasonography) have had limited success.[5 6] Similarly, putative genetic-risk markers of lung cancer susceptibility, such as *CYP2D4*, *GSTM1* and L-*myc* have failed to enhance cessation among adult smokers.[7–10] However, biomarkers that signal disease progression as a result of tobacco use have never been tested as a way to motivate and achieve cessation. One group of such biomarkers is tumour suppressor genes that are silenced via epigenetic alterations.

Epigenomic alterations and abnormalities are viewed as one of the earliest processes of cancer initiation.[11–13] Lung cancer develops

through a multistep process that includes genetic and epigenetic alterations. Among the biochemical pathways whose alterations have been shown to contribute to transformation of a normal to malignant cell is Rb-p16 regulation of the G1-S transition of the cell cycle. In lung cancers and preinvasive lesions of bronchial epithelium, *p16* is commonly inactivated and the most common mechanism of inactivation is transcriptional silencing associated with methylation of the promoter region.[14] The presence of methylation within the promoter region of *p16* can be detected among an excess of normal (unmethylated) *p16* DNA.

There is an association between carcinogens in tobacco smoke and DNA methylation of the promoter region, suggesting that methylation of *p16* should be found more often in current compared with non-smokers or ever smokers.[15 16] This is the case.[17–20] In a prospective analysis, methylation of the *p16* gene in the sputum of heavy smokers with forced expiratory volume in 1 s (FEV_1) <75% was associated with a nearly twofold increased risk of developing lung cancer.[21]

We report the results of a pilot study involving patients from general pulmonary and internal medicine clinics at a large veterans administration hospital who were approached for testing of *p16* methylation. Our hypothesis was that presenting epigenetic evidence of progression towards development of lung cancer via methylation of *p16* would motivate smokers to quit. The primary goals were to determine participant acceptance and understanding of *p16* testing, with secondary goals of assessing the effect of test feedback on perceived risk of developing lung cancer, desire to quit and smoking behaviour.

METHODS AND MATERIALS

Study design

This study was approved by the Durham Veterans Administration IRB Committee. In order to increase the likelihood of finding an adequate number of participants with methylated p16, recruitment was restricted to those with a minimum 30 pack-year smoking history and evidence of airway obstruction on spirometry with a FEV_1 to forced vital capacity (FVC) ratio of ≤70%. Exclusion criteria were a history of respiratory tract malignancy, inability to understand English and inability to provide informed consent.

After informed consent was obtained and spirometry performed, qualifying participants received an educational slideshow about p16 function (see Baseline procedures section). Participants then received a baseline survey to assess recall and understanding of the educational material, feelings about undergoing testing, current desire to stop smoking, current smoking behaviour and demographic information. Participants were provided with a mailing tube containing 10 mL of Saccomano's fixative and instructions to provide three samples of morning sputum and to return them by mail. Sputum samples were assayed for methylated p16. Participants were notified by mail of their testing result. Two weeks after the result, participants completed a survey over the phone assessing recall and interpretation of their test result, and smoking behaviour. A final battery of questions was administered over the phone 2 months after the first follow-up call regarding perceptions of smoking-related risks, smoking-related health effects and smoking behaviour.

Participant recruitment

Participants were recruited from internal and pulmonary medical clinics at the Durham Veterans Administration Medical Center. The study coordinator reviewed clinic notes from patients to identify active smokers with at least a 30 pack-year smoking history. Identified patients were approached during their clinic visits to assess their interest in a study to see whether information about smoking-related changes in their genes would motivate them to stop smoking. They were offered $40 for completing the study.

A total of 55 patients were approached from April 2009 to April 2010 to participate in this study. Eight patients were not enrolled due to: inadequate smoking history (<30 pack-year history; 1 patient), not currently smoking (1 patient), history of lung cancer (1 patient), excessive anxiety regarding testing (2 patients), too great a distance to travel (1 patient) and not interested in participating (2 patients). An additional 12 participants consented but were excluded because their FEV_1/FVC ratio was >70%. This left 35 participants of the 55 initially screened who formed the study cohort. Two participants expired prior to the first follow-up call, and 2 participants could not be reached for either follow-up call, leaving complete data on 31 participants. None of the four participants lost to follow-up had methylated *p16*.

Baseline procedures

After obtaining informed consent, spirometry was performed using a calibrated, hand-held spirometer by a trained study coordinator to ensure participants met enrolment criteria. Participants were then presented with a 20 min educational slideshow on the function of the *p16* gene and the role of methylation of *p16* in the development of lung cancer (educational materials are available on request). Participants were told that *p16* acts like a switch that controls the duplication of cells. Additionally, participants were informed that when the switch is turned off by methylation, the cells duplicate in an uncontrolled fashion, leading to a doubling of their risk of developing lung cancer. Participants were then provided with a survey assessing their understanding of the educational material, interest in being tested, current smoking behaviour, perceived lung cancer risk and desire to quit. All participants were offered smoking cessation assistance (including a referral to a stop-smoking clinic) at the time of enrolment, although none of them elected to pursue this resource during the study period.

Measures

In addition to collecting demographic and smoking-history information, measures assessing understanding of the educational material were collected at the baseline assessment. Assessment of the participant's recall and interpretation of their test result was performed at the first follow-up call, while questions about their desire to quit smoking and smoking behaviour were administered at the baseline assessment, first and second follow-up calls.

Understanding of p16 function at baseline

Participants were asked nine questions assessing their recall and understanding of the presented educational material. Eight of these questions addressed the function of p16 in relation to developing lung cancer. "*P16* is a: (gene, protein, chemical, group of cells causing lung cancer)"; "Smokers with the *p16* switch off have more lung cells copied than normal (true, false, don't know; for this and remaining questions in the section)."; "Smokers are less likely than nonsmokers to have the *p16* gene turned off"; "Whether a person gets lung cancer involves more than *p16*"; "Smokers who have more lung cancer cells being made than normal are at higher risk for getting lung cancer"; "*P16* can tell you how hard it would be for you to quit smoking"; "If your *p16* switch is turned on, you can still get lung cancer due to your smoking"; and, "Getting the *p16* test can tell you if you now have lung cancer ?"

An additional question addressed understanding of *p16* as a marker of risk for developing lung cancer: "If you have the *p16* switch turned on, what is your risk for getting lung cancer compared with someone who had the *p16* switch turned off? (7 point scale with 1=much lower risk to 7=much higher risk)." Finally, to assess their test result expectations, participants were asked: "If you were to take the p16 test, what do you think it would show? (I am in the higher risk group, I am in the average risk group)."

Desire to quit smoking at baseline, first, and second follow-up

The participants' desire to stop smoking was assessed at all three time points by asking "How strong is your desire to stop smoking at this time? (7 point scale with 1=no desire to quit to 7=extremely strong desire)."

Perceived personal risk of developing lung cancer at baseline, first, and second follow-up calls

Participants were asked, "What do you think is your chance of getting lung cancer from your smoking if you don't quit? (very unlikely, unlikely, moderately likely, likely, very likely)." Scores were converted to a five-point scale and reported as averages.

Notification of test results and first follow-up survey

Participants were notified of their test result by letter an average of 115±79 days after submitting their sputum samples. Notification letters stated the following, "We are writing to inform you of the results of your recent sputum evaluation of the p16 switch. Your switch was found to be on/off (one or the other was reported). As you recall, having the p16 switch in the 'on' position suggests that you may have similar risk of developing lung cancer compared with other smokers. This risk is higher than the risk among non-smokers or former smokers. If the p16 gene is in the 'off' position, then your risk for developing lung cancer is higher than the average smoker. Currently, the only way to reduce your risk for developing lung cancer is to stop smoking."

Recall, interpretation and evaluation of p16 results at first follow-up

Participants were asked two questions about their recall of their test result and interpretation of the risks associated with the result: "What was the result of your *p16* test: (*p16* switch is on, *p16* switch is off, don't know)," and "Based on your *p16* result, your risk of getting lung cancer compared with the average smoker if you continue to smoke is: (much lower than average, slightly lower than average, average, slightly higher than average, much higher than average)."

Participants' evaluation of their test result was rated on five dimensions: accuracy, credibility, trustworthiness, usefulness, relevance and understandability (1=not at all to 7=completely). Participants were also asked: "How well does p16 predict getting lung cancer? (1=predicts poorly to 7=predicts well)." Finally, they were asked, "How has the *p16* result affected, if at all, your smoking?" (made me less likely to want to quit, did not affect my desire to quit, made me more likely to want to quit, I have quit smoking).

Smoking behavior at first and second follow-up

Participants were asked whether they, "Had smoked a cigarette, even a puff, in the last 7 days? (yes, no)." If participants responded yes they were then asked how many cigarettes they smoked on average per day during the last week. Participants who continued to smoke were then asked, "Have you seriously tried to stop smoking for at least 24 hours since we first spoke with you? (no, yes)."

Nucleic acid isolation and methylation-specific PCR

Identification of methylated *p16* was determined using a modification of the assay as described by Belinsky *et al*.[14] Briefly, sputum samples were pelleted, washed and stored at −20°C in Saccomano's fixative until time of analysis. DNA was isolated from the thawed pellets using a phenol/chloroform/isoamyl alcohol extraction. DNA was bisulfite modified and purified using Qiaex II (Qiagen) gel extraction kit per manufacturers instructions. Methylation identification was performed using a nested two-step PCR analysis to optimise sensitivity of the assay in samples with limited presence of methylated *p16*. Primers were selected as previously described.[14] Methylated-positive controls were taken from H1752 cells with appropriate negative controls obtained from samples of normal human lung

from never smokers. Analysis of the amplified samples was performed by gel electrophoresis.

Statistics

All statistical analysis was performed using Matlab V.7.4 service pack b, with the statistical workbook installed. Continuous variables were analysed using Student t test, while non-continuous variables were analysed using Fisher's exact test. Significance was defined as a p value of <0.05.

RESULTS

Demographics

All but one of the participants were male, most were elderly, and Caucasian (table 1). There were no differences between the methylated and non-methylated group in terms of educational levels, age at first cigarette, number or types of cigarettes smoked, family history of lung cancer or lung function as assessed by spirometry (table 1).

Understanding of the educational material at baseline assessment

As reported in table 2, participants demonstrated good understanding of the educational material: 29 (83%) correctly identified *p16* as a gene; 30 (86%) recognised that *p16* methylation resulted in additional cells being copied, but only 17 (49%) correctly identified the false statement that *p16* is more commonly methylated in non-smokers. Twenty-eight of the participants (80%) correctly noted that developing lung cancer involves more than *p16* methylation, and 30 participants (86%) correctly answered that producing additional cells was more likely to lead to lung cancer, with the same percentage of participants correctly noting that without *p16* methylation it is still possible to develop lung cancer. Only 16 participants (46%) knew that *p16* does not inform whether someone has lung cancer. When comparing the methylated and non-methylated subgroups, there were no statistically significant differences in response with the exception of whether the presence of methylated *p16* is diagnostic of lung cancer. The

Table 1 Demographics of participants consented for sputum testing for p16 methylation

	Total participants (N=35)		Non-methylated participants (N=28)		Methylated participants (N=7)	
Variables	**N**	**%**	**N**	**%**	**N**	**%**
Gender						
Male	34	97	23	96	7	100
Age						
Mean (SD)	61.3 (7.8)		61.6 (6.6)		59.9 (12.2)	
Ethnicity						
White	26	74	21	75	5	71
Black	7	20	6	21	1	14
Multiple	2	6	1	4	1	14
Education						
<High school	9	36	8	29	1	14
High school	6	17	6	21	0	0
Trade school	5	14	3	11	2	29
Some college	10	29	6	21	4	57
College	4	11	4	14	0	0
Postgraduate	1	3	1	4	0	0
Age started smoking						
Mean (SD)	14.8 (6.2)		15.4 (6.2)		14.5 (2.3)	
Cigarettes/day						
Mean (SD)	19.2 (10.0)		20.5 (9.7)		13.8 (9.9)	
Type of cigarette smoked						
Regular	15	43	14	50	1	14
Light	15	43	9	32	6	86
Ultralight	5	14	5	18	0	0
Spirometry						
FEV_1	1.41 (0.67)		1.31 (0.65)		1.79(0.68)	
FVC	3.08 (0.95)		2.91 (0.92)		3.75 (0.78)	
FEV_1/FVC	44.96 (12.38)		44.29 (12.17)		47.54 (13.84)	
Lung cancer in the family						
Yes	6	17	4	14	2	29
No	25	74	20	74	5	71
Don't know	3	9	3	11	0	0

FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity.

Table 2 Comprehension of *p16* educational material at baseline assessment

Variables	All participants (N=35)		Non-methylated participants (N=28)		Methylated participants (N=7)	
	N	%	N	%	N	%
Understanding of p16 function P16 is a: (gene)	29	83	22	79	7	100
Smokers with the *p16* switch off have more lung cells copied than normal (true)	30	86	25	89	5	71
Smokers are less likely than non-smokers to have the *p16* gene turned off (false)	17	49	12	43	5	71
Whether a person gets lung cancer involves more than *p16* (true)	28	80	21	75	7	100
Smokers who have more lung cancer cells being made than normal are at higher risk for getting lung cancer (true)	30	86	24	86	6	86
P16 can tell you how hard it would be for you to quit smoking (false)	28	80	21	75	7	100
If your *p16* switch is turned on, you can still get lung cancer due to your smoking (true)	30	86	23	82	7	100
Getting the *p16* test can tell you if you now have lung cancer (false)	16	46	9	32	7	100
Understanding of risk associated with p16 methylation*	3.71 (2.48)		3.96 (2.47)		2.71 (2.43)	
What do you think your p16 test will show (higher risk group)†	27	77	21	75	6	86

*Results based on a seven-point scale with higher scores indicating higher perceived risk.
†One participant did not answer this question and so totals do not equal 35.

methylated group provided the correct response in 100% of participants vs 32% of the non-methylated group (p<0.005, table 2).

Understanding of risk associated with p16 methylation at baseline assessment

Participants had good comprehension of the risk of developing lung cancer associated with *p16* methylation. They displayed appropriate appreciation of the relative risk when comparing a smoker with functional *p16* versus one without, with an average risk score of 3.71±2.48 (table 2), indicating that participants associated p16 methylation with an intermediate level of risk for the future development of lung cancer.

Recall, interpretation and evaluation of test results at first follow-up

Seven of the 35 participants (20% of the initial cohort) had methylation of *p16* in their submitted sputum samples, which is similar to previously reported prevalence in this population with airway obstruction and history of heavy tobacco use.[14 22] Thus, with respect to their test expectations, participants overestimated the likelihood of their test result showing *p16* methylation,

Table 3 Recall of *p16* testing results and attitudes towards testing at first follow-up

Variables	Non-methylated All (N=24)		Accurate recall (N=17)		Methylated all (N=7)	
	N	%	N	%	N	%
Accurate recall of test result	17	71			6	86
Interpretation of risk conferred by result						
Much lower than average	0	0	0	0	1	14
Slightly lower than average	3	13	3	18	0	0
Average	8	33	6	35	1	14
Slightly higher than average	6	25	4	24	2	28
Much higher than average	7	29	4	24	3	43
Average value (SD)	3.7 (1.0)		3.5 (1.1)		3.9 (1.5)	
Evaluation of test mean (SD)*						
Accurate	6.38 (1.0)		6.35 (1.11)		6.57 (1.13)	
Credible	6.24 (1.12)		6.41 (1.06)		6.86 (0.38)	
Trustworthy	6.25 (1.18)		6.35 (1.11)		6.43 (0.98)	
Useful	6.33 (1.27)		6.35 (1.37)		6.43 (0.98)	
Relevant	6.21 (1.31)		6.17 (1.42)		6.43 (0.98)	
Understandable	6.29 (1.23)		6.29 (1.16)		6.57 (1.13)	
P16 testing predicts lung cancer*	5.33 (1.58)		5.33 (1.82)		4.83 (1.83)	

*Results based on a seven-point scale with higher score indicating greater agreement with the statement.

Table 4 Smoking behaviour and desire to quit

	Non-methylated					
	All (N=24)		Accurate recall (N=17)		Methylated all (N=7)	
Variables	N	%	N	%	N	%
Effect of test result on motivation to quit smoking at first follow-up						
Less likely to quit	0	0	0	0	0	0
No change	5	21	3	18	2	28
More likely to quit	16	67	13	77	4	57
No cigarette in 7 days	3	13	1	6	1	14
Have you tried to stop for 24 h since you entered study						
First follow-up						
Yes	17	71	12	71	4	43
No	7	29	5	29	3	57
Second follow-up						
Yes	17	71	12	71	3	43
No	7	29	5	29	4	57
Desire to quit* N (SD)						
Baseline	5.6 (1.7)		N/A		5.7 (1.5)	
First follow-up	5.9 (1.23)		6.1 (1.05)		5.4 (1.62)	
Second follow-up	6.0 (1.47)		6.2 (1.09)		5.0 (2.0)	

*Results based on a seven-point scale with seven representing a strong desire to quit.

with 77% reporting that they would expect that their test result would place them in the higher risk group (table 2). Overall, 6/7 participants (86%) and 17/24 participants (71%) had accurate recall of their test result in the methylated versus non-methylated groups, respectively. Participants in both groups were confident in *p16* test results with high scores for accuracy, credibility, trustworthiness, usefulness, relevance and understandability (table 3). There were no statistically significant differences between groups in the first follow-up assessment.

Among the methylated group, 5/7 participants (71%) correctly categorised themselves at above average risk of developing lung cancer compared with the average smoker. Among the non-methylated group, only 8/24 participants (33%) correctly identified themselves as average risk, 13/24 participants (54%) thought their test result placed them at increased risk and 3/24 participants (13%) thought their test result placed them at decreased risk (p<0.39). Participants believed that *p16* was a fair predictor of the development of lung cancer, irrespective of participant methylation status, with scores of 5.14±1.86 and 5.33 +1.58 on a seven-point scale in methylated and non-methylated participants, respectively (p<0.81, table 3).

Perceived personal risk of developing lung cancer at baseline, first and second follow-up

Concern for developing lung cancer due to continued smoking was high among participants at baseline with average scores of 4.1±1.2 and 4.6±0.5 on a five-point scale for non-methylated and methylated participants, respectively. Risk perceptions remained stable after notification of test results with average scores of 4.1±0.9 and 4.1±0.9 for non-methylated and methylated participants at the first follow-up call, respectively, and scores of 4.1 ±0.9 and 4.2±1.2 for non-methylated and methylated participants at the second follow-up call, respectively (results non-significant for all comparisons). In essence, these findings suggest that the test result did not modify perceptions of personal lung cancer risk.

Desire to quit and smoking behaviour at first and second follow-up

At the first follow-up call, over half of the participants felt that they were more likely to quit smoking because of their *p16* result with no difference between non-methylated and methylated participants (67% and 57%, respectively, p<0.84). No participants reported that they were less likely to quit due to their test result.

Among participants in the non-methylated group, 17/24 (71%) stated at the first as well as second follow-up calls that they had tried to stop smoking for 24 h since entering the study, while only 4/7 (57%, p<0.65) and 3/7 (43%, p<0.21) of the methylated participants stated they had tried to stop at the first and second follow-up calls, respectively. A total of three participants (13%) in the non-methylated group and one participant (14%) in the methylated group stated they had quit smoking by the study's conclusion (table 4).

DISCUSSION

We have demonstrated the feasibility of using a genetically based assessment tool to provide feedback to high-risk smokers. Previous studies using biomarker feedback have presented smokers with information regarding increased susceptibility to smoke exposure such as with

GSTM1 polymorphisms,[23] or evidence of adverse effects of smoking on lung function.[24]

This study is novel in that it provided information to participants showing direct epigenetic injury related to smoke exposure with clear increases in risk for developing lung cancer. Despite concerns that anxiety regarding genetic testing for lung cancer risk would deter patients from study participation, only two patients cited this as their reason for not joining the study. The compliance rate of the study was high with 89% of participants completing the study. Furthermore, the *p16* test result was rated accurate and reliable by most participants. Hence, there was little evidence that participants discounted their test result.

At baseline, participants had a good understanding and recall of the material presented about *p16*. For example, they knew how *p16* functions to influence the development of lung cancer. However, there is room for improvement, demonstrated by the large proportion of participants who were unclear about whether this was a test of risk for developing lung cancer versus a test for the diagnosis of lung cancer. Surprisingly, the lack of understanding of the relation between a positive *p16* test and the diagnosis of lung cancer was not equally distributed between groups. Hundred per cent of the methylated participants correctly answered that the test was not diagnostic for lung cancer, while only 32% of the non-methylated participants answered this question correctly. Despite this difference in understanding, cessation rates were similar between groups. In addition, participants accurately identified that the presence of *p16* methylation confers an intermediate increase in risk for the future development of lung cancer (table 2). Furthermore, many viewed *p16* methylation as a fair predictive marker for developing lung cancer (table 3). With regard to test expectations, most participants overestimated their personal risk of methylated *p16* relative to the average smoker (table 2), and those in the non-methylated group also overestimated their risk of developing lung cancer based on their *p16* test result (table 3).

Our hypothesis was that *p16* test feedback would increase perceptions of lung cancer risk in smokers with methylated *p16*. However, this did not occur. In part, this reflects the baseline findings that the majority of smokers already expressed heightened perceptions of lung cancer risk (eg, mean scores above 4 on a five-point scale). Thus, there was little room to heighten these perceptions further. An important future direction is to determine whether test results may affect emotional reactions to risk (eg, worry, fear, anxiety, etc), which may spur the motivation to quit.

Although participants with methylated *p16* displayed good understanding of *p16* function, with high levels of accurate recall, and appropriately associated their test result with the development of lung cancer, they did not act on the biomarker feedback. Instead of increasing quit rates, rates of self-reported quitting were not affected by *p16* methylation status. Furthermore, there was a trend (although not statistically significant) towards fewer of the methylated participants attempting to stop smoking for even 24 h compared with non-methylated participants. Similar trends in decreasing motivation to stop smoking among a higher-risk group of smokers were noted in a study by Sanderson *et al*[23] in which smokers were tested for the absence of *GSTM1*. If notification of increased risk does induce a sense of futility or enhanced depression among smokers, then future studies may require an additional educational session around the time of testing notification to avoid these adverse responses. Notably, it is unknown whether smoking cessation stops or reverses processes of methylation. If not, then the use of this form of epigenetic feedback may indeed cause feelings of futility that can lead to depression and possibly require intensified screening for lung cancer.

Although limited by a small sample size, our findings suggest that the failure to demonstrate evidence of physiological harm does not reinforce an optimistic bias, as demonstrated by no reduction in perceptions of personal risk, or reduction in motivation to attempt smoking cessation among participants without methylated *p16*. Similar behaviour was noted by Parkes *et al*,[24] who measured spirometric lung age in smokers. Quit rates were similar in participants with normal or advanced spirometric lung age, and superior to control group quit rates. It is unclear how the presentation of normal physiological results motivates smokers to quit, although Parkes[24] suggested that normal test results allow the smoker to feel that it is "not too late" to stop smoking. Similar mechanisms may be at work in the present study.

Limitations of this study include a small sample size of older male veterans with extensive tobacco histories who may be more resistant to smoking cessation than younger smokers, a limited assessment of participant motivation after the test results were communicated, and a short duration of follow-up without objective assessment of smoking behaviour. In fact, a non-methylated participant followed clinically by one of the authors (LAB) stopped smoking 1 year after the completion of the study and cited participation in the study as a motivating factor. In addition, the assay itself is challenging to perform, and relatively expensive at almost $500 per sample, although costs are now lower due to improvements in assay technique and this cost would likely be recovered in decreased future healthcare expenditures if the approach proved to be successful in motivating smoking cessation. Finally, this study did not control for prior smoking cessation efforts by the participants, and represented a stand-alone intervention. If included as part of an integrated cessation programme higher cessation rates may occur.

In conclusion, we demonstrated good participant acceptance and understanding of a novel epigenetic risk assessment tool for motivating smoking cessation. Similar to other biomarker studies, identification of

methylated *p16* does not appear to induce smoking cessation in a group of heavy smokers. In future studies, it would be important to assess smoking cessation attempts and success over a longer period of time, with a focus on assessment of depression and additional education regarding the function of *p16* in the development of lung cancer.

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Contributors SS designed and conducted the study, performed the data analysis and wrote the manuscript. MB enrolled the participants, collected and collated the data and maintained the study documents. SL performed the methylation assays. LAB assisted with study design and recruitment strategies and reviewed the manuscript. MMW provided support for the study coordinator and reviewed the manuscript. MK designed the study, oversaw the methylation assay performance and reviewed the manuscript. IML designed the survey tool and the study and assisted with writing the manuscript.

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