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Mendelian Randomization and mediation analysis of leukocyte telomere length and risk of lung and head and neck cancers

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Mendelian Randomization and mediation analysis of leukocyte telomere length and risk of lung and head and neck cancers

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ABSTRACT

Background: Evidence from observational studies of telomere length (TL) has been conflicting regarding its direction of association with cancer risk. We investigated the causal relevance of TL for lung and head and neck cancers using Mendelian Randomization (MR) and mediation analyses.

Methods: We developed a novel genetic instrument for TL in chromosome 5p15.33, using variants identified through deep-sequencing, that were genotyped in 2051 cancer-free subjects. Next, we conducted an MR analysis of lung (16396 cases, 13013 controls) and head and neck cancer (4415 cases, 5013 controls) using 8 genetic instruments for TL. Lastly, the 5p15.33 instrument and distinct 5p15.33 lung cancer risk loci were evaluated using two-sample mediation analysis, to quantify their direct and indirect, telomere-mediated, effects.

Results: The multi-allelic 5p15.33 instrument explained 1.49-2.00% of TL variation in our data ($p=2.6\times10^{-9}$). The MR analysis estimated that a 1000 base pair increase in TL increases risk of lung cancer (OR=1.41, 95% CI: 1.20-1.65) and lung adenocarcinoma (OR=1.92, 95% CI: 1.51-2.22), but not squamous lung carcinoma (OR=1.04, 95% CI: 0.83-1.29), or head and neck cancers (OR=0.90, 95% CI: 0.70-1.05). Mediation analysis of the 5p15.33 instrument indicated an absence of direct effects on lung cancer risk (OR=1.00, 95% CI: 0.95-1.04). Analysis of distinct 5p15.33 susceptibility variants estimated that TL mediates up to 40% of the observed associations with lung cancer risk.

Conclusions: Our findings support a causal role for long telomeres in lung cancer etiology, particularly for adenocarcinoma, and demonstrate that telomere maintenance partially mediates the lung cancer susceptibility conferred by 5p15.33 loci.

KEY MESSAGES

- Genetic predisposition to long telomeres increases risk of lung cancer, predominately lung adenocarcinoma
- Genetic determinants of long telomeres are not associated with squamous carcinomas of the lung or head and neck
- Using two-sample mediation analysis we determined that the novel 5p15.33 instrument for telomere length does not have direct effects on the outcome, and demonstrated that the association between 5p15.33 lung cancer susceptibility variants is partially mediated by telomere length, suggesting the presence of other relevant mechanisms

INTRODUCTION

Telomeres are highly conserved stretches of tandem repeats of the TTAGGG sequence, which protect chromosome ends from degradation and maintain genome stability(1, 2). Due to the incomplete replication of chromosomes during cell division, human telomeres lose between 50 and 200 base pairs with each replication(1-3). In checkpoint proficient cells critically short telomeres trigger senescence, followed by apoptosis, which represents a barrier against cancer initiation by limiting cellular proliferation(4, 5). As telomeres shorten their ability to maintain chromosomal stability also diminishes, which may increase cancer susceptibility(6, 7). However, long telomeres may also promote cancer development through an accumulation of mutations due to prolonged cell survival and proliferation. In fact, cancer cells are characterized by such a proliferative advantage, often through reactivation of telomerase, which is normally silent in somatic cells(4, 5, 8).

Telomere length (TL) has been studied extensively in relation to cancer risk. However, findings of epidemiologic studies have been conflicting (6, 9-11). Observational studies investigating TL measured after cancer diagnosis are particularly vulnerable to reverse causation and residual confounding, therefore shorter TL observed in cancer cases is likely to reflect underlying disease or the impact of cancer treatment (12, 13). It is also difficult to isolate the influence of TL on cancer risk from that of other risk factors that influence both TL and cancer susceptibility, including biological or replicative age (10, 14, 15).

Mendelian Randomization (MR) is an approach for evaluating causality by using single nucleotide polymorphisms (SNPs) in relevant genes as instrumental variables (IVs) (16). Genome-wide association studies (GWAS) identified a number of genetic regions involved in TL regulation, including genes encoding the catalytic subunit of telomerase (*TERT*) in chromosome 5p15.33 and its RNA template (*TERC*) in 3q26.2 (17-21). By leveraging these associations, MR can provide a valid test of the causal hypothesis assuming the genetic IVs only affect cancer risk through TL regulation.

Previous studies using genetic proxies for TL suggest that longer telomeres confer an increased risk of lung cancer, especially adenocarcinoma (22-24), which is consistent with the findings of prospective observational studies (25-27). Lung cancer case-control studies report both increased (28) and inverse (6, 29) associations for long TL, and some implicate high TL variability in lung cancer susceptibility (30). For head and neck cancers (HNC), which are predominantly squamous carcinomas, short TL is consistently associated with increased risk in case-control studies (6, 31, 32), whereas a recent MR analysis (24) did find evidence supporting a causal relationship.

The overarching aim of this study is to investigate the causal relationship between TL and risk of lung and upper aero-digestive tract cancers. First, we developed a novel genetic instrument for TL in chromosome 5p15.33, given the extensive pleiotropy in this region and potential for violating MR assumptions (22, 33). Next, we conducted the largest two-sample MR analysis of lung and HNC risk to date. Lastly, we quantified the direct and telomere-mediated effects of 5p15.33 genetic variants on cancer risk using a two-sample mediation analysis approach (Figure 1).

METHODS

Study populations

We used individual-level data from 23 pooled studies of lung cancer, with 16396 cases (5690 adenocarcinoma, 4045 squamous carcinoma) and 13013 controls; and 11 HNC studies with 4415 cases and 5013 controls, all part of the OncoArray collaboration (34) (Supplementary Tables 1-2). Descriptions of studies and genotyping methods have been previously published (34, 35) (details in Supplementary File 1). Analyses were restricted to individuals of predominantly European ancestry (≥80% lung, >70% HNC)(34, 36). Studies received approval from institutional research ethics review boards and informed consent was obtained from the participants.

The novel 5p15.33 instrument was developed using data from two studies: the cancer-free controls from the Mount Sinai and Princess Margaret Hospital (MSH-PMH) case-control study in Toronto(37), and cancer-free individuals from the Copenhagen General Population Study

(CGPS)(38), a population-based prospective cohort (Table 1). TL was measured in DNA from peripheral blood leukocytes using previously described quantitative polymerase chain reaction assays performed in MSH-PMH (37) and CGPS (23, 38) (details in Supplementary File 2).

Statistical Analysis

Mendelian randomization analysis

The genetic instruments for TL included independent SNPs showing strong prior evidence of association with TL, such as $p<5\times10^{-8}$ in the discovery stage of at least one GWAS and replication in a separate GWAS or meta-analysis (17-21). In addition to the new 5p15.33 instrument described below, we selected 7 additional loci involved in telomere maintenance: rs10165485 (proxy for rs11125529, r^2=1.0) in *ACYP2* (2p16.2), rs6772228 in *PXK* (3p14.3), rs10936599 in *TERC* (3q26.2), rs11100479 (proxy for rs7675998, r^2=0.99) in *NAF1* (4q32.2), rs9420907 in *OBFC1* (10q24.3), rs10419926 in *ZNF676* (19p12), and rs755017 near *RTEL1* and *ZBTB46* (20q13). Only genotyped, non-imputed variants were used.

For the purpose of developing a new instrument in the 5p15.33 region, TL values were converted to Z-scores in MSH-PMH (n=879) and CGPS (n=1172) studies separately, and pooled to increase statistical power. Linear regression was used to estimate the association between 899 variants in 5p15.33 and TL, adjusting for age, sex, study, and the top 5 genetic ancestry principal components (PCs).

Selection of variants for the 5p15.33 instrument was based on statistical significance, consistency across the two studies, and instrument strength, measured by the *F* statistic, which depends on the variance in TL explained by the genetic predictors (R^2), sample size (n), and number of instruments (k): $F=\left(\frac{n-k-1}{k}\right)\left(\frac{R^2}{1-R^2}\right)$. Variants were considered for inclusion in the 5p15.33 instrument if they met the following criteria:

i. F≥5 and p<0.05 in the Toronto and Copenhagen combined dataset (n=2051)

ii. F<5 and p<0.05 overall (n=2051) and F>5 among never smokers (n=848)

iii. Consistent direction of allelic effects in MSH-PMH and CGPS

iv. Minor allele detected in at least 2 individuals

Independent genetic variants (r^2<0.2) that met the selection criteria were combined into an allele score representing the 5p15.33 region to increase the power of the resulting instrument (39, 40).

The MR analysis combined summary statistics across the genetic IVs to estimate the causal parameter β_{IV}, which is the log odds ratio (OR) describing the causal effect of increasing TL on cancer risk (Supplementary Figure 1). Parameters for the MR analysis included β_{TL} and β_Y, where β_{TL} is a vector of SNP-TL associations and β_Y is a vector of per-allele cancer log ORs for each instrument. For genetic instruments outside of 5p15.33, β_{TL} and corresponding standard errors (SE) were obtained from the literature and scaled to represent a 1000 base pair (kbp) increase in leukocyte TL, a proxy for TL in relevant tissues(19-21). For all instruments, β_Y and corresponding SE were estimated directly using individual-level OncoArray lung and HNC data. Logistic regression models were adjusted for age, sex, study, and 10 PCs.

The causal parameter β_{IV} was estimated using the maximum likelihood-based (ML) approach and the inverse-variance weighted (IVW) method (41, 42). This was complemented by sensitivity analyses using the weighted median estimator (WME), which provides valid estimates of the causal parameter even when up to 50% of the statistical weights are contributed by genetic instruments violate MR assumptions (43).

Mediation analysis

The aim of the mediation analysis was to quantify how much of the lung cancer association in the 5p15.33 region is mediated by TL. First, we validated the 5p15.33 instrument by decomposing its total effect on lung cancer into direct and indirect effects, mediated by TL. Next, we

extended this analysis to independent (r^2<0.20) variants that capture the lung cancer association signal in 5p15.33 (details in Supplementary File 3).

Our mediation approach is based on the counterfactual framework(44, 45) and extends the sensitivity analysis using two randomized controlled trials proposed by Vanderweele, which allows the mediator-outcome (θ_2) and exposure-mediator (β_1) relationships to be estimated in separate studies (46). Application of this approach in the present context assumes that a valid estimate for the mediator-outcome relationship can be obtained from an independent MR or cohort studies. Based on previously published formulas for mediation analysis (44, 45), the total effect (TE) of increasing the exposure from reference level a^* to level a on lung cancer (Y) conditional on covariates *c* can be decomposed into natural direct effects (NDE) and natural indirect effects (NIE):

$$OR^{TE}_{a,a^*|c} = \frac{P(Y_a = 1 \mid c) / \{1 - P(Y_a = 1 \mid c)}{P(Y_{a^*} = 1 \mid c) / \{1 - P(Y_{a^*} = 1 \mid c)} = OR^{NIE}_{a,a^*|c} \times OR^{NDE}_{a,a^*|c} \quad (1)$$

Assuming a rare outcome and absence of exposure-mediator interaction, mediated effects are given by:

$$OR^{NIE}_{a,a^*|c} \approx \exp\{\theta_2 \times \beta_1 (a - a^*)\} \quad (2)$$

where θ_2 is log-OR per one unit increment in TL and β_1 is the effect of the 5p15.33 instrument on TL. Based on equation 1, NDE can be obtained by subtracting the NIE from the total effect:

$$\log(OR^{NDE}_{a,a^*|c}) \approx \log(OR^{TE}_{a,a^*|c}) - \log(OR^{NIE}_{a,a^*|c}) \quad (3)$$

In the presence of interaction between the exposure and mediator, the NIE is given by:

$$OR^{NIE}_{a,a^*|c} \approx \exp\{(\theta_2 \times \beta_1 + \theta_3 \times \beta_1 a) \times (a - a^*)\} \quad (4)$$

where θ_2 now represents the main effect of the mediator, TL, and θ_3 is the exposure-mediator interaction parameter, with NDE having a more complicated form given by Valeri and VanderWeele(45). Formulas for a dichotomized mediator are provided in Supplementary File 4.

The β_1 parameter for the 5p15.33 instrument is equivalent to β_{TL} estimated in the cancer-free subset of the MSH-PMH and CGPS studies, adjusting for appropriate covariates. For 5p15.33 cancer susceptibility variants, β_1 estimates were selected from Bojesen et al. (47), the largest fine-mapping analysis of common 5p15.33 loci and TL with 15567 cancer-free controls. Per allele associations were reported as percent increase in TL and base-pair change. OR^{TE} for all variants was estimated in 23 lung cancer OncoArray studies, and is equivalent to β_Y for the 5p15.33 instrument.

External estimates of the mediator-outcome relationship (θ_2) were substituted into the equation (2) to avoid estimating the effect of TL on lung cancer risk directly using MSH-PMH case-control data, which are likely to be biased due to the post-diagnostic timing of TL measurement. The effect of TL on lung cancer risk was obtained from two studies: an MR analysis TL by Zhang et al.(22), and a meta-analysis of prospective studies by Zhu et al. (11) (Supplementary Figure 2).

Since interaction between the 5p15.33 instrument and TL is plausible, we conducted sensitivity analyses under different magnitudes of θ_3 (details in Supplementary File 4). Confidence intervals for the NIE and NDE were approximated as Bayesian credible intervals. Analyses were conducted using R version 3.3.3.

RESULTS

Characteristics of the combined Toronto and Copenhagen dataset (n=2051), used to develop the 5p15.33 instrument, are summarized in Table 1. The cancer-free participants in the MSH-PMH and CGPS studies were of similar mean age, 61.0 and 61.30 years, respectively. Age was the strongest predictor of TL ($p=2.6\times10^{-30}$), while sex, smoking status, and cigarette pack-years among smokers were not associated with relative TL (Supplementary Table 3).

Novel 5p15.33 instrument for telomere length

The 5p15.33 variants comprising this instrument were not used in any previous MR studies of TL. After excluding 17 singletons and other SNPs that did not meet our criteria, 14 variants were

included in the multi-allelic instrument for 5p15.33 (Table 2; regional plot and LD illustrated in Supplementary Figure 3). Most variants were located in non-coding intronic regions of several genes, including *SLC6A3*, *TERT*, *LPCAT1*, and a long-noncoding RNA (*LINC01511*) except for rs35033501, a synonymous *TERT* variant. The resulting multi-allelic 5p15.33 IV accounted for 1.49% of variation in the telomere Z-score in all subjects (F = 35.83; β_{TL}= 0.14, SE=0.02) and 2.00% in never smokers (F = 20.81), but was not predictive of smoking status (p=0.19) or cigarette pack-years among smokers (p=0.59) (Table 3). The 5p15.33 instrument was positively associated with lung cancer (OR=1.04, 95% CI: 1.01-1.07) and lung adenocarcinoma (OR=1.06, 1.03-1.10), but not squamous lung carcinomas (OR=1.03, 0.98-1.07). An inverse association was observed for HNC (OR=0.95, 0.90-1.00) and oral cavity cancer (OR=0.93, 0.87-0.98).

Telomere length and cancer risk

Results of the MR analysis based on 8 genetic instruments are presented in Table 4 and Figure 2. The likelihood-based model estimated a 41% increase in lung cancer risk per kbp increase in TL (OR_{ML}=1.41, 95% CI: 1.20-1.65). Estimates of the causal OR for lung cancer remained consistent across MR estimation methods. Genetic determinants of TL were predominantly associated with adenocarcinoma (OR_{ML}=1.92, 1.51-2.45), and appeared unrelated to squamous carcinoma (OR_{ML}=1.04, 0.83-1.29) and small cell carcinoma (OR_{ML}=1.03, 0.76-1.39).

The effect of long TL on lung cancer risk was larger in magnitude among never smokers (OR_{ML}=1.78, 1.22-2.61) compared to smokers (OR_{ML}=1.36, 1.14-1.63), although the former was attenuated in sensitivity analyses (OR_{WME}=1.55, 95% CI: 0.98-2.46). Effects on adenocarcinoma risk were also substantial in never smokers (OR_{ML}=2.68, 1.70-4.24). Genetic determinants of long telomeres conferred a 68% increase in lung cancer risk (OR_{ML}=1.68, 1.07-2.62) in subjects aged 50 years or younger. In contrast to lung cancer, genetic predisposition for longer TL did not seem related to risk of HNC overall (OR_{ML}= 0.90, 0.70-1.05), oral cavity (OR_{ML}=0.88, 0.65-1.19) and oropharynx cancers (OR_{ML}=0.83, 0.59-1.16).

Several additional sensitivity analyses were undertaken to further interrogate the MR results. Since smoking is an established risk factor for both HNC and lung cancer, MR analyses were repeated with adjustment for cigarette pack-years and smoking status. No appreciable changes were observed in the causal effect estimates for lung cancer overall (OR_{ML}=1.50, 1.27-1.78), lung adenocarcinoma (OR_{ML}=1.95, 1.53-2.49), HNC (OR_{ML}=0.91, 0.67-1.23), oral cavity (OR_{ML}=0.82, 0.57-1.18) or oropharynx cancers (OR_{ML}=0.86, 0.57-1.31).

The potential for directional pleiotropy was evaluated by checking for asymmetry in the plots depicting ratio estimates for each instrument, β_Y / β_{TL}, plotted against instrument strength, $\beta_{TL} / SE(\beta_Y)$ (Supplementary Figure 4). These results were not suggestive of pleiotropy and none of the genetic instruments were associated with cigarette smoking status or pack-years (Supplementary Table 4). Lastly, selected causal effects were re-estimated using the weighted mode-based estimator (MBE), which is robust to horizontal pleiotropy when the largest number of similar causal effect estimates are based on valid instruments, even if the majority of instruments are invalid (48). Estimates for lung cancer overall (OR_{MBE}=1.34, 1.08-1.66), lung adenocarcinoma (OR_{MBE}=1.55, 1.14-2.12), and adenocarcinoma in never smokers (OR_{MBE}=2.04, 1.04-4.04), were consistent with the primary results in Table 4.

Mediation analysis of the 5p15.33 instrument

We conducted mediation analyses to quantify direct (OR^{NDE}) and indirect effects (OR^{NIE}) of the 5p15.33 instrument on lung cancer. The OR^{NIE} we report is the proportional change in the odds of lung cancer for a change in TL that occurs when the 5p15.33 allele score increases by one from the reference level, corresponding to the mean of the allele score distribution. The estimate of the TL effect on lung cancer (θ_2) was selected from the strict model reported by Zhang et al.(22) (OR per kbp increase: 1.37, 95% CI: 1.12-1.68), which excluded rs2736100 (*TERT*). OR^{TE} for the 5p15.33 IV was re-estimated after removing overlapping subjects (n=3498) between the OncoArray and Zhang et al.(22). Assuming no interaction between the 5p15.33 IV and TL, the lung cancer effect appeared to be almost entirely mediated by TL (OR^{NIE}=1.05, 1.01-1.08), whereas the direct

effects of the 5p15.33 IV appeared null (OR^{NDE}=1.00, 0.95-1.04) (Figure 3; Supplementary Table 5). For lung adenocarcinoma, the 5p15.33 effects mediated by TL were larger in magnitude (OR^{NIE}=1.11, 1.05-1.18) than direct effects, which were close to unity (OR^{NDE}=0.97, 0.90-1.03).

Interaction sensitivity analyses for the NIE and NDE were carried out across three levels of θ_3: 0.10, 0.20 and 0.30. As the magnitude of the interaction parameter increased, so did the NIE, while TL-independent effects were not observed (Figure 3). Indirect effects on lung cancer risk mediated by TL ranged from OR^{NIE}=1.06 (95% CI: 1.03-1.10) for θ_3=0.10, to OR^{NIE}=1.09 (95% CI: 1.05-1.15) for θ_3= 0.30. For adenocarcinoma, increasing the magnitude of interaction between the 5p15.33 IV and TL was also associated with increasing NIE and diminishing direct effects.

The prospective meta-analysis estimate of θ_2 from Zhu et al.(11) reported an OR of 1.28 (95% CI: 1.09-1.50) for lung cancer comparing long vs. short TL. Based on this binary mediator, the NIE mediated by TL was attenuated, but remained statistically significant (OR^{NIE}=1.01, 1.00-1.03). A positive direct effect on lung cancer risk was also observed (OR^{NDE}=1.03, 1.00-1.06). Assuming interaction between the 5p15.33 instrument and TL, the mediated effects ranged from OR^{NIE}=1.02 (95% CI: 1.01-1.03) when θ_3=0.10, to OR^{NIE}=1.03 (95% CI: 1.01-1.05) when θ_3=0.30, while the direct effects decreased (Figure 3; Supplementary Table 5).

Mediation analysis of 5p15.33 lung cancer susceptibility loci

Five common (MAF>0.05), independent (r^2 <0.20) variants were selected to represent the lung cancer susceptibility signal in 5p15.33 (details in Supplementary File 3): rs7705526 ($P_{Adeno}=4.6\times10^{-13}$; $P_{Lung}=8.0\times10^{-7}$), rs2736108 ($P_{Adeno}=1.7\times10^{-12}$; $P_{Lung}=1.8\times10^{-11}$), rs421629 ($P_{Adeno}=6.2\times10^{-9}$; $P_{Lung}=1.2\times10^{-16}$), rs13167280 ($P_{Adeno}=1.4\times10^{-8}$; $P_{Lung}=1.1\times10^{-6}$), and rs56345976 ($P_{Adeno}=2.2\times10^{-7}$; $P_{Lung}=3.6\times10^{-9}$). These variants have been associated with lung cancer and lung adenocarcinoma in previous studies (37, 49-51), and are representative of the genetic susceptibility architecture in this region.

Estimates of β_1 were obtained from Bojesen et al.(47), and three *TERT* lung cancer risk variants were significantly associated with TL: rs7705526 (P_{TL}=2.3×10^{-14}), rs2736108 (P_{TL}=5.8×10^{-7}), and rs13167280 (P_{TL}=1.2×10^{-5}). Estimates of θ_2 were selected from the MR analysis (22) and OR^{TE} were re-estimated for each variant after removing the overlapping subjects. For all variants, the TL-increasing allele was positively associated with cancer risk, and both direct and indirect, TL-mediated effects were significant (Supplementary Table 6).

For lung cancer, the proportion mediated (PM) by TL was the largest for rs13167280 (OR^{NIE}=1.05, 1.03-1.07; PM=40.5%), followed by rs7705526 (OR^{NIE}=1.03, 1.01-1.05; PM=28.7%) and rs2736108 (OR^{NIE} 1.02, 1.01-1.03; PM=13.7%). The magnitude and proportion of the SNP effects that were mediated by TL were larger for adenocarcinoma compared to lung cancer overall: rs7705526 (OR^{NIE}=1.07, 1.04-1.10; PM=36.5%), rs13167280 (OR^{NIE}=1.05, 1.03-1.07; PM=24.8%), and rs2736108 (OR^{NIE}=1.04, 1.03-1.06; PM=22.9%).

DISCUSSION

We observed an association between genetic determinants of long telomeres and increased risk of lung, but not head and neck cancers. Our findings lend support to a causal relationship between longer leukocyte TL and increased risk of lung adenocarcinoma, but not squamous or small cell carcinoma. The magnitude of the increased risk was larger in never smokers and participants aged 50 or younger, consistent with a stronger influence of genetic susceptibility in individuals with a lower burden of modifiable risk factors (52). Although histology and smoking status are closely linked, our results suggest that the associations were histology-specific for adenocarcinoma (53, 54). Lastly, our mediation analysis demonstrated that mechanisms resulting in long telomeres mediate a proportion of the increase in lung cancer and lung adenocarcinoma risk conferred by 5p15.33 loci, and that the proportion of genetic susceptibility attributed to telomere maintenance differs between distinct 5p15.33 susceptibility loci.

Other analyses using multi-SNP telomere scores have also observed excess risks of lung cancer(22-24) and lung adenocarcinoma(22, 24), but did not observe an effect of TL on oral cancer

risk (23, 24). Opposite directions of effect for the 5p15.33 instrument on lung and HNC are consistent with earlier reports of opposing allelic effects for 5p15.33 SNPs on lung and oral cancer, respectively (35, 55). Leukocyte TL and functional *TERT* variants were previously reported to be unrelated to squamous HNC risk(56), although one study linked short TL to increased HNC risk based on rs2736100, which may be an invalid instrument(22, 57). With the exception of the 5p15.33 IV, the instruments used in this study overlap with those used in other MR analyses of TL (22-24).

Our findings lend support to the hypothesis that a greater number of telomere-increasing alleles increase lung cancer susceptibility. Although the precise molecular mechanisms remain to be elucidated, telomere maintenance may promote carcinogenesis by enabling prolonged cell survival and accumulation of mutations. This is supported by the hallmark observation that telomerase is overexpressed in 85-90% of adult tumors(8, 58), as well as recent data showing that long telomeres increase chromosomal instability(59) and promote immortalization of cancer cells(60). Excessively long telomeres may also be more fragile and dysfunctional, which is supported by the observation that *TERT* not only replenishes telomeres, but also regulates a trimming process to maintain TL homeostasis (61-63).

Differences in the effect of TL persisted after stratifying by smoking status, suggesting that underlying mechanisms differ across tissues and histological types. Longer TL does not appear to increase risk of small cell lung cancer or squamous lung carcinoma, the histology that also comprises 90% of HNC tumours, and for which the causal effect of tobacco smoking is the strongest(64). Since our genetic instruments are unrelated to smoking, confounding is unlikely to account for these differences. It is plausible that genetic predisposition for telomere maintenance offers some protection against genomic instability due to oxidative stress, declining regenerative capacity and immune function(7, 65, 66). Although human papillomavirus (HPV), a known cause of oropharynx cancer(67), has been reported to correlate with TL(31), the similarity of associations observed for oropharynx and oral cancers, only 2% of which are attributed to HPV(68), suggests that HPV infection is unlikely to modify the influence of TL.

This analysis has several important strengths. Genetic instruments represent are unaffected by reverse causality and are more likely to reflect causality due to the independence of genotypes from confounding factors. In addition to the large sample size, our analysis leveraged rich genetic data in 5p15.33, including rare sequence variations, to develop a robust, novel instrument. Furthermore, the use of multiple genetic instruments from essential genes for telomere maintenance mitigates the possibility for weak instruments bias and genetic confounding due to pleiotropy. The association between genetic predisposition to long TL and increased lung cancer risk persisted in analyses using the weighted median and mode-based estimators, which further supports the causal interpretation of these results.

Our mediation analysis offers insight not only by validating the new 5p15.33 instrument, by demonstrating an absence of direct effects, but also by formally quantifying the contribution of telomere-related mechanisms to the observed association between the established lung and adenocarcinoma susceptibility loci and lung cancer risk in this region. Although we confirmed that TL is an important molecular mechanism underlying the associations observed for 5p15.33 lung cancer risk loci, our results also indicated that only a fraction of these genetic effects operate through telomere maintenance. For instance, only 3-8% of the total effect of rs421629 (*CLPTM1L*) was mediated TL, and approximately half of the association between the *TERT* loci and lung cancer risk can be attributed to telomere mechanisms.

These findings are consistent with our knowledge that 5p15.33 is a complex susceptibility locus for multiple cancers(33, 55, 69) and GWAS peaks in this region also encompass non-cancer traits, such as red blood cell counts, prostate-specific antigen levels, and lung diseases(69-72). In addition, non-canonical functions of *TERT*, related to proliferation and differentiation via regulation of Wnt/β-catenin and Myc signaling, have been proposed(73). Therefore, although telomere maintenance is clearly an important 5p15.33 mechanism, cancer susceptibility loci in this region likely invoke additional pathways.

Several limitations of this work should be acknowledged. The time lag between genotype assignment at conception and the assessment of genetic effects on TL and cancer risk, as well as

the time-varying nature of TL, pose challenges for interpreting MR estimates of the causal effect (74). However, while genetic instruments do not recapitulate all aspects of telomere function and dynamics, they can still provide a valid test of the causal hypothesis that inherited predisposition to telomere maintenance increases lung cancer susceptibility (75). Secondly, genetic instruments for leukocyte TL may not be accurate proxies for TL in target tissues, which would reduce the power of our genetic instruments. However, the validity of instruments based on leukocyte TL is supported by correlation between TL in leukocytes and other tissues, including lung, and comparable rates of telomere shortening across somatic tissues (76-78). Thirdly, our MR analysis may be affected by winner's curse, with the magnitude and strength of association with TL observed in the discovery dataset likely to be exaggerated, particularly the 5p15.33 instrument. However, since the instrument discovery and MR analysis populations are independent, any potential bias in the causal parameter due to winner's curse or limited instrument strength will be towards the null (79). A related concern involves our ability to detect subtle effects of TL on cancer risk due to the modest proportion of variation in TL explained by our genetic instruments (approximately 5%), which is comparable to most genetic instruments for complex phenotypes (80-82). Based on our power calculations, this analysis was adequately powered (>80%) to detect effects with OR of 1.5 and above for all lung and HNC histological subtypes and smoking-stratified analyses.

Lastly, the validity of our mediation analysis depends in part on the validity of the published estimates of the mediator-outcome relationship. MR-based estimates of the mediator-outcome relationship are likely to satisfy the assumption of no unmeasured confounding, but must assume that all instruments used in Zhang et al. (22) were valid. While observational studies are more susceptible to confounding and bias due measurement error in the molecular mediator (83), a synthesis of prospective studies provides complementary evidence that does not depend on MR assumptions, and is less vulnerable to reverse causation than case-control designs.

In summary, we demonstrated that genetic determinants of long telomeres are associated with an increased risk of lung cancer, particularly adenocarcinoma. The associations observed for HNC were less consistent with a causal relationship, however we cannot preclude the possibility of

a very subtle telomere effects (OR<1.5). Using mediation analysis that incorporates independent published data, we validated the novel 5p15.33 instrument and quantified the proportion of the lung cancer association signal in 5p15.33 that is mediated by TL. While this work provides insight into the role of TL in cancer etiology, further research is needed to identify appropriate ways of utilizing this complex biomarker in the context of disease prevention or clinical intervention.

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Table 1: Characteristics of the Toronto (MSH-PMH) and Copenhagen (CGPS) OncoArray studies that comprise the dataset for the development of genetic instruments for telomere length in chromosome 5p15.33

Characteristic and description		Toronto (MSH-PMH)		Copenhagen (CGPS)		Total	
		N	(%)	N	(%)	N	(%)
Age (years)	<50	135	(17.4)	287	(24.5)	422	(20.6
	50 to 59	241	(28.6)	259	(22.1)	500	(24.4)
	60 to 69	313	(35.0)	264	(22.5)	577	(28.1)
	70 to 79	143	(14.7)	237	(20.2)	380	(18.5)
	≥80	47	(4.3)	125	(10.7)	172	(8.4)
	Mean (SD)	61.0	(11.7)	61.3	(12.8)	61.2	(12.3)
Sex	Males	436	(49.6)	470	(40.1)	906	(44.2)
	Females	443	(50.4)	702	(59.9)	1145	(55.8)
Smoking status	Never smokers	438	(50.1)	410	(36.4)	848	(41.3)
	Ever smokers	436	(49.6)	717	(61.2)	1153	(56.2)
	Former smokers	366	(41.7)	717	(61.2)	1083	(52.8)
	Current smokers	59	(6.7)	0	(0)	59	(2.9)
	Unknown	5	(0.6)	45	(3.8)	50	(2.4)
	Mean cigarette pack-years (SD)	8.7	(17.2)	14.4	(20.2)	12.0	(19.2)
Total		879		1172		2051	(100.0)

Abbreviations:

CGPS	Copenhagen General Population Study
MSH-PMH	Mount Sinai Hospital-Princess Margaret Hospital study
SD	Standard deviation

Table 2: Genetic variants included in the novel 5p15.33 instrumental variable and their associations with the telomere length Z-score in the combined dataset (n=2051)

Variant	Gene	Alleles		EAF	Per-allele estimate		P-value
		Long TL	Other		β[a,b]	(SE)	
rs956942	*LINC01511*	A	G	2.4×10^{-3}	1.11	(0.29)	1.7×10^{-4}
Chr5:1383486	*CLPTM1L-SLC6A3*	A	G	4.9×10^{-4}	2.09	(0.65)	1.4×10^{-3}
Chr5:1404329	*SLC6A3*	T	C	9.8×10^{-4}	1.28	(0.46)	5.8×10^{-3}
Chr5:1501109	*LPCAT1*	A	G	7.4×10^{-4}	1.46	(0.53)	6.1×10^{-3}
Chr5:1297379	*TERT*	A	C/G	1.5×10^{-3}	0.68	(0.27)	0.01
rs80022192	*LINC01511*	G	A	4.9×10^{-4}	1.60	(0.65)	0.01
rs35033501	*TERT*	A	G	0.03	0.22	(0.09)	0.01
rs28363089	*SLC6A3*	A	G	0.03	0.23	(0.02)	0.01
Chr5:1434327	*SLC6A3*	A	T	0.99	0.89	(0.38)	0.02
Chr5:1402812	*SLC6A3*	T	C	4.9×10^{-4}	1.49	(0.65)	0.02
rs79717857	*CLPTM1L*	A	C	0.02	0.21	(0.09)	0.02
rs35334674	*TERT*	G	A	0.97	0.19	(0.08)	0.02
rs7733853	*LPCAT1*	A	G	0.24	0.08	(0.03)	0.02
rs72715516	*SLC6A3*	G	A	0.96	0.21	(0.10)	0.04

Abbreviations:

EAF	Effect allele frequency, where the effect allele is the long telomere allele
SE	Standard error
LINC01511	Long intergenic non-protein coding RNA 1511
CLPTM1L	Cleft lip and palate associated transmembrane protein 1-like
SLC6A3	Solute carrier family 6 member 3
LPCAT1	Lysophosphatidylcholine acyltransferase 1
TERT	Telomerase reverse transcriptase

[a] Linear regression models adjusted for age, sex, study, and ethnicity principal components

[b] Regression coefficients are standardized and correspond to a 1 standard deviation (1 unit) change in the telomere length Z-score, approximately 1000 base pairs

Table 3: Per-allele associations for the 5p15.33 genetic instrument and relevant telomere and cancer endpoints

Outcome	Sample size (Cases, Controls)		β[a] / OR[b]	(SE) / 95% CI	P-value	F statistic	R^2 (%)
Telomere Length		2051	0.14	(0.02)	2.6×10^{-9}	35.83	1.49
Telomere length in never smokers		848	0.18	(0.04)	7.0×10^{-6}	20.81	2.02
Smoking status (ever/never)		2051	-0.08	(0.06)	0.19	-	-
Cigarette pack-years		1101	0.40	(0.73)	0.59	0.29	0.00
Lung cancer	16396	13013	1.04	1.01, 1.07	4.89×10^{-3}	-	-
Adenocarcinoma	5690	13013	1.06	1.03, 1.10	1.4×10^{-3}	-	-
Squamous cell carcinoma	4045	13013	1.03	0.98, 1.07	0.23	-	-
Head and neck cancer	4415	5013	0.95	0.90, 1.00	0.04	-	-
Oral cavity	2284	5013	0.93	0.87, 0.98	0.01	-	-
Oropharynx	1849	5013	0.96	0.90, 1.03	0.26	-	-
Never smokers							
Lung cancer	1619	3923	1.06	0.99, 1.14	0.08	-	-
Adenocarcinoma	836	3923	1.12	1.02, 1.22	0.02	-	-
Head and neck cancer	773	1827	0.85	0.77, 0.95	3.8×10^{-3}	-	-
Alcohol non-drinkers							
Head and neck cancer	614	795	0.86	0.74, 0.99	0.04	-	-

Abbreviations:

R^2 Coefficient of determination estimating the proportion of the variance in the telomere length Z-score that is explained by the 5p15.33 genetic instrument

SE Standard error

TL Telomere length

[a] Linear regression models were adjusted for age, sex, study, and top 5 ethnicity principal components

[b] Logistic regression models were adjusted for age, sex, study, and top 10 ethnicity principal components

Table 4: Mendelian Randomization estimates of the causal odds ratios for lung and head and neck cancers per 1000 base pair increase in telomere length

Outcome	Cases	Controls	Estimation Method								
			Maximum Likelihood			Inverse Variance Weighted			Weighted Median Estimator		
			OR[a]	95% CI	P-value	OR[a]	95% CI	P-value	OR[a]	95% CI	P-value
Lung cancer	16396	13013	1.41	1.20, 1.65	2.0×10^{-5}	1.39	1.21, 1.60	3.7×10^{-6}	1.37	1.12, 1.67	2.0×10^{-3}
Adenocarcinoma	5690	13013	1.92	1.51, 2.45	1.3×10^{-7}	1.83	1.51, 2.22	5.5×10^{-10}	1.63	1.23, 2.16	6.5×10^{-4}
Squamous	4045	13013	1.04	0.83, 1.29	0.74	1.04	0.83, 1.29	0.74	1.09	0.82, 1.46	0.57
Small cell	1846	13013	1.03	0.76, 1.39	0.86	1.03	0.76, 1.38	0.86	0.96	0.66, 1.38	0.82
Head and neck cancer	4415	5013	0.90	0.70, 1.15	0.39	0.90	0.70, 1.15	0.41	0.71	0.51, 0.98	0.04
Oral cavity	2284	5013	0.88	0.65, 1.19	0.40	0.88	0.65, 1.19	0.40	0.67	0.44, 1.03	0.07
Oropharynx	1849	5013	0.83	0.59, 1.16	0.28	0.83	0.60, 1.16	0.28	0.72	0.46, 1.12	0.14
Ever smokers											
Lung cancer	14498	8815	1.36	1.14, 1.63	5.3×10^{-4}	1.36	1.15, 1.60	2.6×10^{-4}	1.31	1.05, 1.63	0.02
Adenocarcinoma	4754	8815	1.72	1.33, 2.24	4.2×10^{-5}	1.66	1.33, 2.07	5.2×10^{-6}	1.71	1.26, 2.32	6.1×10^{-4}
Squamous	3835	8815	1.06	0.84, 1.35	0.60	1.06	0.84, 1.35	0.61	1.08	0.80, 1.47	0.63
Head and neck	3108	2865	1.12	0.79, 1.58	0.54	1.11	0.79, 1.56	0.54	0.91	0.60, 1.39	0.69
Never smokers											
Lung cancer	1619	3923	1.78	1.22, 2.61	3.1×10^{-3}	1.76	1.23, 2.52	2.0×10^{-3}	1.55	0.98, 2.46	0.06
Adenocarcinoma	836	3923	2.68	1.70, 4.24	2.4×10^{-5}	2.68	1.70, 4.24	2.4×10^{-5}	2.24	1.18, 4.27	0.01
Squamous	149	3923	0.72	0.26, 1.97	0.52	0.72	0.26, 1.95	0.51	0.80	0.22, 2.90	0.75
Head and neck	773	1827	0.72	0.42, 1.22	0.22	0.72	0.42, 1.22	0.22	0.71	0.32, 1.55	0.39
Early onset (≤50 years)											
Lung cancer	1868	1557	1.68	1.07, 2.62	0.02	1.67	1.08, 2.59	0.02	1.76	0.98, 3.22	0.06
Alcohol non-drinkers											
Head and neck	614	795	0.76	0.37, 1.56	0.45	0.76	0.37, 1.57	0.46	0.45	0.17, 1.16	0.10

Abbreviations:

CI Confidence Intervals

OR Odds ratio

[a] Regression models for each genetic instrument were adjusted for age, sex, study, and the top 10 ethnicity principal components

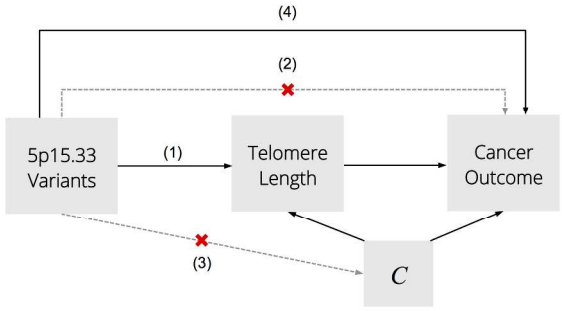

Figure 1: Conceptual diagram of Mendelian Randomization and mediation analyses. Mendelian Randomization is based the following assumptions (1–3): the genetic variant is strongly associated with telomere length; there is no direct association between the instrument and cancer outcome, except through telomere length; the genetic instrument is independent of any confounders (C). Mediation analyses of the 5p15.33 instrument for telomere length and 5p15.33 susceptibility variants test for the presence of direct effects (4), and quantify how much of the total genetic effect on lung cancer risk is mediated by telomere length

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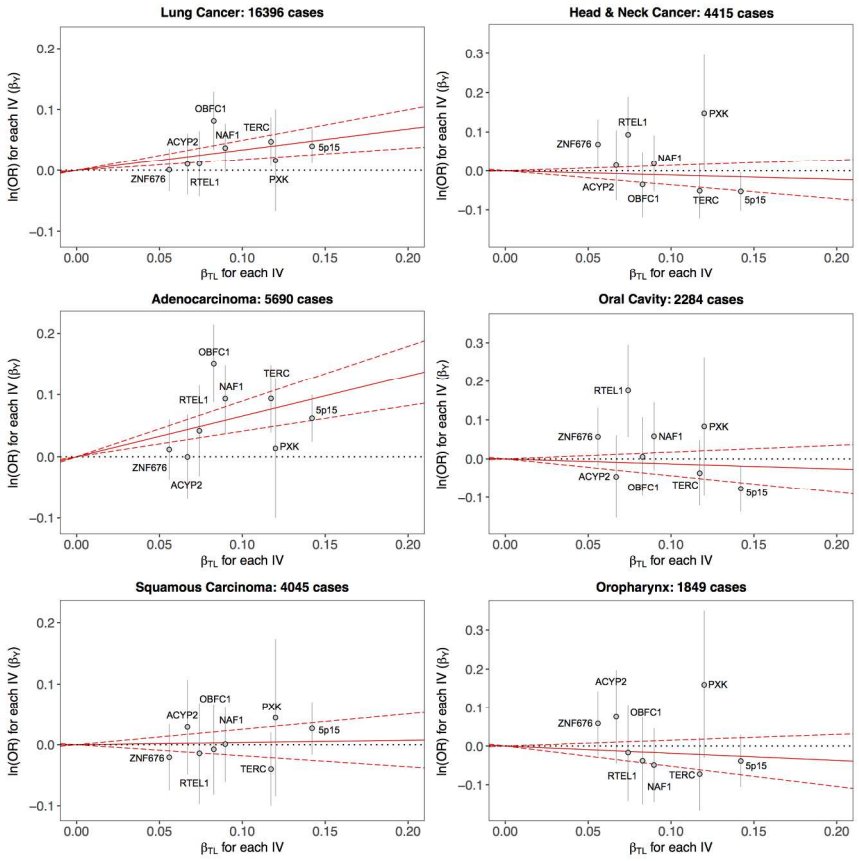

Figure 2: Scatter plots showing the association estimates for telomere length (β_{TL}) and cancer risk (β_Y) for each instrumental variable (IV), overlaid on the causal log odds ratio for the effect of increasing telomere length on cancer risk (solid red line) and corresponding 95% confidence intervals (dotted red lines), estimated using the likelihood-based method

190x254mm (300 x 300 DPI)

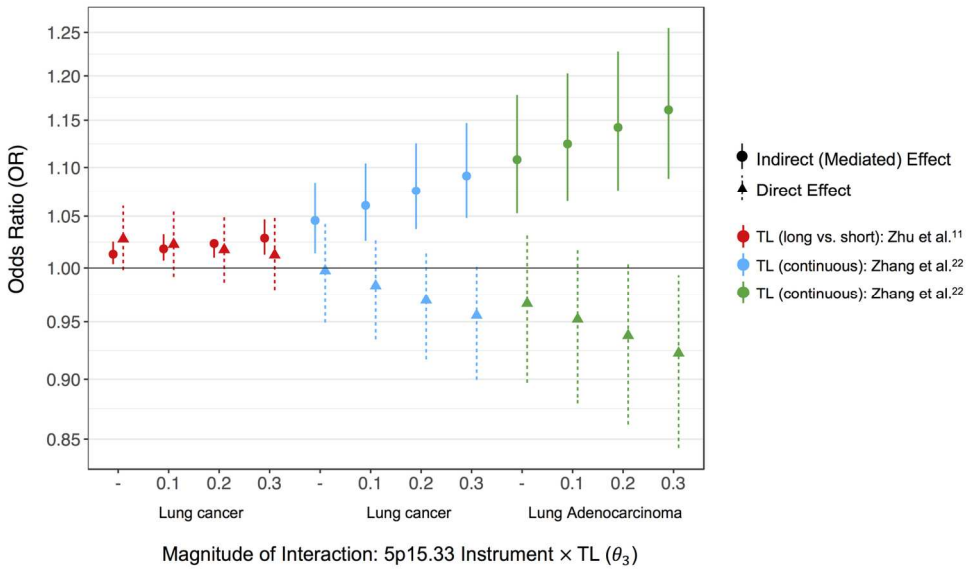

Figure 3: Odds ratio (OR) plot summarizing the direct effects (triangle, dotted line) and indirect effects (circle, solid line) of the 5p15.33 genetic instrument on lung cancer risk. Estimates of the direct and indirect effects are presented across different levels of interaction and for different versions of the mediator (dichotomous and continuous), indicated by different colours.

149x91mm (300 x 300 DPI)