Whole exome sequencing characterization of individuals presenting extreme phenotypes of high and low risk of developing tobacco-induced lung adenocarcinoma

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Background: Tobacco is the main risk factor for developing lung cancer. Yet, some heavy smokers do not develop lung cancer at advanced ages while others develop it at young ages. Here, we assess for the first time the genetic background of these clinically relevant extreme phenotypes using whole exome sequencing (WES).

Methods: We performed WES of germline DNA from heavy smokers who either developed lung adenocarcinoma at an early age (extreme cases, n=50) or did not present lung adenocarcinoma or other tumors at an advanced age (extreme controls, n=50). We selected non-synonymous variants located in exonic regions and consensus splice sites of the genes that showed significantly different allelic frequencies between both cohorts. We validated our results in all the additional extreme cases (i.e., heavy smokers who developed lung adenocarcinoma at an early age) available from The Cancer Genome Atlas (TCGA).

Results: The mean age for the extreme cases and controls was respectively 49.7 and 77.5 years. Mean tobacco consumption was 43.6 and 56.8 pack-years. We identified 619 significantly different variants between both cohorts, and we validated 108 of these in extreme cases selected from TCGA. Nine validated variants, located in relevant cancer related genes, such as *PARP4*, *HLA-A* or *NQO1*, among others, achieved statistical significance in the False Discovery Rate test. The most significant validated variant (P=4.48×10^{-5}) was located in the tumor-suppressor gene *ALPK2*.

Conclusions: We describe genetic variants associated with extreme phenotypes of high and low risk for the development of tobacco-induced lung adenocarcinoma. Our results and our strategy may help to identify high-risk subjects and to develop new therapeutic approaches.

Keywords: Cancer risk; extreme phenotypes; whole exome sequencing (WES); tobacco; lung adenocarcinoma; *ALPK2*; *HLA-A*; *PARP4*; *NOQ1*

Introduction

Lung neoplasms account for the majority of cancer deaths worldwide (1), and their development is strongly linked to tobacco (2,3). Yet, despite the robustness of this association, some heavy smokers develop lung cancer at young ages, while others do not develop it at advanced ages, suggesting that large interindividual differences in the susceptibility to develop tobacco-induced lung cancer exist. The characterization of the potential molecular causes underlying these characteristic phenotypes could allow high-risk populations, that could benefit from prevention and screening programs, to be identified. Most importantly, it could lead to the discovery of the biological mechanisms that explain the existence of individuals presenting phenotypes of increased and decreased susceptibility to developing this disease.

Here, we report for the first time the results of a whole exome sequencing (WES) study of the germline DNA of individuals presenting clinical phenotypes of very high and very low risk of developing tobacco-induced lung adenocarcinoma. For this purpose, we selected individuals who were heavy smokers and either developed lung adenocarcinoma at an early age; or did not develop lung adenocarcinoma -as well as any other tumors, related or unrelated to tobacco- at an advanced age. We aimed to identify new susceptibility variants associated with these clinically relevant phenotypes. We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/tlcr-20-1197).

Methods

Study design

We performed an extreme phenotype study, with the aim of increasing the efficiency of discovering genetic alterations associated with the risk of developing lung adenocarcinoma induced by tobacco. We hypothesized that risk-related genetic alterations would be enriched in the phenotypic extremes, and consequently, a careful selection of individuals presenting the extreme phenotypes of interest would facilitate the identification of such alterations (4-6). This methodology has allowed highly relevant cancer biomarkers to be identified, as reviewed elsewhere (7,8).

The cancer cohort subjects (extreme cases) were selected from heavy smokers (≥15 pack-years) presenting a histologically confirmed diagnosis of lung adenocarcinoma at an early age (≤55 years). We also included one patient who developed lung adenocarcinoma at an extremely young age (37 years) and presented tobacco consumption below 15 pack-years (6), given his phenotypic relevance, and because we assumed that he was too young to achieve the selected threshold of tobacco consumption. Patients with known driver genetic alterations susceptible of targeted therapy (*EGFR*, *ALK*, *ROS1*, *BRAF* genetic alterations, etc.) were not included in the study, in order to increase the molecular homogeneity of the selected individuals.

The cancer-free cohort individuals (extreme controls) were selected from heavy smokers (≥15 pack-years) who had not developed lung adenocarcinoma -or any other cancer, related or not to tobacco- at an advanced age (≥72 years).

The thresholds for age and tobacco consumption were set in order to identify from our series those individuals presenting the most extreme phenotypes regarding the individual risk of developing tobacco-induced lung adenocarcinoma.

Extreme cases and controls were recruited among 3,631 subjects included in the databases of the Center for Applied Medical Research (CIMA, Pamplona, Spain) and the University Clinic of Navarra (Pamplona, Spain), from the University of Navarra.

Samples and data from patients included in the study

were provided by the Biobank of the University of Navarra and were processed following standard operating procedures approved by the Ethics and Scientific Committees.

DNA extraction and genotyping by WES

Genomic DNA was obtained from peripheral EDTA-blood using the QIAamp DNA Mini Kit (Qiagen Iberia, Madrid, Spain) following the manufacturer's instructions, and were stored at –20 ℃ until use. Genotyping was performed with a low input protocol using 800 ng of germline DNA after analysis in a TapeStation system (Agilent, Santa Clara, USA) and sequencing with the Agilent Human Exome Capture v5 kit at 2×100 bp and medium coverage at 75× in a NovaSeq system (Illumina, San Diego, USA). All exome data files are available from the authors upon reasonable request.

Statistical analysis

After quality control (FastQC) and trimming of the reads (trimmomatic) (9), we performed the read alignment using a BWA-MEM aligner and the hg38 human assembly as a reference (10). The resulting BAM files were processed using an analysis pipeline of variant calling based on the GATK best practices (11). Several filters were applied such us the variant score normalized by allele depth for a variant (DP <20), the root mean square of the mapping quality of reads across all samples and the strand biases estimated by both Fisher's Exact Test (FS >60) and Symmetric Odds Ratio Test (SOR >40) among others.

The detected mutations were then annotated using ANNOVAR with different databases of genome localization, variant effect prediction, population SNPs (ExAC and 1000genomes) and clinical association of variants (ACMG, ClinVar, dbSNP and COSMIC) (12). All the results (point mutations and indels) were integrated and analyzed using statistical methods in R/Bioconductor (13). We selected nonsynonymous variants located in exonic regions and in the intronic splice site flanking regions. Types of selected variants included nonsynonymous single nucleotide variants (SNVs), frameshift insertions and deletions, non-frameshift insertions and deletions and stop gains and losses. The comparison of allele frequencies between the experimental groups was performed using *"allelic"* R package (14) and the plots were developed with "ComplexHeatmap" R package (15). In the case of genes harboring 3 or more variants that showed a significant difference in their allele frequencies (P<0.05), a burden analysis at the gene level was

performed using "REBET" R package (16).

Validation of results of extreme cases using data from The Cancer Genome Atlas (TCGA)

We downloaded WES germline data from lung cancer patients included in TCGA (17) from the dbGaP database (https://www.ncbi.nlm.nih.gov/gap/), and we selected all the available patients presenting phenotypes similar to our extreme controls, i.e., heavy smokers (≥15 pack-years) diagnosed with lung adenocarcinoma at a young age (≤55 years). In these TCGA patients we assessed the germline variants that were significantly different between our extreme cases and controls, and we compared these data with our study groups.

Data processing and statistical were similar to the methodology applied in our study groups and have been described in the previous section. We considered that a given variant was validated if it maintained statistical significance (P<0.05) when extreme TCGA cases were compared to our extreme controls, while being non-significant (P>0.1) in the comparison against our extreme cases (*Figure 1*).

We explored the diseases and biological pathways related to the genes harboring the variants that were validated using the Reactome Pathway Database (18) and Ingenuity Pathway Analysis (https://www.qiagenbioinformatics.com), which include manually curated and fully traceable data derived from literature sources.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Ethics Board of Clinical Universidad de Navarra (reference number 165/2015) and informed consent was obtained from all the patients.

Results

We performed germline WES in 100 Caucasian individuals presenting extreme phenotypes of very high and very low risk of developing tobacco-induced lung adenocarcinoma, 50 extreme cases and 50 extreme controls (*Figure 1*). The mean age for the cancer and cancer-free cohorts was respectively 49.7 (range, 34–55) and 77.5 years (range, 72–90). Mean tobacco consumption was respectively 43.6 (range, 6–129.5) and 56.8 pack-years (range, 20–123.8). Additional characteristics are displayed on Table S1.
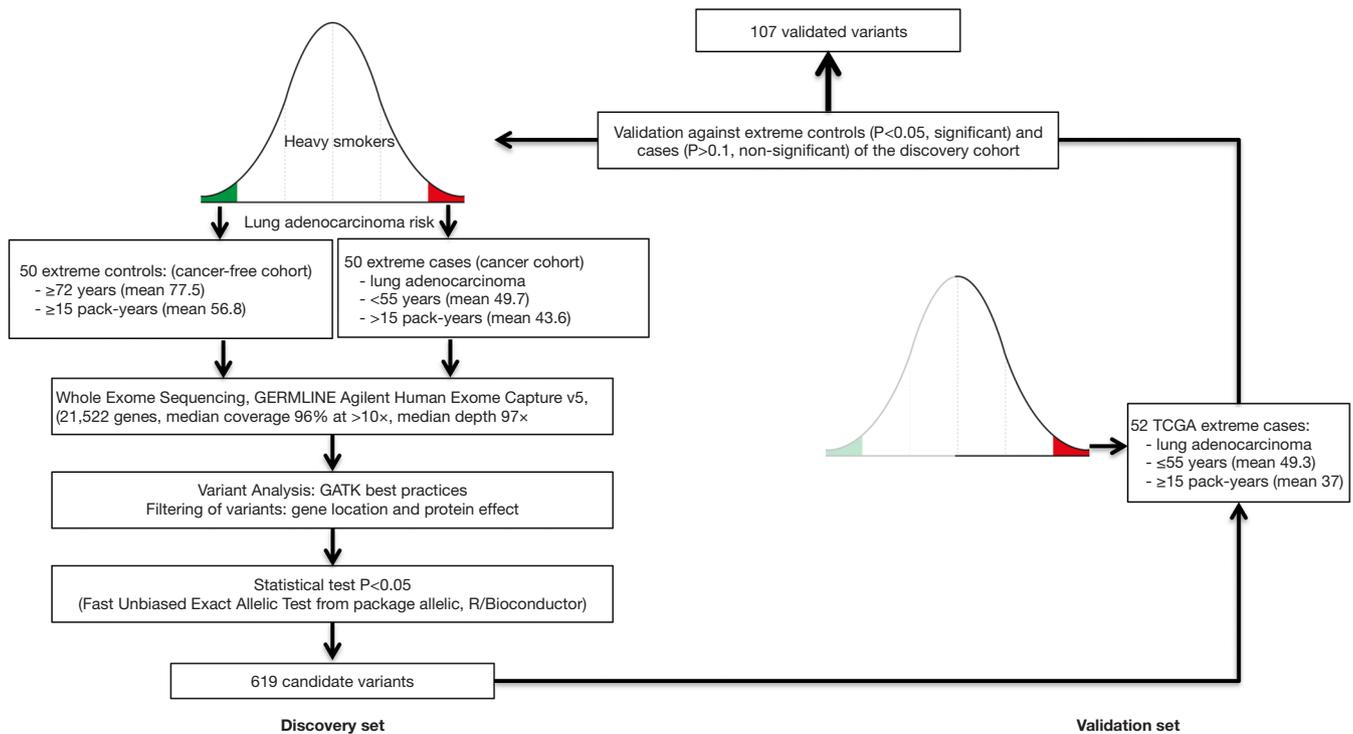
Figure 1 Study design. Left panels (discovery set): we selected individuals presenting extreme phenotypes of high and low risk of developing lung adenocarcinoma induced by tobacco and we analyzed their germline DNA with WES, yielding 619 variants differentially represented (P<0.05) between extreme cases and extreme controls according to their genotype frequencies. Right panel (validation set): significant variants were assessed between TCGA cases presenting the same phenotypes as our extreme cases, and our identification groups. We considered that a given variant was validated if it retained statistical significance (P<0.05) between extreme TCGA cases and our extreme controls, while being non-significant (P>0.01) in the comparison against our extreme cases. TCGA, The Cancer Genome Atlas; WES, whole exome sequencing.

Median exome sequencing coverage was 96% at >10× and median depth was 97×. We identified 619 variants that were differentially represented in their genotype frequencies (P<0.05) between both cohorts, located in 475 genes in Supplementary online file (https://cdn.amegroups.cn/static/application/3d42322f0a2368cd1833caaa0d1ccb48/tlcr-20-1197-1.xlsx).

The most significant variants were located in *ALPK2* (P=4.48×10^{-5}), *HLA-A* (2 variants, presenting respectively P=7.68×10^{-5} and P=1.53×10^{-4}) and *CRIPAK* (P=2.24×10^{-4}). The 50 most significant variants are represented on *Figure 2*.

Twenty-three genes included ≥3 significantly different genetic variants (range, 3–11, Table S2). The genes harboring more significant variants were *PRAMEF2* (11 variants) and *GBP4* (8 variants). Among the genes harboring more than three significant variants, those obtaining the

most significant P values at the gene level were *ANKRD36C* (P=2.28×10^{-14}) and *PRAMEF2* (P=1.2×10^{-12}) (Table S2).

Validation of results using extreme cases obtained from TCGA

We validated our results in all the individuals available from TCGA who presented the same clinical characteristics as our extreme cases, i.e., heavy smokers (≥15 pack-years) presenting a histologically confirmed diagnosis of lung adenocarcinoma at an early age (≤55 years), according to the established criteria (*Figure 1*). We identified 52 extreme cases, who presented a mean age of 49.3 years (range, 33–55) and a mean tobacco consumption of 37 pack-years (range, 15–120).

From the 619 variants previously identified, 547 were assessable in the TCGA germline data, 108 of which
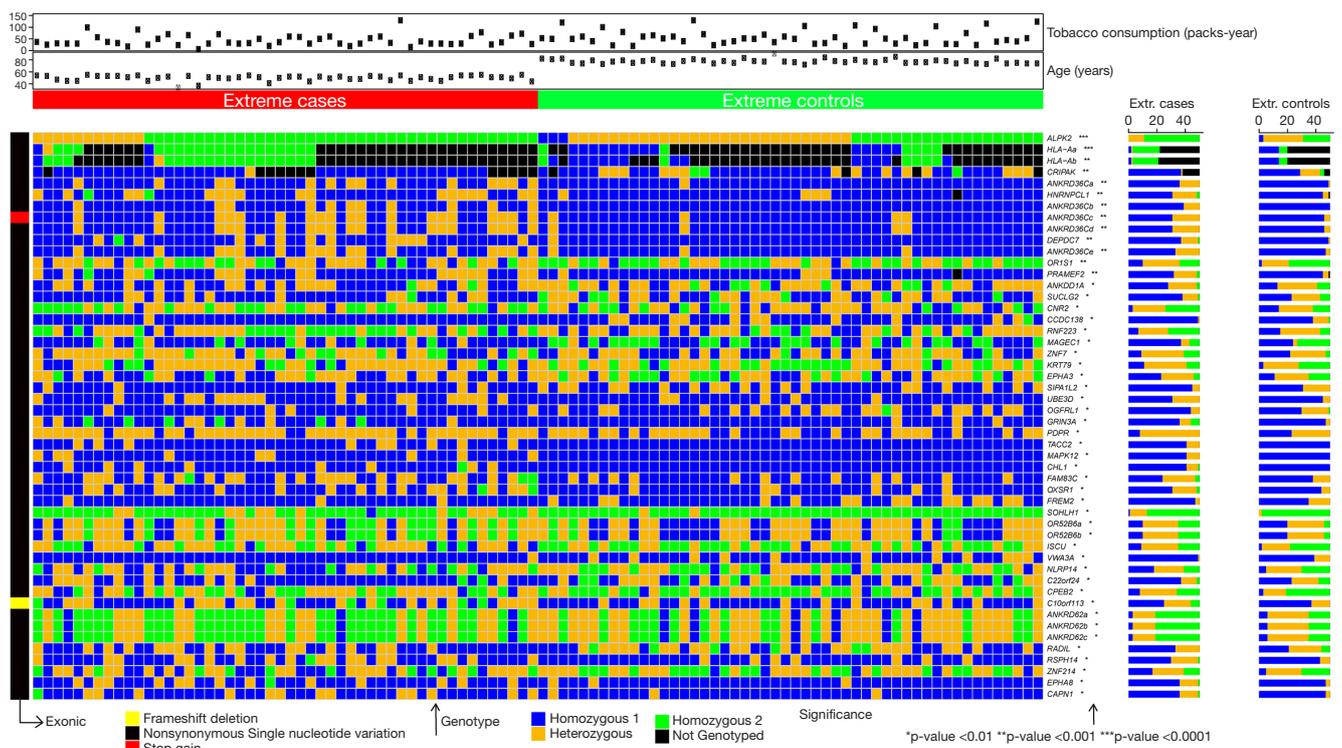
Figure 2 Representation of the 50 variants with the most significantly different allelic frequencies between individuals presenting extreme phenotypes of high and low risk of developing tobacco-induced lung adenocarcinoma in the identification series.

were validated, according to the previously stated criteria: statistical significance (P<0.05) when extreme TCGA cases were compared to our extreme controls, while being non-significant (P>0.1) in the comparison against our extreme cases. All the validated variants are presented in Table S3.

Table 1 represents the variants that obtained the most significant P values in the validation study (19-24). These variants achieved statistical significance in the False Discovery Rate (FDR) test for the comparison of TCGA extreme cases with our extreme controls. From all the validated variants, those presenting the highest significance in the identification study were located in *ALPK2* and *HNRNPCL1*.

Three genes harbored ≥3 validated variants: *PRAMEF19* (6 variants), *RFPL2* (5 variants) and *PRAMEF2* (3 variants). Genes harboring more than 1 validated variants are presented in Table S4.

We explored the diseases related to the genes harboring the validated variants using Ingenuity Pathway Analysis. Forty-eight of the most significant 50 disease categories obtained for such genes were related to cancer, with P values ranging from 2.18×10^{-9} to 3.88×10^{-5} (data not

shown).

We performed a Reactome Pathway Database analysis which indicated that the most significant biological pathways associated with the genes harboring the validated variants were related to immune regulation, interferon and cytokine signaling, and antigen processing and presentation, achieving significant P values in the global analysis and in the FDR test (*Table 2*).

Discussion

In this study we report for the first-time novel germline variants associated with individuals presenting extreme phenotypes of very high and very low risk for developing lung adenocarcinoma induced by tobacco, assessed by WES. We validated our results in an independent group including all the available patients from TCGA who presented the same clinical characteristics as our extreme cases. Many of the most significant validated variants belong to genes associated with relevant pathways related to cancer initiation and development, such as *ALPK2*, *HLA-A*, *PARP4* or *NQO1*, among others. The genes harboring the validated

Table 1 Most significant validated variants, according to the validation P value

Gene	Gene family and function	Variant	P identification	P validation	FDR P validation
PARP4 (NM_006437)	Poly-ADP-ribose polymerases. Maintenance of genomic stability (19)	c.T3194C/ p.V1065A	0.044238228	0.000229805	0.017957651
GSDMB (NM_018530)	Gasdermins. Regulation of cell proliferation and differentiation, and programmed cell death (20)	c.C865T/p.P289S	0.04946443	0.000294914	0.020164734
ZNF761 (NM_001289951)	Zinc fingers. Transcriptional regulation, ubiquitin mediated protein degradation, signal transduction, actin targeting, DNA repair, cell migration, etc. (21)	c.G1582A/p.G528S	0.014883284	0.000523869	0.028640922
VWA3A (NM_173615)	Von Willebrand factor. Regulation of hemostasis and thrombosis (22)	c.G1637A/p.C546Y	0.003787218	0.000574843	0.028640922
ZNF717 (NM_001128223)	Zinc fingers. Transcriptional regulation, ubiquitin mediated protein degradation, signal transduction, actin targeting, DNA repair, cell migration, etc. (21)	c.1298T/p.S433I	0.048581632	0.00061091	0.028640922
ISCU (NM_001301140)	Iron-sulfur cluster assembly enzyme). p53 regulated maintenance of iron homeostasis (23)	c.C35T/p.A12V	0.00374117	0.00062832	0.028640922
NQO1 (NM_001286137)	NAD(P)H: Quinone Oxidoreductase. Detoxification and bioactivation of quinones and reactive oxygen species (24)	c.C343T/p.P115S	0.030727149	0.000696929	0.029324634
ZNF761 (NM_001289951)	Zinc fingers. Transcriptional regulation, ubiquitin mediated protein degradation, signal transduction, actin targeting, DNA repair, cell migration, etc. (21)	c.G1807C/p.E603Q	0.017176972	0.000867587	0.033897847
HLA-A (NM_001242758)	Human leukocyte antigen. HLA mediated antigen presentation	c.C453A/p.N151K	0.009672224	0.001077238	0.039283293

FDR, false discovery rate.

Table 2 Reactome Pathway Database analysis of pathways related to the genes that harbor the validated variants

Pathway name	P value	FDR
Antigen Presentation: Folding, assembly and peptide loading of class I MHC	1.11E-16	3.44E-15
Endosomal/Vacuolar pathway	1.11E-16	3.44E-15
ER-Phagosome pathway	1.11E-16	3.44E-15
Antigen processing-Cross presentation	1.11E-16	3.44E-15
Interferon gamma signaling	1.11E-16	3.44E-15
Interferon alpha/beta signaling	1.11E-16	3.44E-15
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	4.3E-14	1.12E-12
Interferon Signaling	3.46E-13	7.95E-12
Class I MHC mediated antigen processing & presentation	9.95E-12	1.99E-11
Adaptive Immune System	1.72E-6	3.09E-5
Cytokine Signaling in Immune system	1.31E-4	2E-3

FDR, false discovery rate.

variants were markedly associated with cancer disease categories, according to the Ingenuity Pathway Analysis; and they were strongly related to antigen presentation and immune regulation pathways, according to the Reactome Pathway Analysis.

Tobacco-induced lung cancer is one of the most relevant challenges to public health. The identification of molecular factors associated with either increased risk, or intrinsic protection from developing this disease, could allow to identify high-risk populations, in which tobacco cessation and screening programs may be most beneficial. Moreover, it could improve our understanding of the mechanisms of carcinogenesis and physiological protection against developing cancer, which may guide new approaches. Remarkably, although our study was specifically designed for lung adenocarcinoma, the individuals in the cancer-free cohort did not develop any other tumors. Consequently, our results may also be relevant to other neoplasms, especially those related to tobacco.

ALPK2, mapped to 18q21.31, is a tumor suppressor gene regulated by oncogenic *KRAS*. (25) The dependence on *KRAS* is of particular relevance, since lung adenocarcinoma frequently presents *KRAS* mutations (26). *ALPK2* is crucial for luminal apoptosis in normal colonic crypts and plays a critical role in the up-regulation of DNA repair genes in the normal colonic epithelium, including *RAD51*, the Fanconi anemia complementation genes *FANCA*, *FANCE* and *FANCG* and the Bloom syndrome gene (*BLM*) (25). *ALPK2* is down-regulated in

human colonic adenomas, as compared to normal colonic mucosa, thus suggesting its involvement in early neoplastic transformation (25). Lawrence *et al.* found that *ALPK2* was a novel mutated gene in human cancers in a large-scale genomic analysis of 4,742 human neoplasms and their matched normal tissue samples (27). In mice xenograft models, knockdown of *ALPK2* inhibits the development and progression of ovarian cancer (28) and renal cell cancer (29), thus supporting its relevance in cancer initiation and development.

Interestingly, four of the variants identified involve the alpha1 and alpha2 domains of HLA-A molecules in areas oriented to the antigenic peptide binding cleft. HLA-A is a highly polymorphic locus incorporating over 450 allelic protein variants. The amino acid sequence variants discovered in this study are shared by different HLA-A allele subgroups generating diallelic polymorphisms that could be important for antigen presentation to CD8 T-cells and thereby could play a role in immune surveillance against cancer. This is especially relevant in tumors induced by carcinogens, (i.e., tobacco) potentially giving rise to multiple neoantigens. The aminoacid sequences found to be protective in homozygosis are shared by multiple HLA-A alleles, but at least three of the protective aminoacids in positions 86, 101 and 151 are shared by alleles HLA-A23 and HLA-A24. No in-depth studies exist on the correlation between cancer susceptibility and HLA-A allelic variants, and these findings warrant detailed analysis to address the potential influence of such polymorphisms on immune

surveillance.

We validated additional variants in genes related to functions that are relevant for cancer initiation and development (*Table 1*). Moreover, an Ingenuity Pathway Analysis of the genes harboring the validated variants revealed that they are strongly linked to cancer-related diseases; and the Reactome Pathway Database analysis showed that their significant association to immune regulation, interferon and cytokine signaling and antigen processing and presentation. These findings support the notion that the variants identified and validated in our study are linked to genes that are markedly associated with cancer development, and consequently, they represent appealing targets for future research.

Increased risk of developing cancer has been firmly linked to specific genetic alterations, such as *BRCA1/2* (30) or *TP53* mutations (31,32), among others. These and other similar alterations were discovered through the identification of individuals presenting cancer at early ages and/or with familial aggregation. Here, we have followed a similar methodology to define the genetic alterations related with high risk of developing lung adenocarcinoma, maintaining age as a selection factor and substituting familial aggregation for cancer presentation following heavy exposure to a well-known risk factor, such as tobacco.

To our knowledge, no genetic alteration has ever been confirmed to induce protection against cancer development in human beings. Nevertheless, protective genetic alterations have been described in other fields of Medicine. For example, alterations in the chemokine coreceptor *CCR5* confer complete protection against certain strains of the Human Immunodeficiency Virus (HIV) (33,34); *APOE3* homozygote mutations have been reported to confer protection against autosomal dominant Alzheimer's disease (35); and nonsense mutations in *PCSK9*, are associated with low levels of serum low density lipoproteins (LDL) and low risk of cardiovascular disease (36). Remarkably, the first two examples were discovered through the study of individuals highly exposed to well-known risk factors for developing the disease under study, i.e., high exposure to HIV-1 (37); and presence of presenilin 1 (*PSEN1*) mutations, which are associated with autosomal dominant Alzheimer disease (38). As for *PCSK9* mutations, they were discovered through the study of individuals presenting extreme phenotypes consisting of very low serum LDL levels. The success of these approaches supports the methodology that we have developed for the present study.

In a previous study we applied the same methodology, studying as extreme cases individuals with non-small lung cancer, rather than exclusively adenocarcinoma; and assessing the germline DNA through Genome Wide Association Study (GWAS) (39). We identified and validated two new genetic variants in *ATP10D* and *PDE10A* which were differentially expressed in individuals presenting the extreme phenotypes assessed, and we confirmed the prognostic relevance of the associated proteins in early stage non-small cell lung cancer. We defined that protected individuals presented proficient cancer-risk phenotypes (PROCARPs) and high-risk individuals showed deficient cancer-risk phenotypes (DECARPs). For the present study, we focused exclusively on patients presenting lung adenocarcinoma, in order to increase the homogeneity of the phenotypes studied; and we used WES to maximize the chance of identifying gene variants that impact protein synthesis.

The main limitation of our study is that, despite our efforts to recruit highly selected individuals, phenotypic heterogeneity may persist from a clinical, pathological and molecular standpoint. Regarding clinical variables, phenotype selection may be improved by including individuals presenting even more extreme ages and increased tobacco exposure (i.e., higher tobacco consumption and/or shorter tobacco cessation intervals, active smokers, …). Additional inclusion criteria could increase homogeneity, such as presenting a similar stage of the disease (i.e., either early or advanced tumors). From a pathological and molecular perspective, heterogeneity could be further reduced by analyzing patients presenting exclusively specific lung carcinoma subtypes, and/or specific tumor molecular profiles, such as either *KRAS* positive or negative tumors, as well as other molecular alterations. These and other variables were not controlled for in our design because of sample size limitations. We hypothesize that further ongoing efforts to increase the sample size while improving the selection of extreme phenotypes will maximize the power of this strategy. Demographical variables, such as the ethnic background should also be taken into consideration in future studies. In this sense, there are emerging initiatives for developing population-specific genetic databases that can address the local genetic component and the population stratification level, such as the Collaborative Spanish Variability Server (CSVS) (40). Due to sample availability, the individuals included in our study were mainly of Caucasian origin, which may limit the applicability of our findings.

An additional limitation is the possibility that extreme controls may develop lung adenocarcinoma in the future. Yet, this limitation is controlled for by the large differences between the mean ages of both groups, which indicate at least an appreciable delay in the development of clinically assessable tumors. Also, the unlikely possibility that lung adenocarcinoma may have been induced by causes other than tobacco in some extreme cases, must be considered. In addition, while the relevant literature supports the relevance in cancer development of the genes that we validated, the biological effects associated with the variants that we report must be established in functional studies. Importantly, our study reveals which variants were overrepresented in each group of individuals, but it must be determined whether their effects may be associated either with the increased risk observed in cases, or with the increased protection observed in controls. Indeed, additional research is needed to further develop this methodology, including the optimal definition of extreme phenotypes, the determination of the sample size, or the extrapolation of the results to general patient populations (e.g., extreme phenotype *vs.* control population designs) (41).

Conclusions

In summary we have characterized for the first time with WES the germline background of individuals presenting extreme phenotypes of very high and very low risk for developing tobacco-induced lung adenocarcinoma. Our findings may allow individuals presenting high and low risk of developing this tumor to be identified and the molecular mechanisms that explain these clinically relevant phenotypes to be characterized. Consequently, our results and our methodology warrant further development through the comprehensive molecular characterization with different techniques of larger groups of individuals presenting these well-defined and carefully selected extreme phenotypes.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at http://dx.doi.org/10.21037/tlcr-20-1197

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Data Sharing Statement: Available at http://dx.doi.org/10.21037/tlcr-20-1197

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Ethics Board of Clinical Universidad de Navarra (reference number 165/2015) and informed consent was obtained from all the patients.

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Table S1 Subject characteristics

Subject number	Extreme case/extreme control	Age	Packs-year	Sex	Stage	KRAS status
1	Case	34	23.5	Female	IV	Positive
2	Case	37	6	Male	IV	Unknown
3	Case	41	20	Female	IV	Unknown
4	Case	44	30	Female	IV	Unknown
5	Case	44	28	Female	IV	Positive
6	Case	45	30	Female	IV	Positive
7	Case	45	30	Male	IV	Unknown
8	Case	45	30	Female	IIB	Unknown
9	Case	45	25	Female	IV	Unknown
10	Case	45	15	Female	IV	Positive
11	Case	46	33	Male	IV	Unknown
12	Case	47	31	Male	III	Unknown
13	Case	48	30	Male	IV	Positive
14	Case	48	30	Male	IV	Unknown
15	Case	48	19	Male	IV	Negative
16	Case	49	64	Male	IV	Unknown
17	Case	49	60	Female	IIIA	Positive
18	Case	49	34	Male	IV	Unknown
19	Case	50	70	Male	IV	Unknown
20	Case	50	50	Male	IV	Unknown
21	Case	50	36	Male	IV	Unknown
22	Case	51	50	Male	IV	Positive
23	Case	51	39	Female	IV	Positive
24	Case	51	35	Male	IV	Negative
25	Case	51	30	Male	IV	Unknown
26	Case	51	30	Male	IV	Negative
27	Case	51	28	Female	I	Negative
28	Case	51	26	Male	I	Unknown
29	Case	51	17	Male	IV	Negative
30	Case	52	70	Male	IV	Unknown
31	Case	52	60	Male	IV	Negative
32	Case	52	60	Female	IV	Negative
33	Case	52	58.5	Female	IV	Negative
34	Case	52	50	Male	I	Positive
35	Case	52	30	Male	IV	Unknown
36	Case	53	66	Male	IV	Negative
37	Case	53	57	Male	IIIAB	Negative
38	Case	53	53	Male	IV	Unknown
39	Case	53	37	Female	IV	Unknown
40	Case	53	32	Female	IV	Negative
41	Case	53	25	Male	IV	Positive
42	Case	54	129.5	Female	IIIB	Unknown
43	Case	54	90	Male	IV	Positive
44	Case	54	62.5	Male	IIIA	Positive
45	Case	54	37	Female	IV	Negative
46	Case	54	32	Male	III	Negative
47	Case	54	30	Female	IV	Positive
48	Case	55	99	Male	I	Unknown
49	Case	55	78	Male	IIIB	Negative
50	Case	55	72	Male	IIIB	Positive
Mean/distribution (%)		49.72	43.56	Female: 36%; male: 64%	I-II: 10%; II: 16%; IV: 74%	Positive: 28%; Negative: 28%; Unknown: 44%
1	Control	90	36	Male		
2	Control	85	30	Male		
3	Control	84	32	Male		
4	Control	83	52	Male		
5	Control	82	53	Male		
6	Control	82	120	Male		
7	Control	82	37.8	Male		
8	Control	81	50	Male		
9	Control	81	150	Male		
10	Control	80	51	Male		
11	Control	80	65	Male		
12	Control	80	107.5	Male		
13	Control	79	40	Male		
14	Control	79	70	Male		
15	Control	79	104	Male		
16	Control	79	79.5	Male		
17	Control	78	75	Male		
18	Control	78	60	Male		
19	Control	78	33	Male		
20	Control	78	28.5	Male		
21	Control	78	40	Male		
22	Control	78	50	Male		
23	Control	78	57	Male		
24	Control	77	60	Male		
25	Control	77	30	Female		
26	Control	77	22.2	Male		
27	Control	77	29.1	Male		
28	Control	77	68	Male		
29	Control	76	92.5	Male		
30	Control	76	50	Male		
31	Control	76	32	Male		
32	Control	76	22.5	Female		
33	Control	76	20	Female		
34	Control	75	54	Male		
35	Control	75	50	Female		
36	Control	75	40	Male		
37	Control	75	39	Male		
38	Control	75	20	Male		
39	Control	74	123.8	Male		
40	Control	74	60	Male		
41	Control	74	53	Male		
42	Control	74	52.1	Male		
43	Control	74	45	Male		
44	Control	74	27.5	Male		
45	Control	74	24	Male		
46	Control	74	23	Male		
47	Control	73	60	Male		
48	Control	73	100	Male		
49	Control	73	115.5	Male		
50	Control	72	105	Male		
Mean/distribution (%)		77.5	56.79	Female: 8%; Male: 92%		

Table S2 Genes harboring several identified variants

Gene	Hits	Gene-adjusted P value
PRAMEF2	11	1.20E-12
GBP4	8	1.97E-06
ANKRD36C	7	2.28E-14
PRAMEF19	6	1.42E-07
RP1L1	6	0.000146459
TTN	6	2.74E-06
APOL4	5	4.36E-05
RFPL2	5	6.11E-05
ZNF717	5	5.36E-05
ANKRD62	4	1.44E-07
HLA-A	4	6.01E-06
MUC3A	4	0.002443697
SLC22A20	4	3.79E-05
ALPK2	3	3.83E-06
CRIPAK	3	2.21E-05
EPB41L4A	3	0.000948708
FYCO1	3	0.000358811
HNRNPCL1	3	1.25E-05
HSPG2	3	0.001280069
ISCU	3	0.00016821
KCNK15	3	0.000981465
OR51A2	3	0.000588876
SAMD9	3	9.88E-05
ADAMTS13	2	NA
ADAMTS7	2	NA
AFAP1L2	2	NA
ALPK3	2	NA
ANKRD36	2	NA
C10orf113	2	NA
C2CD6	2	NA
C7orf31	2	NA
CACNA1A	2	NA
CCDC65	2	NA
CELSR1	2	NA
CRELD2	2	NA
CROCC	2	NA
CRYBG3	2	NA
DNAH17	2	NA
FAM71A	2	NA
FAM83B	2	NA
FCGBP	2	NA
FHL5	2	NA
FMN1	2	NA
FTSJ3	2	NA
FUT3	2	NA
ICE1	2	NA
KANK4	2	NA
KIAA1161	2	NA
KIF24	2	NA
KRT27	2	NA
KRT76	2	NA
MAGEC1	2	NA
NOC3L	2	NA
NWD1	2	NA
OR1B1	2	NA
OR1S1	2	NA
OR2M2	2	NA
OR52B6	2	NA
OR7G1	2	NA
PARP4	2	NA
PERM1	2	NA
PLIN4	2	NA
RBBP8NL	2	NA
REXO4	2	NA
RNF212	2	NA
RNF223	2	NA
RTN3	2	NA
SCN1B	2	NA
SHROOM3	2	NA
SLC14A1	2	NA
SLC26A8	2	NA
SPATA31E1	2	NA
SPINK5	2	NA
STEAP2	2	NA
STKLD1	2	NA
SUCLG2	2	NA
TCP10	2	NA
UBAP2	2	NA
VWA3A	2	NA
ZNF214	2	NA
ZNF761	2	NA

Table S3 Validated variants

Gene	Transcript	Nucleotide	Protein	P value, identification	P value, validation	P value FDR	ExAC_NFE	1000g_eur
PARP4*	NM_006437	c.T3194C	p.V1065A	0.044238228	0.000229805	0.017957651	0.6021	NA
GSDMB*	NM_018530	c.C865T	p.P289S	0.04946443	0.000294914	0.020164734	0.4531	0.4662
ZNF761*	NM_001289951	c.G1582A	p.G528S	0.014883284	0.000523869	0.028640922	0.4165	0.3956
VWA3A*	NM_173615	c.G1637A	p.C546Y	0.003787218	0.000574843	0.028640922	0.0728	0.0626
ZNF717*	NM_001128223	c.C1300T	p.H434Y	0.048581632	0.00061091	0.028640922	0.0097	NA
ISCU*	NM_001301140	c.C35T	p.A12V	0.00374117	0.00062832	0.028640922	0.6284	0.6799
NQO1*	NM_001286137	c.C343T	p.P115S	0.030727149	0.000696929	0.029324634	0.188	0.2107
ZNF761*	NM_001289951	c.G1807C	p.E603Q	0.017176972	0.000867587	0.033897847	0.4169	0.3986
HLA-A*	NM_001242758	c.C453A	p.N151K	0.009672224	0.001077238	0.039283293	0.44	0.4672
PCDH15	NM_001142763	c.T55G	p.S19A	0.021783253	0.001577643	0.053935675	0.2486	0.2485
EFCAB5	NM_001145053	c.T541G	p.L181V	0.039061049	0.001715852	0.055210047	0.5077	0.4622
CCDC170	NM_025059	c.C806T	p.A269V	0.047859492	0.001895921	0.057614932	0.1046	0.0795
DNAH5	NM_001369	c.G71A	p.G24E	0.041733278	0.002402288	0.069160621	0.2959	0.2833
RFPL2	NM_006605	c.C686G	p.T229S	0.034035563	0.00266233	0.072814729	0.4604	0.493
ZNF717	NM_001128223	c.G1298T	p.S433I	0.048581632	0.002876172	0.074917435	0.009	NA
SCN1B	NM_199037	c.C744A	p.S248R	0.043825599	0.003158097	0.078521767	0.1818	0.1461
ADAMTS13	NM_139025	c.C1852G	p.P618A	0.009488575	0.00336158	0.0786577	0.1014	0.1034
CAPRIN2	NM_001319842	c.A556G	p.M186V	0.008851883	0.003594959	0.0786577	0.6009	0.5736
GRIN3B	NM_138690	c.T2896A	p.Y966N	0.02740015	0.004438778	0.090976832	0.4863	0.4622
IQCC	NM_001160042	c.T866G	p.F289C	0.026661076	0.00449063	0.090976832	0.0593	0.0567
ADAMTS7	NM_014272	c.A3302G	p.H1101R	0.043882539	0.005067793	0.096671997	0.4311	0.4483
GRIN3A	NM_133445	c.G3122A	p.R1041Q	0.002156188	0.005125206	0.096671997	0.1174	0.1173
RFPL2	NM_006605	c.A787G	p.S263G	0.034035563	0.005420003	0.098687083	0.4706	0.493
SAMD9	NM_001193307	c.G1645T	p.V549L	0.019798804	0.005679533	0.098687083	0.1025	0.0974
AFDN	NM_001207008	c.A1264G	p.I422V	0.005770162	0.006016632	0.098687083	0.0264	0.0328
PIGT	NM_001184730	c.A464C	p.D155A	0.030857823	0.006016632	0.098687083	0.0272	0.0298
DCST1	NM_001143687	c.A1459C	p.M487L	0.045800969	0.006134115	0.098687083	0.3485	0.3569
RFPL2	NM_001098527	c.C148T	p.R50C	0.045136896	0.006448596	0.099206134	0.4863	0.4801
ZZEF1	NM_015113	c.C6151T	p.P2051S	0.040340489	0.006529106	0.099206134	0.1322	0.1282
OR1S1	NM_001004458	c.A547G	p.N183D	0.037324377	0.007145987	0.100211877	0.4135	NA
CCDC138	NM_001351545	c.G280T	p.D94Y	0.001675799	0.007152	0.100211877	0.0592	0.0527
RFPL2	NM_006605	c.G239A	p.C80Y	0.049009454	0.007322857	0.100211877	0.4567	0.4642
RFPL2	NM_006605	c.C838T	p.R280C	0.034035563	0.007661038	0.100211877	0.4434	0.4781
SUCO	NM_016227	c.T31C	p.S11P	0.011425725	0.007878678	0.100211877	0.2543	0.2515
SHROOM3	NM_020859	c.C1405G	p.P469A	0.049425871	0.008211575	0.100211877	0.6119	0.6064
STEAP2	NM_001244945	c.T50G	p.F17C	0.04946443	0.00821912	0.100211877	0.5455	0.5119
STEAP2	NM_152999	c.G1367A	p.R456Q	0.04946443	0.00821912	0.100211877	0.5451	0.5119
IP6K3	NM_054111	c.G1039A	p.E347K	0.027746259	0.008244121	0.100211877	0.0114	0.0239
SPATA31E1	NM_178828	c.C2112G	p.D704E	0.037849687	0.008923026	0.106106421	0.0988	0.1103
ZNF212	NM_012256	c.G9T	p.E3D	0.006439433	0.010008181	0.115813375	0.2827	0.1352
EPB41L5	NM_001184937	c.G1384A	p.A462T	0.013378109	0.010162782	0.115813375	0.7266	0.7266
PMF1	NM_001199653	c.A224G	p.Q75R	0.023403507	0.010597929	0.117504701	0.3702	0.3708
OGFRL1	NM_001324266	c.T139C	p.S47P	0.002119722	0.010982935	0.117504701	0.1288	0.1044
MS4A5	NM_023945	c.G152A	p.G51E	0.010279638	0.011169385	0.117504701	0.054	0.0507
RTN3	NM_001265589	c.C17A	p.A6E	0.037849687	0.011170465	0.117504701	0.1459	0.1332
HERC1	NM_003922	c.A6658G	p.I2220V	0.024220389	0.011682191	0.119179625	0.541	0.5368
C15orf40	NM_001160113	c.C31T	p.L11F	0.049560072	0.011765447	0.119179625	0.2519	0.1918
LRIG1	NM_015541	c.A3158C	p.Q1053P	0.006487146	0.012588631	0.122306627	0.2007	0.1958
OTOP3	NM_001272005	c.G1430A	p.R477H	0.012479653	0.012870761	0.122306627	0.0199	0.0219
TNFRSF19	NM_001204459	c.C388T	p.P130S	0.012479653	0.012870761	0.122306627	0.0201	0.0179
C2CD6	NM_001168216	c.T1128A	p.H376Q	0.00880454	0.012968527	0.122306627	0.4128	0.4553
RP1L1	NM_178857	c.C4484G	p.P1495R	0.046794096	0.014193774	0.130687541	0.261	0.2555
ZNF407	NM_001146189	c.A2915C	p.N972T	0.04941747	0.014426711	0.130687541	0.1347	0.1451
PRSS35	NM_153362	c.G671A	p.R224Q	0.016361524	0.014573931	0.130687541	0.8911	0.8936
SCN1B	NM_199037	c.G749C	p.R250T	0.043825599	0.015170203	0.133022262	0.1828	0.1461
CEACAM18	NM_001278392	c.G139A	p.V47M	0.047373275	0.015320663	0.133022262	0.2562	0.2594
UBE3D	NM_001350603	c.G574A	p.V192M	0.001923148	0.015847169	0.13544377	0.1542	0.1571
SHROOM3	NM_020859	c.T973G	p.S325A	0.049425871	0.016241128	0.136675343	0.0882	0.0716
ALPK2	NM_052947	c.A6469G	p.I2157V	4.48E-05	0.016940915	0.140404247	0.8158	0.835
SAMD9	NM_001193307	c.T428C	p.I143T	0.013369963	0.017279771	0.141075148	0.125	0.1292
CEP72	NM_018140	c.A1525G	p.T509A	0.017195383	0.018753848	0.148745972	0.2357	0.2286
DOPEY1	NM_001199942	c.G1760A	p.R587Q	0.022604387	0.019113685	0.148745972	0.126	0.1431
RNF223	NM_001205252	c.C725A	p.P242H	0.040340489	0.020805745	0.148745972	0.2038	0.163
ZNF518A	NM_001278525	c.A2932G	p.M978V	0.036148746	0.021061792	0.148745972	0.7286	0.7386
MAPK12	NM_001303252	c.C308T	p.T103M	0.002634204	0.022425767	0.148745972	0.0539	0.0596
EIF2AK1	NM_001134335	c.A1670G	p.K557R	0.026661076	0.022425767	0.148745972	0.0501	0.0606
HHLA2	NM_001282556	c.C1032A	p.N344K	0.026661076	0.022425767	0.148745972	0.0436	0.0417
RP2	NM_006915	c.C844T	p.R282W	0.026661076	0.022425767	0.148745972	0.0299	0.0209
PRAMEF19	NM_001099790	c.T943C	p.W315R	0.026661076	0.022425767	0.148745972	0.0722	0.0388
PRAMEF19	NM_001099790	c.A829G	p.I277V	0.026661076	0.022425767	0.148745972	–	NA
PRAMEF19	NM_001099790	c.G822A	p.M274I	0.026661076	0.022425767	0.148745972	–	NA
PRAMEF19	NM_001099790	c.T802A	p.Y268N	0.026661076	0.022425767	0.148745972	–	NA
PRAMEF19	NM_001099790	c.C665T	p.A222V	0.026661076	0.022425767	0.148745972	–	NA
PRAMEF19	NM_001099790	c.G604A	p.V202I	0.026661076	0.022425767	0.148745972	–	NA
PRAMEF2	NM_023014	c.G247A	p.E83K	0.026661076	0.022425767	0.148745972	0.0463	NA
OR52B4	NM_001005161	c.121delC	p.L41fs	0.047102298	0.022869093	0.148745972	0.3464	0.3499
PPIL3	NM_032472	c.T450G	p.D150E	0.02658216	0.023588294	0.148745972	0.1247	0.1441
HNRNPCL1	NM_001013631	c.G694C	p.D232H	0.026661076	0.023657952	0.148745972	0.0469	NA
PRAMEF2	NM_023014	c.T612G	p.I204M	0.026661076	0.023657952	0.148745972	0.0473	0.0388
PRAMEF2	NM_023014	c.G631T	p.E211X	0.026661076	0.023657952	0.148745972	0.0472	NA
CACNA1A	NM_001127221	c.G2754C	p.E918D	0.027086483	0.025085417	0.155928672	0.1574	0.1471
COL6A3	NM_057166	c.G6670C	p.D2224H	0.007121036	0.026199237	0.159233141	0.0893	0.0716
OR2B2	NM_033057	c.A701G	p.Q234R	0.027721865	0.026199237	0.159233141	0.0582	0.0596
MYOZ3	NM_001122853	c.T481C	p.S161P	0.026661076	0.027245095	0.160248031	0.9852	0.9811
CDH2	NM_001308176	c.A2441G	p.N814S	0.026661076	0.027245095	0.160248031	0.0289	0.0338
MYL5	NM_002477	c.G516C	p.E172D	0.026661076	0.027245095	0.160248031	0.0078	0.0159
CACNA1E	NM_001205294	c.G5806A	p.A1936T	0.031927993	0.030991739	0.180211835	0.4682	0.4314
SMTN	NM_001207018	c.G1807C	p.A603P	0.025882578	0.031298216	0.180211835	0.6645	0.672
NEB	NM_004543	c.C18242T	p.S6081L	0.030857823	0.031892656	0.181721697	0.0382	0.0189
THAP9	NM_024672	c.G897T	p.L299F	0.038877388	0.033109782	0.186711866	0.5653	0.5507
RP1L1	NM_178857	c.A5666T	p.D1889V	0.047319194	0.034513968	0.192644289	0.2154	0.2147
NUDT6	NM_007083	c.T340C	p.C114R	0.03612642	0.037507497	0.207238391	0.0421	0.0497
ZNF777	NM_015694	c.G1534A	p.A512T	0.049488575	0.038526459	0.210739732	0.0938	0.0825
ATP6AP2	NM_005765	c.C268G	p.P90A	0.026389176	0.039056658	0.211182975	0.063	0.0783
HNRNPCL1	NM_001013631	c.A764G	p.D255G	0.000461102	0.039379641	0.211182975	0.194	0.1352
PHACTR2	NM_001100164	c.C526T	p.P176S	0.049560072	0.040171853	0.21333384	0.1579	0.174
TIMELESS	NM_001330295	c.T3047C	p.I1016T	0.014677691	0.042809418	0.223408604	0.0549	0.0537
SLC22A20	NM_001004326	c.G244A	p.E82K	0.006115612	0.04288465	0.223408604	0.4686	0.4165
ZFP28	NM_001308440	c.C128G	p.A43G	0.014066167	0.044733968	0.230844155	0.3444	0.2654
ELN	NM_001081752	c.G1234A	p.G412S	0.012011833	0.045239475	0.231270961	0.4168	0.4205
EXOC3L4	NM_001077594	c.C2053G	p.Q685E	0.021484233	0.046480592	0.233478992	0.3102	0.2435
PI3	NM_002638	c.C50T	p.T17M	0.03674669	0.047388568	0.233478992	0.1784	0.162
FUCA1	NM_000147	c.A857G	p.Q286R	0.007508067	0.047588449	0.233478992	0.3386	0.332
TACC2	NM_001291876	c.G2157T	p.E719D	0.002634204	0.049123108	0.233478992	0.0323	0.0318
AIFM3	NM_001018060	c.G493A	p.V165I	0.005770162	0.049123108	0.233478992	0.0241	0.0239
ZNF57	NM_001319083	c.C974T	p.T325M	0.026661076	0.049123108	0.233478992	0.0223	0.0209
ALPK3	NM_020778	c.C1241G	p.T414S	0.020334339	0.049810099	0.233478992	0.2769	0.2575
BANF2	NM_001014977	c.C233G	p.T78S	0.010544779	0.049967306	0.233478992	0.2747	0.2604

*, P<0.05 in FDR analysis for the validation P value. FDR, false discovery rate.

Table S4 Genes harboring several validated variants

Gene	Hits
PRAMEF19	6
RFPL2	5
PRAMEF2	3
HNRNPCL1	2
RP1L1	2
SAMD9	2
SHROOM3	2
STEAP2	2
ZNF717	2
ZNF761	2