Genetic Variants in Cell Cycle Control Pathway Confer Susceptibility to Lung Cancer

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Abstract

Purpose: To test the hypothesis that common sequence variants of cell cycle control genes may affect lung cancer predisposition.

Experimental Design: We explored lung cancer risk associations of 11 polymorphisms in seven cell cycle genes in a large case-control study including 1,518 Caucasian lung cancer patients and 1,518 controls.

Results: When individuals with variant-containing genotypes were compared with homozygous wild-type carriers, a significantly increased lung cancer risk was identified for polymorphisms in *p53* intron 6 [rs1625895; odds ratio (OR), 1.29; 95% confidence interval (95% CI), 1.08-1.55] and in *p27* 5′ untranslated region (UTR; rs34330; OR, 1.27; 95% CI, 1.01-1.60). Compared with homozygous wild-types, the homozygous variant genotypes of *STK15* F31I and *CCND1* G870A were associated with a significantly altered lung cancer risk with ORs of 0.58 (95% CI, 0.37-0.90) and 1.26 (95% CI, 1.03-1.53), respectively. To assess the cumulative effects of all the investigated polymorphisms on lung carcinogenesis, we conducted a combined analysis and found that compared with low-risk individuals with few adverse alleles, individuals with more adverse alleles had an increased risk in a significant dose-dependent manner (P_{trend} = 0.041). This pattern was more evident in ever smokers (P_{trend} = 0.037), heavy smokers (P_{trend} = 0.020), and older subjects (P_{trend} = 0.011). Higher-order gene-gene interactions were evaluated using the classification and regression tree analysis, which indicated that *STK15* F31I and *p53* intron 6 polymorphisms might be associated with lung carcinogenesis in never/light-smokers and heavy smokers, respectively.

Conclusions: Our results suggest that cell cycle gene polymorphisms and smoking may function collectively to modulate the risk of lung cancer.

Lung cancer is the leading cause of cancer-related death in the United States. Although tobacco smoking is the predominant risk factor for lung cancer, a consensus is emerging that gene-gene and gene-environment interactions in multiple cancer-related pathways may play a crucial role in this multi-step disease (1).

The intricate and versatile cell cycle regulatory network is essential for mammalian cells to undergo an orderly series of critical cellular events including replication, division, proliferation, and differentiation. Mammalian cell cycle progression is primarily regulated by interactions between cyclins, a family of periodically expressed proteins, and their binding partners, cyclin-dependent kinases (CDK; ref. 2). Among the eight well-studied cyclins, *CCND1* (cyclin D1) is the most important cyclin that promotes cell cycle transition from the G_1 to the S phase through interacting with either *CDK4* or *CDK6* (3). The interaction between CCND1 and CDK4/CDK6 is negatively regulated by two families of CDK inhibitors—the KIP family including p21, p27, and p57, and the INK4 family including p15, p16, p17, and p19 (4). Both p21 and p27 are transcriptionally activated by the tumor-suppressor protein p53, which is also involved in G_1-S transition and S-phase progression (5). In addition, p21 and p53 also play important roles in G_2-M cell cycle checkpoint regulation (6, 7).

Anomalies of cell cycle regulation genes have been frequently observed in a variety of human malignancies, including lung cancer (8–10). Dysfunctions of proto-oncogenes, such as *CCND1* and *STK15*, and tumor suppressor genes, such as *p53*, *p21*, and *p27*, are commonly associated with increased cell proliferation, defective apoptosis, elevated cancer risk, and poor survival (4, 11–14).

It has been proposed that a stepwise accumulation of multiple genetic alterations in the cell cycle control network may precede the commencement of malignant transformation (15, 16). The conventional single-gene–based approach to study the role of genetic variants in carcinogenesis has been unable to yield consistent data across different studies even for the same single nucleotide polymorphisms (SNP). Beyond reasons such as flawed study design and inadequate statistical power, this phenomenon may also be partially attributable to the low penetrance of most highly prevalent SNPs. Recently, the concept of pathway-based polygenic approaches is emerging as

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advantageous over the single-gene strategy to detect modest cancer risk through examining the aggregated influence of multiple SNPs (17–19). However, no study using such an approach to evaluate cell cycle polymorphisms and lung cancer risk has yet been conducted. In this study, we investigated a panel of 11 common sequence variants in seven cell cycle control genes in a case-control study of 1,518 lung cancer patients and 1,518 controls. The selected variants included three well-studied *p53* polymorphisms (a 16-bp insertion/deletion polymorphism in intron 3, a SNP in intron 6, and a nonsynonymous SNP in exon 4), two SNPs of *p21* (a SNP in the 3′ UTR and a nonsynonymous SNP in exon 2), two nonsynonymous SNPs of *STK15* (both in exon 4), a SNP in the 5′ UTR of *p27*, a SNP in exon 4 of *CCND1*, a SNP in the promoter region of *CDK4*, and a SNP in the 3′ UTR of *CDK6*. These SNPs were selected from published association studies and the dbSNP database of The National Center for Biotechnology Information based on their location (promoter, coding regions, or untranslated regions) and minor allele frequencies (>5%; refs. 20–22). We examined the individual and combined effects of these polymorphisms and evaluated higher-order gene-gene and gene-smoking interactions using classification and regression tree (CART) analysis. The objective was to expand our current understanding of the role of cell cycle control genetic variations in lung cancer susceptibility.

Materials and Methods

Study population and epidemiologic data. Lung cancer patients were accrued at The University of Texas M.D. Anderson Cancer Center for a molecular epidemiologic study. These cases were newly diagnosed and histologically confirmed lung cancer patients without previous chemotherapy or radiotherapy. There were no recruitment restrictions on age, gender, ethnicity, or cancer stage. Healthy controls were accrued from the Kelsey-Seybold Clinics, the largest multispecialty physician group in the Houston metropolitan area. Controls had no previous history of cancer (except nonmelanoma skin cancer) and were frequency matched to cases on the basis of age (±5 years), gender, ethnicity, and smoking status. Both cases and controls were interviewed by trained M. D. Anderson staff interviewers using a structured questionnaire to collect information on demographic characteristics (age, gender, ethnicity, etc.), work history, tobacco use history, and family history of cancer. Each participant had 40-mL blood drawn into a coded, heparinized tube that was sent to laboratory for immediate DNA extraction. Laboratory personnel were blinded to case control status. All patients signed written informed consent, and human subject approval was obtained from both M.D. Anderson and Kelsey-Seybold institutional review boards.

Genotyping. All polymorphisms were genotyped using a 5′ nuclease assay–based *TaqMan* SNP genotyping assay except for *p53* intron 3, which was genotyped using a PCR-restriction fragment length polymorphism (22). Sequences for genotyping probes and primers were either obtained from the SNP500Cancer database or designed using the PrimerExpress 2.0 software (Applied Biosystems). The probes were labeled fluorescently with either 6-FAM or VIC on the 5′ end and a nonfluorescent minor groove binder quencher on the 3′ end. The genotyping procedure was described previously (23). Briefly, genomic DNA was extracted from peripheral blood lymphocytes using the Human Whole Blood Genomic DNA Extraction Kit (Qiagen). The PCR amplification mix (5 μL) included sample DNA (5 ng), 1× TaqMan buffer A, 200 μmol/L deoxynucleotide triphosphates, 5 mmol/L $MgCl_2$, 0.65 units of AmpliTaq Gold, 900 nmol/L of each primer, and 200 nmol/L of each probe. The PCR conditions included one cycle for 10 min at 95°C, 40 cycles for 15 s at 95°C, and 1 min at 60°C. ABI PRISM 7900HT sequence detection system (Applied Biosystems) and SDS 2.1 software (Applied Biosystems) were used to read and analyze the end-point genotyping data. Internal quality controls and negative controls were used to ensure genotyping accuracy, and 5% of all samples were randomly selected and genotyped in duplicate with 100% concordance. Sequences of probes and primers are available upon request.

Statistical analysis. Statistical analyses were done using either SAS software (SAS Institute, Inc.) or Intercooled Stata 8.0 statistical software package (Stata Corp.). Pearson's χ^2 test was used to assess the differences of categorical variables such as gender and smoking status between cases and controls. Student's t test was used to test the differences between cases and controls for continuous variables including age and pack-years. Hardy-Weinberg equilibrium was determined using the goodness-of-fit χ^2 test to compare the observed frequency with the expected frequency in both cases and controls. The lung cancer risks were calculated as odds ratios (OR) and 95% confidence intervals (95% CI) using unconditional multivariate logistic regression adjusted for age, gender, smoking status (never and ever smoking), and pack-years (light and heavy smoking), where appropriate. The definitions of smoking status and pack-years were as previously described (24). To assess the combined effects, we defined the minor allele as the adverse allele except for those minor alleles associated with a significant ($P < 0.05$) reduction in cancer risk. In this case, the wild-type allele was defined as the adverse allele. For those genes with multiple SNPs assayed, only one SNP was included in the combined analyses because all tested SNPs in the same gene were in linkage disequilibrium (data not shown). The adverse alleles were identified from main effects and categorized by quartiles of the number of adverse alleles in controls. Using the quartile with the lowest number of adverse alleles as a reference, the ORs and 95% CIs were calculated for each of the other quartiles using multivariate logistic regression adjusted for age, gender, and smoking status. We also examined the combined effects by the number of unfavorable genotypes identified from the main effects analysis of single SNPs. Higher-order gene-gene interaction were evaluated using CART analysis implemented by the HelixTree Genetics Analysis Software (version 4.1.0, Golden Helix). CART is a nonparametric decision tree–based data mining approach to identify specific combinations of genetic and environmental factors associated with disease risk (18). The recursive-partitioning algorithm in HelixTree starts at the first node (with the entire data set) and uses a statistical hypothesis-testing method, formal inference-based recursive modeling, to determine the first locally optimal split and each subsequent split of the data set, with multiplicity-adjusted P values to control tree growth ($P < 0.05$). This process continues until the terminal nodes have no subsequent statistically significant splits or the terminal nodes reach a prespecified minimum size (at least 10 subjects for each terminal node in our analysis). Subgroups of individuals with differential risk associations were identified in the different order of nodes of the tree structure, indicating the presence of gene-gene and gene-environment interactions. Logistic regression was used to calculate the OR and 95% CI in each terminal node of the tree. In this study, all statistical analyses were two-sided and $P < 0.05$ was considered as the statistically significant threshold.

Results

Subject characteristics. There were 1,518 white lung cancer patients available for this analysis. We identified 1,518 cancer-free controls from the control database of 2,098 subjects matched to cases on age, gender, and smoking status. The matching was adequate on age (cases versus controls: 61.7 ± 11.1 years versus 61.4 ± 9.4 years, $P = 0.422$), gender (in both cases and controls: males, 52.17%, females, 47.83%, $P = 1.000$), and smoking status (cases: never smoker, 17%; ever smoker, 83%; controls: never smoker, 15.42%, ever smoker, 84.58%, $P = 0.237$). Among ever smokers, cases reported a significantly higher level of cigarette consumption than

controls, as assessed by the mean value of pack-years (cases versus controls: 43.5 ± 35.0 versus 39.0 ± 32.6, $P = 0.0003$).

Main effects on lung cancer risk by individual polymorphisms. The overall lung cancer risks associated with the individual polymorphisms are listed in Table 1. All polymorphisms conformed to Hardy-Weinberg equilibrium in the controls. When heterozygotes and rare homozygotes were combined to compare with the common homozygotes, two SNPs, *p53* intron 6 (rs1625895) and *p27* 5′ UTR (rs34330), exhibited significantly increased lung cancer risks (OR, 1.29; 95% CI, 1.08-1.55 and OR, 1.27; 95% CI, 1.01-1.60, respectively). In addition, when compared with the homozygous wild-type reference group, a significantly altered lung cancer risk was observed for the homozygous variant genotype of both *STK15* F31I (OR, 0.58; 95% CI, 0.37-0.90) and *CCND1* G870A (OR, 1.26; 95% CI, 1.03-1.53). We further analyzed the effects of these SNPs on cancer risk stratified by smoking intensity (Table 2). We found that compared with the wild-type genotype, the variant-containing genotypes of *p53* intron 6 and *CCND1* G870A were significantly associated with an increased cancer risk in heavy smokers with ORs of 1.48 (95% CI, 1.15-1.89) and 1.40 (95% CI, 1.06-1.84), respectively. The homozygous variant genotype of *p27* 5′ UTR was significantly associated with an increased cancer risk in never smokers with OR of 1.83 (95% CI, 1.10-3.15; Table 2).

Lung cancer risk by smoking intensity for polymorphisms of p53 intron 6, p27 5′ UTR, CCND1 G870A, and STK15 F31I. Table 3 summarizes the effects of smoking intensity on lung cancer risk

Table 1. Allelic and genotypic frequencies and risks for lung cancer in Caucasians

Genes (reference no.)	Major/minor allele		Minor allele frequency	Common homozygote (*n*)	Heterozygote (*n*)	Rare homozygote (*n*)	Heterozygote and rare homozygote (*n*)	HWE *P*
CCND1 G870A (rs603965)	G/A	Case	0.47	368	638	284	922	0.81
		Control	0.44	369	645	227	872	0.06
		OR (95% CI)*		Reference	0.99 (0.82-1.18)	1.26 (1.03-1.53)	1.06 (0.89-1.25)	
p53 Intron 3 (16 bp deletion)	Deletion/—	Case	0.14	1,044	348	20	368	0.14
		Control	0.13	1,036	301	26	327	0.45
		OR (95% CI)		Reference	1.15 (0.97-1.38)	0.77 (0.42-1.38)	1.12 (0.94-1.33)	
p53 R72P (rs1042522)	G/C	Case	0.25	756	492	92	584	0.33
		Control	0.26	702	481	90	571	0.54
		OR (95% CI)		Reference	0.95 (0.81-1.12)	0.93 (0.68-1.27)	0.95 (0.81-1.11)	
p53 Intron 6 (rs1625895)	G/A	Case	0.15	956	347	21	368	0.10
		Control	0.12	960	260	25	285	0.14
		OR (95% CI)		Reference	1.34 (1.11-1.61)	0.83 (0.46-1.49)	1.29 (1.08-1.55)	
CDK4 promoter (rs2072052)	A/C	Case	0.31	287	252	61	313	0.61
		Control	0.30	294	248	58	306	0.59
		OR (95% CI)		Reference	1.04 (0.82-1.33)	1.06 (0.71-1.58)	1.04 (0.83-1.32)	
CDK6 3′ UTR (rs42309)	G/T	Case	0.27	321	238	41	279	0.73
		Control	0.25	342	233	38	271	0.84
		OR (95% CI)		Reference	1.1 (0.87-1.40)	1.22 (0.76-1.96)	1.12 (0.89-1.41)	
p27 5′ UTR (rs34330)	C/T	Case	0.28	324	222	40	262	0.81
		Control	0.22	369	207	33	240	0.58
		OR (95% CI)		Reference	1.25 (0.98-1.59)	1.39 (0.85-2.27)	1.27 (1.01-1.60)	
p21 3′ UTR (rs1059234)	C/T	Case	0.07	539	83	2	85	0.76
		Control	0.07	541	83	1	84	0.35
		OR (95% CI)		Reference	0.96 (0.69-1.34)	1.78 (0.16-19.7)	0.97 (0.70-1.35)	
p21 S31R (rs1801270)	C/A	Case	0.07	536	84	1	85	0.35
		Control	0.07	535	86	1	87	0.35
		OR (95% CI)		Reference	0.96 (0.69-1.33)	0.90 (0.06-14.5)	0.95 (0.69-1.32)	
STK15 F31I (rs2273535)	A/T	Case	0.20	692	373	36	409	0.09
		Control	0.22	594	320	51	371	0.36
		OR (95% CI)		Reference	1.03 (0.85-1.24)	0.58 (0.37-0.90)	0.97 (0.81-1.16)	
STK15 I57V (rs1047972)	G/A	Case	0.15	916	321	26	347	0.73
		Control	0.16	819	304	31	335	0.66
		OR (95% CI)		Reference	0.95 (0.79-1.14)	0.74 (0.44-1.27)	0.93 (0.78-1.11)	

Abbreviation: HWE, Hardy-Weinberg equilibrium.
*Adjusted for age, gender, smoking status, and pack-years.

Table 2. Modulating effect of *p53* intron 6, *p27* 5′ UTR, *CCND1* G870A, and *STK15* F31I genotypes on lung cancer risk by smoking intensity

Smoking intensity	Genotype	*p53* intron 6		*p27* 5′ UTR		*CCND1* G870A		*STK15* F31I	
		Cases/controls	OR (95% CI)*	Cases/controls	OR (95% CI)	Cases/controls	OR (95% CI)	Cases/controls	OR (95% CI)
Never	*Genotype 1* †	163/128	Reference	61/80	Reference	164/136	Reference	174/151	Reference
	Genotype 2 †	48/41	0.91 (0.56-1.47)	63/46	1.83 (1.10-3.15)	46/35	1.06 (0.64-1.74)	7/4	1.08 (0.69-1.67)
Light	*Genotype 1*	279/380	Reference	114/160	Reference	307/409	Reference	325/394	Reference
	Genotype 2	114/122	1.27 (0.94-1.72)	76/115	0.94 (0.65-1.38)	77/91	1.11 (0.79-1.56)	7/21	0.87 (0.64-1.17)
Heavy	*Genotype 1*	514/452	Reference	149/129	Reference	535/469	Reference	566/369	Reference
	Genotype 2	206/122	1.48 (1.15-1.89)	123/79	1.35 (0.93-1.95)	161/101	1.40 (1.06-1.84)	25/23	0.98 (0.75-1.28)

*Adjusted for age, gender, and smoking status.
†Genotype 1: WW for *p53* intron 6 and *p27* 5′ UTR, WW+WM for *CCND1* G870A and *STK15* F31I; Genotype 2: WM+MM for *p53* intron 6 and *p27* 5′ UTR, MM for *CCND1* G870A and *STK15* F31I (WW, common homozygotes; WM, heterozygotes; MM, rare homozygotes).

as stratified by genotypes of the four significant SNPs identified from the main effect analysis. As expected, compared with light smokers, heavy smokers had an increased lung cancer risk for all three genotypes for each SNP. The effect was more striking in individuals with the homozygous variant genotypes for all four SNPs, especially *p53* intron 6 and *p27* 5′ UTR. The increased lung cancer risk conferred by heavy smoking was apparently higher in carriers of the homozygous variant genotype of *p53* intron 6 (OR, 8.65; 95% CI, 2.01-37.17) than that in the wild-type (OR, 1.52; 95% CI, 1.24-1.87) or heterozygous (OR, 1.61; 95% CI, 1.13-2.31) carriers. Similarly, the heavy smoking–conferred risk in the homozygous variant carriers of *p27* 5′ UTR (OR, 6.24; 95% CI, 1.67-23.34) was much higher than that in individuals carrying the heterozygous (OR, 2.05; 95% CI, 1.31-3.21) or the homozygous wild-type genotype (OR, 1.54; 95% CI, 1.09-2.19).

Combined effects of adverse alleles. We evaluated the combined effects of multiple adverse alleles in the cell cycle control pathway on lung cancer risk. A tertile analysis based on the number of adverse alleles in controls revealed an increased risk of lung cancer in a dose-dependent fashion: Relative to the reference group of subjects with one variant allele, a 1.53-fold increased risk (95% CI, 0.97-2.41) was noted for subjects with two variant alleles and a 1.59-fold increased risk (95% CI, 1.07-2.34) for those with three or more variant alleles (P_{trend} = 0.041; Table 4). Similar results were observed in quartile analysis: Compared with the reference group of subjects with ≤1 adverse allele, the ORs for individuals with two, three, and four adverse alleles were 1.50 (95% CI, 0.95-2.38), 1.58 (95% CI, 1.02-2.44), and 1.59 (95% CI, 1.05-2.39), respectively (P_{trend} = 0.074; data not shown).

We also evaluated the combined adverse allelic effects on lung cancer risk by host characteristics (Table 4). When the subjects were stratified by smoking status, the gene-dose effect of the combined variant alleles was only evident in ever smokers but not in never smokers. Compared with the reference group (one variant allele), both the medium-risk (two variant alleles) and the high-risk (three or more variant alleles) groups exhibited increased lung cancer risks in ever smokers (OR, 1.60; 95% CI, 0.96-2.67; OR, 1.68; 95% CI, 1.09-2.58, respectively; P_{trend} = 0.037). Similar findings were noted in heavy smokers with ORs of 2.07 (95% CI, 1.00-4.28) for the medium-risk group and 2.23 (95% CI, 1.21-4.11) for the high-risk group (P_{trend} = 0.020) but not in light smokers, and in older persons with ORs of 1.63 (95% CI, 0.84-3.19) for the medium-risk group and 2.08 (95% CI, 1.17-3.70) for the

Table 3. Modulating effect of smoking intensity on lung cancer risk by *p53* Intron 6, *p27* 5′ UTR, *CCND1* G870A, and *STK15* F31I genotypes

Genotype	*p53* Intron 6		*p27* 5′ UTR		*CCND1* G870A		*STK15* F31I	
	Cases/controls	OR (95% CI)	Cases/controls	OR (95% CI)	Cases/controls	OR (95% CI)	Cases/controls	OR (95% CI)*
WW								
Light	279/379	Reference	114/159	Reference	119/142	Reference	214/252	Reference
Heavy	514/452	1.52 (1.24-1.87)	149/129	1.54 (1.09-2.19)	191/187	1.24 (0.89-1.71)	375/247	1.83 (1.42-2.35)
WM								
Light	110/107	Reference	65/98	Reference	188/266	Reference	111/141	Reference
Heavy	190/115	1.61 (1.13-2.31)	102/71	2.05 (1.31-3.21)	344/282	1.68 (1.31-2.16)	191/122	1.88 (1.33-2.66)
MM								
Light	4/15	Reference	11/17	Reference	77/91	Reference	7/21	Reference
Heavy	16/7	8.65 (2.01-37.17)	21/8	6.24 (1.67-23.34)	161/101	1.88 (1.25-2.83)	25/23	2.94 (1.03-8.39)

Abbreviations: WW, common homozygotes; WM, heterozygotes; MM, rare homozygotes.
*Adjusted for age, gender, and smoking status.

Table 4. Combined effects of adverse alleles in the cell cycle control pathway on lung cancer risk

No. adverse alleles	Cases (*n*)/ controls (*n*)	OR (95% CI)*
Overall		
≤1	51/75	Reference
2	102/101	1.53 (0.97-2.41)
≥3	362/347	1.59 (1.07-2.34)
P_{trend}		0.041
Age		
<62 y †		
≤1	28/37	Reference
2	54/47	1.48 (0.79-2.79)
≥3	163/171	1.25 (0.73-2.14)
P_{trend}		0.688
≥62 y		
≤1	23/38	Reference
2	48/54	1.63 (0.84-3.19)
≥3	199/176	2.08 (1.17-3.70)
P_{trend}		0.011
Gender		
Male		
≤1	30/38	Reference
2	56/50	1.52 (0.81-2.85)
≥3	188/153	1.57 (0.92-2.68)
P_{trend}		0.147
Female		
≤1	21/37	Reference
2	46/51	1.61 (0.82-3.15)
≥3	174/194	1.61 (0.91-2.86)
P_{trend}		0.171
Smoking status		
Never smokers		
≤1	11/13	Reference
2	24/24	1.19 (0.44-3.21)
≥3	76/74	1.22 (0.51-2.91)
P_{trend}		0.691
Ever smokers		
≤1	40/62	Reference
2	78/77	1.60 (0.96-2.67)
≥3	286/273	1.68 (1.09-2.58)
P_{trend}		0.037
Smoking intensity		
Light smokers (pack-years <39)		
≤1	30/44	Reference
2	58/68	1.26 (0.70-2.26)
≥3	191/232	1.22 (0.74-2.02)
P_{trend}		0.559
Heavy smokers (pack-years ≥39)		
≤1	21/31	Reference
2	44/33	2.07 (1.00-4.28)
≥3	171/115	2.23 (1.21-4.11)
P_{trend}		0.020

*Adjusted for age, gender, smoking status, and pack-years for the overall analysis; adjusted for age, gender, and pack-years for the analysis stratified with smoking status; adjusted for age, gender, and smoking status for the analysis stratified with pack-years; adjusted for age, smoking status, and pack-years for the analysis stratified with gender; and adjusted for gender, smoking status, and pack-years for the analysis stratified with age.
† The median age of control subjects is 62 y.

high-risk group (P_{trend} = 0.011). No significant association was identified by gender.

We further evaluated the combined effects of high-risk genotypes on lung carcinogenesis by summing the unfavorable genotypes of four risk-conferring SNPs including *p53* intron 6, *p27* 5′ UTR, *STK15* F31I, and *CCND1* G870A. Using the combination of GG, CC, TT, and GG+GA genotypes (for *p53* intron 6, *p27* 5′ UTR, *STK15* F31I, and *CCND1*, respectively) as the reference group, a progressively increased gene-dosage effect was observed when subjects were categorized on the basis of increasing number of unfavorable genotypes (Table 5). The groups with one, two, three, and four unfavorable genotypes all exhibited a significantly increased lung cancer risk with ORs of 3.82 (95% CI, 1.24-11.80), 3.73 (95% CI, 1.21-11.49), 4.70 (95% CI, 1.49-14.78), and 7.55 (95% CI, 1.89-30.17), respectively (P_{trend} = 0.002). This analysis was further stratified by smoking intensity. We observed similar gene-dosage effects in heavy smokers but not in light smokers (data not shown).

CART analysis. Figure 1 depicts the tree structure generated by using CART analysis, which included all investigated genetic variants and the smoking phenotype variable (never/light smokers versus heavy smokers). The smoking phenotype was singled out in the first splitting node (well-matched variables such as age and gender were excluded from the CART analysis). There was a clear distinction in polymorphism profiles between subgroups of never, light, and heavy smokers. In never smokers, individuals with the variant genotypes of both *p27* 5′ UTR and *p21* 3′ UTR exhibited the lowest lung cancer risk with a 27% case rate. Using this terminal node as the reference, never smokers with the variant genotype of *p27* 5′ UTR but wild-type genotype of *p21* 3′ UTR exhibited a 4.1-fold (95% CI, 1.03-16.55) increased risk (Fig. 1). In heavy smokers, the key predictor was the *p53* intron 6 SNP. *CCND1* G870A and *p53* exon4 R72P variants were the most important variables to classify individuals with the variant genotypes of the *p53* intron 6 SNP in heavy smokers. When compared with the reference group of individuals with the variant genotypes of *p27* 5′ UTR and *p21* 3′ UTR in never smokers, heavy smokers with at least one variant allele of *p53* intron 6, at least one wild-type allele of *CCND1*, and the homozygous wild-type genotype of *p53* exon 4 R72P exhibited the highest lung cancer risk (OR, 12.10; 95% CI, 2.34-62.73).

Discussion

In this study, we used a polygenic approach to investigate the combined effects of 11 common variants in seven cell cycle control genes on lung cancer risk. Multivariate logistic

Table 5. Joint effects of unfavorable genotypes in case patients and control subjects in Caucasians

No. unfavorable genotypes	Cases/controls	OR (95% CI)*
Reference group †	4/15	Reference
1	197/208	3.82 (1.24-11.80)
2	229/241	3.73 (1.21-11.49)
3	106/94	4.70 (1.49-14.78)
4	16/9	7.55 (1.89-30.17)
P_{trend}		0.002

*Adjusted for age, gender, smoking status, and pack-years.
†Reference group: *CCND1* G870A: GG+GA, *p53* intron 6: GG, *STK15* F31I: TT, and *p27* 5′ UTR: CC.

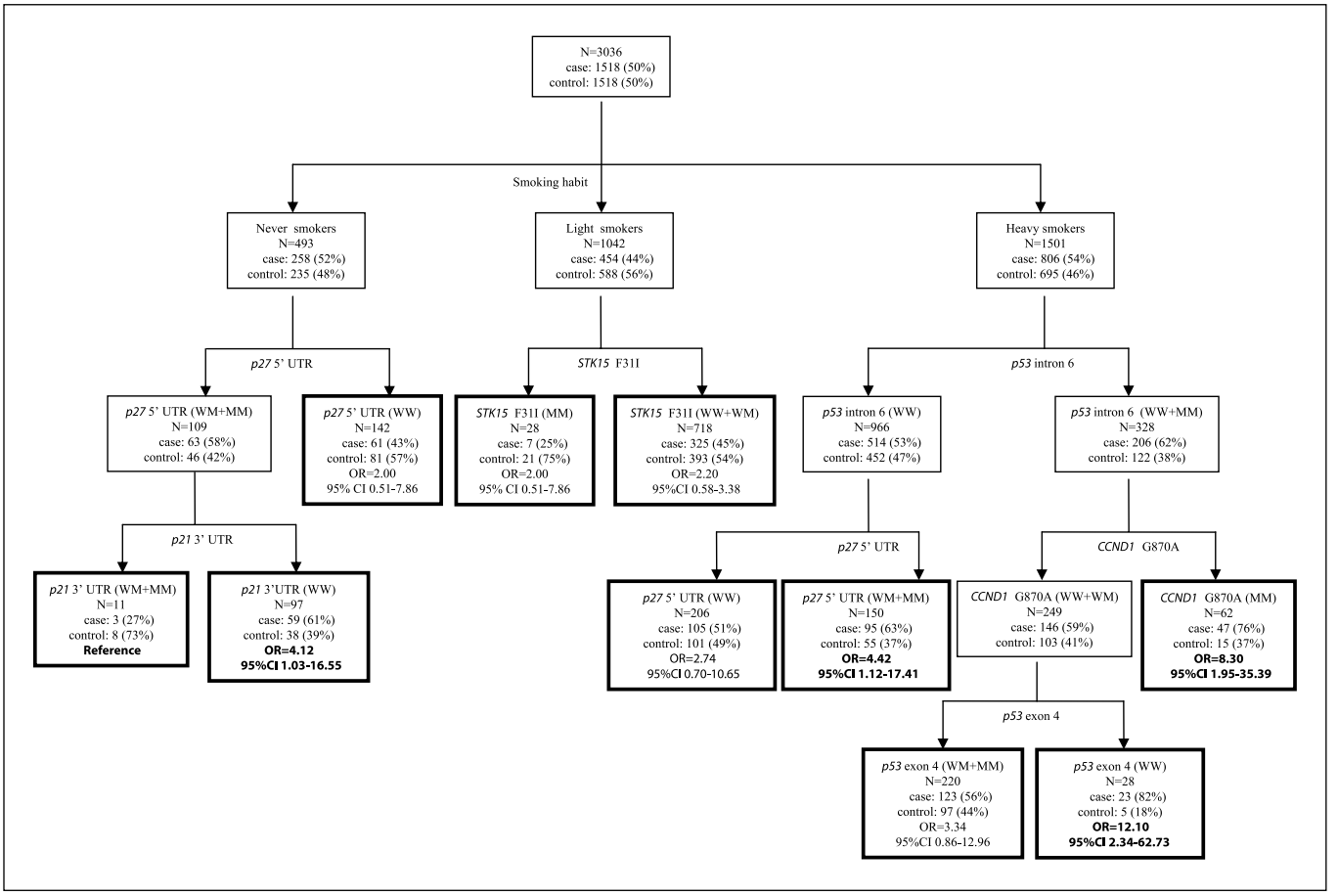

Fig. 1. Classification and regression tree analysis for smoking habit, cell cycle polymorphisms, and lung cancer risk. WW, common homozygotes; WM, heterozygotes; MM, rare homozygotes. OR was adjusted for age and gender.

regression revealed that four SNPs (*p53* intron 6, *p27* 5′ UTR, *CCND1* G870A, and *STK15* F31I) were associated with significantly altered risk in the main analysis. Moreover, combined analysis showed an increasing risk of lung cancer with an increasing number of adverse alleles or high-risk genotypes. To the best of our knowledge, this is the largest study to evaluate the aggregate effect of cell cycle regulatory gene polymorphisms on lung cancer susceptibility.

Main effects on lung cancer risk were observed for the *p53* intron 6 SNP when we combined the genotypes with at least one variant allele as the risk group. This observation recapitulated the findings of a number of previous studies of this SNP in lung cancer risk (22, 25–27). Although no direct evidence has been established to support a functional role of this SNP, we have previously shown that the variant allele is associated with impaired DNA repair capacity in conjunction with other *p53* polymorphisms (22). However, for the *p53* intron 6 polymorphism, we also noticed an opposite direction of risk between the heterozygotes and the rare homozygotes (Table 2). Nonetheless, the opposite result for the homozygous variant genotype did not reach statistical significance. We believe that this fluctuating result is possibly due to the small sample size of the homozygous variant genotype carriers. Further studies with larger sample sizes are warranted to provide additional insights into the association between the *p53* intron 6 homozygous variant genotype and lung cancer risk. We observed that the AA genotype of *CCND1* G807A was associated with a significantly increased risk compared with the genotypes with at least one wild-type allele. This SNP has been associated with increased risk of various malignancies such as colorectal cancer and esophageal cancer (28, 29). The variant allele encodes an alternatively spliced transcript of *CCND1*, which, compared with the transcript encoded by the wild-type allele, exhibits greater nuclear accumulation during G_1-S transition, and thus higher oncogenic potential (30). We also observed that the homozygous variant carriers of the *p27* 5′ UTR SNP exhibited a 1.27-fold increased lung cancer risk. This is the first study reporting a significant implication of this SNP in cancer risk and the exact molecular mechanism remains to be determined. This SNP located at a region coding for a U-rich element in the 5′ UTR of *p27* mRNA, which is essential for translation (31), suggesting that the variant allele may be associated with reduced p27 protein production. This conjecture is substantiated by the observation that deletion of a 127-bp fragment containing this SNP resulted in a reduced translation efficiency through impairment of internal ribosome entry site activity (32). Finally, the homozygous variant genotype of *STK15* F31I conferred a protective effect on lung cancer in the current study, whereas most previous investigations associated this SNP with increased risk of various other types of malignancies (33–35). The reason for this cancer type–specific risk association is unknown; however, the variant

allele has been reported to result in reduced STK15 kinase activity relative to the common allele (20), which is in agreement with our results because increased STK15 kinase activity has been associated with higher cancer occurrence and progression (36).

As expected, we also noticed that compared with light smokers, heavy smokers had an increased lung cancer risk for all three genotypes. Our cases and controls were matched on smoking status but not on smoking intensity, which was significantly different between cases and controls. Although matching on smoking status could affect smoking intensity to some degree, this should not negate our findings in stratified analyses that the lung cancer risk conferred by heavy smoking is more prominent in the homozygous variant genotype than in the wild-type or heterozygous genotype of *p53* intron 6 and *CCND1* G870A.

Because tobacco smoke leads to enhanced DNA damage that may be accumulated with defects of cell cycle control genes, we also evaluated the modifying effects of significant cell cycle polymorphisms on lung cancer risk conferred by smoking. Both *p53* intron and *p27* 5′ UTR SNPs modulated the risk of heavy smoking on lung cancer. The detailed mechanisms underlying these effects need to be elucidated. Because our previous study has shown that apoptotic index and DNA repair capacity of the variant carriers of the *p53* intron 6 SNP were significantly lower than those in individuals with the wild-type alleles (22), it is reasonable to speculate that the observed modifying effect might be due to this influence on DNA repair and apoptosis.

The pathway-based polygenic approach has been successfully applied to identify modest risks in a number of cancer association studies (18, 37, 38). Through such an approach, we identified a trend of increasing level of lung cancer risk with increasing number of adverse alleles or unfavorable genotypes in a dose-dependent manner. This finding recapitulated the notion that lung carcinogenesis is a polygenic process and thus the combined analysis of multiple factors may exhibit higher power to characterize high-risk populations. In addition, it also fits with the hypothesis that multistep genetic mutations may precede carcinogenesis. For instance, simultaneous mutations in the *p53* gene greatly enhanced the oncogenic effects of overexpressed *STK15* alone (39). However, individuals with three and four adverse alleles exhibited similar effects (OR, 1.58 versus OR, 1.59), indicating that there might be a threshold effect in this dose-response association. Moreover, the results of the unfavorable genotype analysis (Table 5) need to be interpreted with caution because the reference group was sparse, which could lead to unstable estimates in all following risk categories.

Interestingly, we also noticed that consistent with the main-effect logistic regression analysis, the *p27* 5′ UTR, *STK15* F31I, *p53* intron 6, and *CCND1* G870A SNPs were also identified in the CART analysis as important genetic variants modulating lung cancer risk. *p27* 5′ UTR was the most prominent SNP to discriminate between cases and controls in never smokers. This information may provide clues as to the genetic factors influencing the risk of lung cancer in new smokers, which accounts for >10% of total lung cancer patients. Although the reasons remain unclear, it is postulated that the interactions between environmental exposures and genetic factors play an essential role (40, 41). *STK15* F31I was the discriminating factor in light smokers. STK15 is a serine/threonine protein kinase modulating G_2-M cell cycle progression through its regulation of mitotic spindle formation and centrosome duplication (42). Dysfunction of STK15 might result in aberrant chromosome segregation, a potential early event of lung carcinogenesis (42, 43). Numerous reports have shown that *p53* is one of the most frequently mutated genes in smoking-associated lung cancers (44, 45). Moreover, interactions and joint effects between *p53* genetic variations and smoking status on the risk of various types of cancer have also been well documented (46, 47). Consistently, the finding in our CART analysis that *p53* intron 6 and *p27* 5′ UTR SNPs characterize different risk groups in heavy smokers confirmed the essential role of both genes in smoking-related lung carcinogenesis. *CCND1*, another SNP critical in the branch of heavy smokers, has also been associated with risks of esophageal and gastric cancers in a smoking-dependent fashion (29). Nonetheless, the ORs obtained from CART analysis reflecting the gene-gene and gene-environment interactions should be interpreted with caution and need to be validated in a larger population.

In summary, our study is one of the first to use a polygenic strategy to evaluate the involvement of cell cycle control polymorphisms in lung cancer. We found that, in the context of specific host characteristics, genetic variations in the cell cycle control signaling cascade may influence lung cancer susceptibility and modulate the risk conferred by tobacco smoking.

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