

Genetic variants of the Wnt signaling pathway as predictors of recurrence and survival in early-stage non-small cell lung cancer patients

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Early-stage non-small cell lung cancer (NSCLC) is potentially curative. Nevertheless, many patients will show disease recurrence after curative treatment. The Wnt signaling pathway is a developmental and stem cell pathway that plays an important role in tumorigenesis and may affect cancer progression. We hypothesize that genetic variants of the Wnt pathway may influence clinical outcome in early-stage NSCLC patients. We genotyped 441 functional and tagging single nucleotide polymorphisms (SNPs) from 54 genes of the Wnt pathway in 535 early-stage NSCLC patients treated with curative intent therapy including surgery and chemotherapy. For validation, 4 top SNPs were genotyped in 301 early-stage NSCLC patients from the Mayo Clinic. Cox proportional hazard model and combined SNP analyses were performed to identify significant SNPs correlated with recurrence-free and overall survival. Results from discovery group showed a total of 40 SNPs in 20 genes correlated with disease recurrence ($P < 0.05$). After correction for multiple comparisons, rs2536182 near *Wnt16* remained significant ($q < 0.1$), which was validated in the replication population. Thirty-nine SNPs in 16 genes correlated with overall survival ($P < 0.05$) in the discovery group, and seven remained significant after multiple comparisons were considered ($q < 0.1$). In patients receiving surgery-only treatment, rs10898563 of *FZD4* gene was associated with both recurrence-free and overall survival. Joint SNP analyses identified predictive markers for recurrence stratified by treatment. Our findings suggest inherited genetic variation in the Wnt signaling pathway may contribute to variable clinical outcomes for patients with early-stage NSCLC.

Introduction

Lung cancer is now the leading cause of cancer mortality for both men and women. More than 200 000 cases of lung cancer are diagnosed each year in the USA with an estimated 160 340 lung cancer deaths in 2012 (1). Only 15% of lung cancer patients are diagnosed with early-stage disease (stage I and II)—the majority of these have localized stage I cancer at presentation, potentially curable disease. Despite appropriate therapy, 30–40% of lung cancer patients with stage I disease and ~50% of patients with stage II disease ultimately relapse and die of their disease (2). Adjuvant cisplatin-based chemotherapy has been shown to improve survival in patients with stage II disease following surgery; however, this results in only a 5.4% cumulative survival benefit (3). No benefit has been shown for adjuvant chemotherapy in stage IA, and the use of adjuvant chemotherapy in stage IB is controversial. Therefore, many patients are subjected to the toxicity of adjuvant chemotherapy despite only a select few benefiting from therapy. Identifying prognostic and/or predictive markers, both tumor and host related, are important to select patients most likely to benefit

from chemotherapy and to develop more targeted and effective management strategies for the highest risk patients.

Tumor-related markers for recurrence have been studied extensively; however, there is little data relating to host genetic variation and relapse in early-stage non-small cell lung cancer (NSCLC). Due to their unique properties, stem cell pathways may contribute to tumor biology within the tumor as well as within the host. Stem cells have been shown to have multiple effects in carcinogenesis, progression, invasion and metastasis (4,5). In solid tumors, a malignant stem cell population has been described, as well as a population of non-malignant host mesenchymal stem cells integral in producing tumor stroma and influencing the behavior of the malignant cells (6,7). The Wnt pathway is a stem cell pathway important in both embryogenesis and tumorigenesis (8,9). Wnt activation results in the stabilization of β -catenin and regulation of the transcription of the downstream members of the pathway. Several of these downstream molecules are important in tumorigenesis, including c-Myc, VEGF-A, cyclin D1 and MMP7 (10–12).

The Wnt pathway has been shown to be predictive of aggressive biology and progression in NSCLC (13–15). Lung adenocarcinomas have recently been shown to have a significant frequency of somatic mutations and copy number alterations within the Wnt pathway suggesting that this pathway plays a role in the pathogenesis of the disease. Specific genomic expression patterns of stem cells with an activated Wnt pathway have been described and tumors with signatures more similar to the stem cell expression pattern have a more aggressive phenotype. In lung cancer as well as several other solid tumor types, this signature has been associated with death from cancer as opposed to long-term remission after treatment for early-stage disease (16). In squamous cell cancers of the head and neck, genomic expression patterns associated with stem cell pathways have been implicated in locoregional failure after chemotherapy and radiation (17).

In this study, we determined whether germline genetic variations within the Wnt pathway correlate with relapse-free survival in patients treated for early-stage NSCLC. Tagging and functional single nucleotide polymorphisms (SNPs) were selected for each gene and genotyped in patients with early-stage NSCLC, and these variants were then correlated with the clinical outcomes of recurrence and survival.

Materials and methods

Patient population

Subjects in this study were histologically confirmed NSCLC patients, who were enrolled into an ongoing epidemiologic lung cancer study at The University of Texas MD Anderson Cancer Center between November 1995 and February 2008. Patients were included if they had clinical stage I or stage II disease and were treated with curative intent. Treatment with curative intent was defined as either surgical resection with negative margins, surgical resection followed by adjuvant chemotherapy and/or adjuvant radiation therapy, neoadjuvant chemotherapy followed by surgical resection or definitive radiation therapy for stage I patients. Patients were excluded if their surgical pathology upstaged them to stage III or IV. Patients were also excluded if they had any malignancy other than superficial skin cancer (except melanoma) within 5 years of enrollment or had any prior chemotherapy, radiation therapy or had been enrolled in a chemoprevention study. In addition, two clinical stage I patients who had pathological stage 0 diagnosis (carcinoma *in situ*) were excluded from further analysis. Although neoadjuvant chemotherapy is not routinely used in stage I and II NSCLC, there were patients included in this study who received neoadjuvant chemotherapy. The majority of these patients were enrolled in a clinical trial investigating neoadjuvant chemotherapy. Those who received neoadjuvant chemotherapy outside of this clinical trial were given this therapy due to a high likelihood of a positive surgical margin as assessed by the surgeon prior to surgery, or to allow for a lobectomy rather than a pneumonectomy. Although there was no restriction in terms of race/ethnicity in our patient accrual, we restricted our study to the non-Hispanic Caucasian subjects due to the small sample sizes of other groups and also to minimize population stratification.

Abbreviations: CI, confidence interval; EMT, epithelial-to-mesenchymal transition; HR, hazard ratio; NSCLC, non-small cell lung cancer; SNP, single nucleotide polymorphism.

For validation, we included an independent group of early-stage NSCLC cases from the Mayo Clinic. Detailed description of the subject recruitment has been described previously (18). In brief, confirmed NSCLC patients without restrictions on age, gender and ethnicity were serially identified by a daily electronic pathology system and then consented for study enrollment from 1997 to 2002. Due to the small number of minorities and patients treated with chemotherapy (<10%), only non-Hispanic white cases with curative surgery-only treatment were included in the replication analysis.

Blood samples were collected for all patients for genotyping analysis. Detailed smoking history was obtained at the time of recruitment and informed consent obtained to collect follow-up clinical data. The study was approved by the institutional review boards at MD Anderson Cancer Center and Mayo Clinic.

Clinical data and outcomes collection

Patients with NSCLC were staged clinically based on the 1997 staging system, and pathologic staging was verified by the pathologic reports. Clinical data including adjuvant chemotherapy, radiation therapy, surgical pathology with margins, histology, date of relapse and site of relapse were abstracted from the medical record. For patients without evidence of disease at last follow-up and with no follow-up within 6 months of time of abstraction, social security death index was used to confirm vital status. For patients with recurrent disease and with no follow-up within 3 months at time of abstraction, social security death index was used to confirm vital status.

The primary endpoint of this study is recurrence-free survival and the secondary outcome is overall survival. Recurrence-free survival was calculated as time from date of first treatment to date of recurrence or local and distant metastasis. Patients were censored at death or last follow-up. Overall survival was calculated from date of first treatment to date of death. Recurrence and survival data was collected from the medical record, social security death index or contact with the patient, family member or primary physician.

In the Mayo population, participants' medical records were abstracted and interviews conducted to obtain demographic and clinical information, including smoking history. Causes of death were determined by reviewing the Mayo Clinic registration database and medical records, correspondence from patients' next of kin, death certificates, obituary documents, the Mayo Clinic Tumor Registry and the social security death index. The same condition was used to define recurrence-free survival, and censors were applied at patient death or last follow-up.

SNP selection and genotyping

We generated a cancer-related gene list for the Wnt signaling pathway using the Gene Ontology database (<http://www.geneontology.org>). Extensive literature review of genes from Gene Ontology database was done using HUGO names and common aliases. A priority score was assigned to each gene based on its relevance and significance to cancer. A total of 54 genes with high priority score in the Wnt pathway were selected and their gene positions annotated using the UCSC Golden Path Genome Browser. Tagging SNPs were identified within 10 kb flanking regions upstream and downstream of each gene with r^2 of 0.8 or higher and a minor allele frequency of >5% in Caucasians using the LDSelect program (<http://droog.gs.washington.edu/LDSelect.html>) based on the HapMap database. Additionally, potentially functional SNPs which may affect gene expression or splicing (SNPs in coding region, promoter region, 5'-untranslated region, 3'-untranslated region or splice sites) were selected. A total of 441 SNPs were selected in these 54 genes. Complete set of SNPs was sent to Illumina for iSelect Infinium BeadChip design using proprietary program developed by Illumina. Genomic DNA was extracted from peripheral blood using QIAamp DNA extraction kit (Qiagen) and genotyped by Illumina's BeadArray platform according to the manufacturer's instruction. Genotypes were auto-called using the Beadstudio software. In the replication phase, the SNPs significant for recurrence in the overall group and surgery-only subgroup [q (false discovery rate-adjusted P value) < 0.1] and the top three SNPs associated with survival were selected for further genotyping using Taqman genotyping assays in 7900HT Sequence Detection System (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. For polymorphisms in the same gene that showed strong linkage disequilibrium, we selected only the most significant SNP for replication.

Statistical analysis

Hazard ratios (HR) were estimated for recurrence and survival endpoints with the Cox proportional hazards model, adjusting for sex, age, race, clinical stage, pack year tobacco history and treatment regimen. We compared three genetic models to determine the significance of each SNP: dominant (common homozygote versus variant allele carrying genotypes), recessive (wild-type allele carrying genotypes versus rare homozygous) and additive (P for trend). Only dominant model was considered if the rare homozygous genotype was observed <5% percent in both patients with event and patients without event.

Differences in host characteristics between treatment groups were assessed by Pearson χ^2 or Fisher exact tests. Predictive marker is defined as a SNP whose association with clinical outcome is at least significant at $P < 0.05$ based on the best statistical model in one treatment group but has risk estimate that is in opposite direction for different treatment group using the same statistical model. Variants with beneficial effects on both groups were not included in the predictive marker analysis since they would not be helpful for treatment selection. For the cumulative analysis of predictive markers, unfavorable genotypes were selected for each significant SNP dependent on the best model, and the total number of unfavorable genotypes was counted for each subject. Specifically, SNPs with significant HR > 1 would have homozygous variant alleles (VV) and heterozygous genotype (WV) as the unfavorable genotypes under the dominant and additive models, and VV as the unfavorable genotype under the recessive model. SNPs with HR < 1 would have homozygous wild-type alleles (WW) and heterozygous genotype (WV) as the unfavorable genotypes under the recessive model, and WW as the unfavorable genotype under the dominant and additive models. Based on the percentage of cases with adverse clinical outcome, the subjects were grouped into tertiles of low, medium and high risks by the number of unfavorable genotypes. Kaplan-Meier survival function and log-rank tests were used to assess differences in progression-free and overall survival times. Meta-analysis was performed to combine the results from discovery and validation groups adjusting for the same covariates. To correct for multiple comparisons, we computed the q value (a false discovery rate-adjusted P value) for each SNP implemented in the R-package. The above analyses were done using STATA software (version 10; STATA Corp., College Station, TX). Survival tree analyses were used to identify higher order gene-gene interactions by STREE program (<http://c2s2.yale.edu/software/stree/>). All tests were two sided and $P < 0.05$ was considered statistical significant.

Results

Host characteristics

A total of 535 patients were treated for stage I or II NSCLC at MD Anderson with curative intent between November 1995 and February 2008 (Table I). The median age of patients was 66 years (range, 29–92) with 49% of patients being male; 87% were current or former smokers with a median history of 45 pack-years. Majority of patients (88%) were white and 8% were black. Adenocarcinoma represented 59% of the patient population, squamous cell represented 28% and 13% of the patients was of other histology or not otherwise specified. More than 80% of the patients had clinical stage I disease with the remainder having clinical stage II disease. Pathological staging was available for 482 patients, and of these patients 75% had stage I disease and 24% had stage II disease. There were 340 patients who had surgical resection with negative margins and no further therapy, 142 patients who received surgery plus either adjuvant/neoadjuvant chemotherapy or adjuvant radiation and 53 patients who were treated with definitive radiation therapy without surgery for stage I disease. In the stratified analysis, there were no statistically significant differences between surgery-only and surgery plus chemotherapy treated groups with regard to age, sex, race, smoking history or histology. However, there were more patients diagnosed with stage I disease in the surgery-only group than those in the surgery and chemotherapy group (86 versus 51%, respectively; $P < 0.0001$). Median follow-up time for the entire cohort of cases was 58.4 months. Twenty-six percent of the patients showed disease recurrence and 40% died at the end of the follow-up period with a median survival time of 77.8 months.

For the Mayo Clinic group, 301 non-Hispanic white patients were treated with curative surgery for stage I or II NSCLC (Supplementary Table S1, available at *Carcinogenesis* Online). The median age was 69 years (range, 42–91) with 51% being male and 91% former or current smokers. Similar to the MD Anderson group, >80% of the patients were diagnosed with stage I NSCLC; however, the median survival time was longer at 133 months with a median follow-up time of 85.4 months, more likely due to the composition of surgery-only patients who usually have more favorable prognosis prior to treatment.

Associations of individual SNPs with recurrence-free survival

Analysis of SNPs in the Wnt pathway associated with recurrence-free survival in patients treated with curative therapy found 40 SNPs with P

Table I. Host characteristics

Variables	Number ^a	Percent
Median survival time (months)	77.80	
Median follow-up time (months)	58.42	
Age, median (range)	66 (29–92)	
Pack year, median (range)	45 (0.1–256) ^b	
Total patients	535	
Sex		
Male	262	49
Female	273	51
Pathological stage		
Stage 0	2	0
Stage 1A	192	40
Stage 1B	169	35
Stage 2A	30	6
Stage 2B	89	18
Clinical stage		
Stage 1A	245	46
Stage 1B	188	35
Stage 2A	26	5
Stage 2B	76	14
Smoking status		
Never	68	13
Former	270	50
Current and recently quit	197	37
Race		
White	469	88
Black	42	8
Others	24	4
Histology		
Adenocarcinoma	315	59
Squamous	149	28
Not otherwise specified	27	5
Other	44	8
Vital status		
Alive	322	60
Dead	213	40
Recurrence ^c		
No	317	75
Yes	103	25
Surgical procedure		
Wedge resection	16	4
Lobectomy	49	11
Lobectomy + MLDd	330	76
Bilobectomy	15	3
Pneumonectomy	26	6
Surgery result		
Complete	470	98
Residual	12	2
Treatment		
Surgery-only	340	64
Surgery + chemotherapy	127	24
Surgery + adjuvant radiation	15	3
Treatment without surgery	53	10

^aNumbers may not add up to total number of patients due to missing clinical information.

^bAmong ever smokers.

^cPatients with recurrent disease at the time of first treatment were not counted in the analysis.

^dMediastinal lymph node dissection.

< 0.05 (Supplementary Table S2, available at *Carcinogenesis* Online). The top three SNPs are listed in Table II with the most significant being rs2536182 near the *WNT16* gene, whose variant allele was associated with 51% decreased recurrence risk compared with wild-type [HR = 0.49, 95% confidence interval (CI): 0.33–0.73, $P = 3.87 \times 10^{-4}$, $q = 0.067$]. When stratified by treatment regimen, rs10898563 of *FZD4*, was the most significant SNP associated with recurrence risk in surgery-only treated patients with borderline significance after adjusting for multiple testing (HR = 2.98, 95% CI: 1.62–5.48, $P = 5.1 \times 10^{-4}$, $q = 0.11$; Table II). In patients treated with surgery and chemotherapy,

there were no significant SNPs associated with recurrence after multiple testing adjustment, although the top SNP, rs11725638 of *LEF1*, was associated with a decreased risk of recurrence (HR = 0.31, 95% CI: 0.14–0.67, $P = 3.05 \times 10^{-3}$, $q = 0.24$; Table II).

Validation of the top SNPs with recurrence

We assessed the association of rs2536182 near *Wnt16* and rs10898563 in *FZD4* (the latter SNP reached borderline significance at $q = 0.11$ with recurrence in stratified analysis) in 301 early-stage, surgery-only treated NSCLC patients from the Mayo Clinic. In recurrence-free survival analysis, only rs2536182 near *Wnt16* but not the *FZD4* variant showed significant association with similar effect as the discovery group (HR = 0.39, 95% CI: 0.18–0.84, $P = 0.01$; meta-analysis HR = 0.44, 95% CI: 0.29–0.68, $P = 2.3 \times 10^{-4}$) (Table III).

Predictive markers for recurrence-free survival

Since recurrence was the primary endpoint of this study, we sought to identify a group of SNPs that are predictive of recurrence-free survival in patients treated with each curative regimen. As seen in Supplementary Tables S3 and S4, available at *Carcinogenesis* Online, 26 SNPs were associated with recurrence risk in surgery-only group and 21 SNPs, including rs11725638 of *LEF1*, were associated with recurrence in surgery- and chemotherapy-treated patients ($P < 0.05$), respectively. Table IV shows the cumulative effects of these predictive markers on the risk for recurrence in early-stage NSCLC patients stratified by treatment. In this analysis, we counted the number of unfavorable genotypes for all predictive markers in each patient. Using patients carrying 0–9 unfavorable genotypes for markers based on surgery-only patients as reference, those with 10–12 unfavorable genotypes and 13 or more favorable genotypes had 2.2-fold (HR = 2.24, 95% CI: 0.81–6.19) and 11-fold (HR = 11.09, 95% CI: 4.59–26.80) increased risk of recurrence, respectively (P trend < 0.0001) in the surgery-only group. Consistent with the single SNP analysis, cumulative analysis of the same predictive markers identified for surgery-only group in the surgery and chemotherapy-treated subjects demonstrated less significant association and opposite risk effect. Similarly for markers based on surgery- and chemotherapy-treated group, individuals with 6–7 unfavorable genotypes and 8 or more unfavorable genotypes had 11-fold (HR = 11.1, 95% CI: 1.36–89.8) and >54-fold (HR = 54.6, 95% CI: 7.2–413.2), respectively (P trend < 0.0001) increased recurrence risk compared with the reference group of 0–5 unfavorable genotypes in patients treated with surgery and chemotherapy. Again, these markers had less significant association and opposite effect on recurrence risk in surgery-only treated patients. The cumulative effects of the predictive markers on recurrence-free survival are graphically shown by the Kaplan–Meier estimates for the three groups of patients in each treatment regimen (Figure 1). There was a dose-dependent decrease in recurrence-free survival for patients with increasing number of unfavorable genotypes for each set of predictive markers. This dose dependence was specific for the markers that were predictive of each therapy. In surgery-only patients, subjects from the high-risk group with >12 unfavorable genotypes from markers based on surgery-only group had recurrence-free median survival time of 51 months compared with >150 months for those with 12 or fewer unfavorable genotypes. Similarly, in patients treated with surgery and chemotherapy, those with >7 unfavorable genotypes from markers based on the same treatment regiment had median survival time of <19.5 months compared with >150 months for patients in the middle- and low-risk groups (Figure 1).

Associations of individual SNPs with overall survival

Variant genotypes were analyzed for association with survival after treatment with curative intent for patients with stage I or II NSCLC. In all our analyses, we minimized false discovery by using a false discovery rate of 10% ($q < 0.1$). There were 39 SNPs in 16 genes that were associated with overall survival at $P < 0.05$ (Supplementary Table S5, available at *Carcinogenesis* Online). After adjustment for multiple testing, seven SNPs had $q < 0.1$ (Table V). Among these, the most significant polymorphisms were rs4135385 of *CTNNB1* (also

Table II. Individual SNPs associated with recurrence risk in NSCLC patients treated with curative therapy

SNP	Gene	Chr	MAF	SNP location	Allelic change	Overall			
						Best model	HR (95% CI) ^a	P value	q value
rs2536182	WNT16	7	0.48	3' flanking	G>C	Dominant	0.49 (0.33–0.73)	3.87 × 10 ⁻⁴	0.067
rs7296283	LRP6	12	0.18	5' flanking	G>A	Dominant	1.84 (1.24–2.72)	2.30 × 10 ⁻³	0.188
rs10898563	FZD4	11	0.37	3' UTR	A>G	Recessive	2.00 (1.26–3.17)	3.30 × 10 ⁻³	0.188

SNP	Surgery-only				Surgery and chemotherapy			
	Best model	HR (95% CI) ^b	P value	q value	Best model	HR (95% CI) ^b	P value	q value
rs2536182	Dominant	0.47 (0.28–0.80)	5.24 × 10 ⁻³	0.154	Dominant	0.64 (0.27–1.53)	0.3554	0.259
rs10898563 ^c	Recessive	2.98 (1.60–5.35)	4.31 × 10 ⁻⁴	0.105	Dominant	0.60 (0.29–1.22)	0.1554	0.259
rs11725638 ^d	Dominant	1.29 (0.75–2.22)	0.3486	0.368	Dominant	0.31 (0.14–0.67)	3.05 × 10 ⁻³	0.242

Underlined bold numbers denote significant association at $q < 0.1$. MAF, minor allele frequency; UTR, untranslated region.

^aAdjusted for sex, age, race, clinical stage, pack year tobacco history and treatment regimen.

^bAdjusted for sex, age, race, clinical stage and pack year tobacco history.

^cTop SNP in surgery-only group.

^dTop SNP in surgery and chemotherapy group.

Table III. Replication result of top SNPs associated with recurrence in NSCLC patients treated with surgery-only

SNP	MD Anderson	P	MOI ^a	Mayo Clinic recur (yes/no)			Mayo Clinic	P	Meta	Meta P
	HR ^b (95% CI)			WW	WV	VV	HR ^b (95% CI)		HR ^b (95% CI)	
rs2536182	0.47 (0.28–0.80)	5.24 × 10⁻³	Dom	12/67	9/146	5/57	0.39 (0.18–0.84)	0.0162	0.44 (0.29–0.68)	2.30 × 10⁻⁴
rs10898563 ^c	2.98 (1.60–5.35)	0.0004	Rec	12/11	100/129	3/38	0.81 (0.24–2.75)	0.7409	2.31 (1.33–3.96)	0.0246

Significant P values ($P < 0.05$) in bold type. VV, homozygous variant genotype;

WW, homozygous wild-type genotype; WV, heterozygous variant genotype.

^aMOI: model of inheritance, the model with the smallest P value for both discovery and replication groups; Dom, dominant; Rec, recessive.

^bAdjusted by age, sex, smoking status, cancer stage and treatment regimen.

^cInformation for three samples from the Mayo Clinic was missing due to failed genotyping assay.

Table IV. Cumulative effect of treatment-based exploratory predictive markers on recurrence risk in NSCLC patients stratified by treatment

Markers based on surgery-only

Number of unfavorable genotypes	Surgery-only					Surgery and chemotherapy				
	Recur, n (%)	No recur, n (%)	HR	P value	Log-rank P	Recur, n (%)	No recur, n (%)	HR	P value	Log-rank P
0–9	6 (10.3)	78 (37.5)	1 (reference)			14 (45.2)	20 (27.4)	1 (reference)		
10–12	12 (20.7)	87 (41.8)	2.24 (0.81–6.19)	0.1186		11 (35.5)	27 (37.0)	0.56 (0.25–1.24)	0.1523	
≥13	40 (69.0)	43 (20.7)	11.09 (4.59–26.80)	8.93 × 10 ⁻⁸	4.77 × 10 ⁻¹³	6 (19.4)	26 (35.6)	0.30 (0.11–0.82)	0.0184	0.1119

Markers based on surgery and chemotherapy

Number of unfavorable genotypes	Surgery-only					Surgery and chemotherapy				
	Recur, n (%)	No recur, n (%)	HR	P value	Log-rank P	Recur, n (%)	No recur, n (%)	HR	P value	Log-rank P
0–5	30 (52.6)	68 (32.2)	1 (reference)			1 (3.2)	35 (47.9)	1 (reference)		
6–7	17 (29.8)	78 (37.0)	0.57 (0.31–1.04)	0.0682		8 (25.8)	24 (32.9)	11.07 (4.38–79.05)	0.0244	
≥8	10 (17.5)	65 (30.8)	0.41 (0.20–0.85)	0.0171	0.0201	22 (71.0)	14 (19.2)	54.56 (7.21–413.2)	1.08 × 10 ⁻⁴	9.95 × 10 ⁻⁹

Unfavorable genotypes for surgery-only markers: rs10898563 (VV), rs757558 (WV + VV), rs4074947 (WV + VV), rs3781586 (WW + WV), rs3765351 (WV + VV), rs11922919 (WW + WV), rs557077 (WW), rs4257529 (WV + VV), rs3923087 (WV + VV), rs7591 (WV + VV), rs7526484 (VV), rs566926 (WV + VV), rs288326 (WV + VV), rs648872 (VV), rs8192633 (WV + VV), rs10127943 (WV + VV), rs12816278 (WV + VV), rs2163910 (WV + VV), rs8068404 (WV + VV), rs11650531 (WV + VV), rs12632968 (WV + VV), rs4968281 (WV + VV), rs314756 (WV + VV), rs640569 (WV + VV), rs4919139 (VV) and rs390258 (WV + VV).

Unfavorable genotypes for surgery and chemotherapy markers: rs11725638 (WV + VV), rs10891310 (VV), rs4919139 (VV), rs9864832 (WV + VV), rs9675157 (WV + VV), rs4958216 (WV + VV), rs2163910 (WV + VV), rs1722846 (VV), rs395901 (WV + VV), rs2039826 (VV), rs7478323 (VV), rs2707761 (WV + VV), rs2277268 (WV + VV), rs2240511 (WV + VV), rs9872276, (VV), rs4730775 (WV + VV), rs4956157 (VV), rs7301320 (WV + VV), rs10917155 (WV + VV) and rs7296858 (WV + VV). V, variant; W, wild-type.

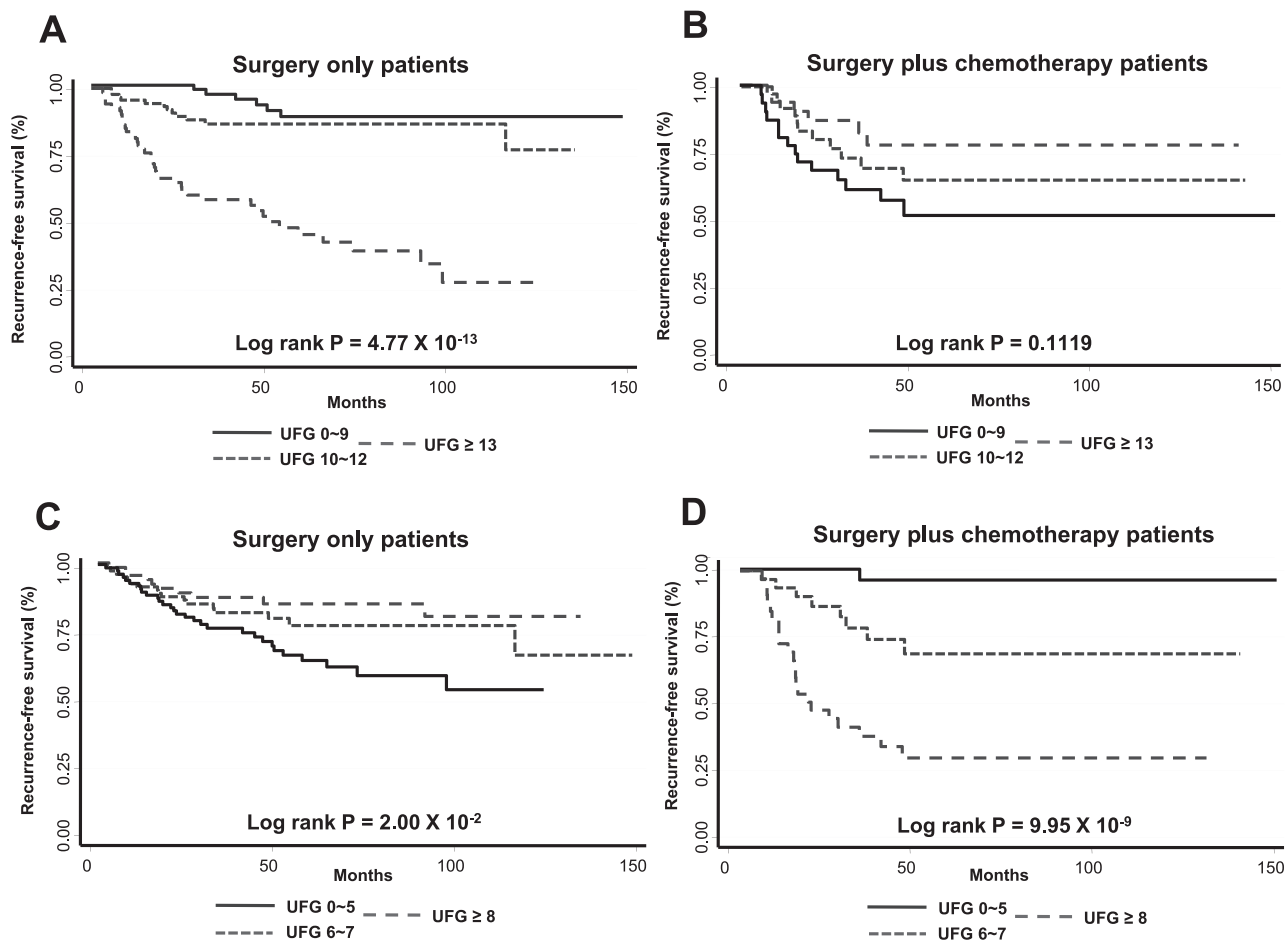


Fig. 1. Kaplan–Meier estimates of recurrence-free survival stratified by treatment for early-stage NSCLC patients grouped by the number of unfavorable genotypes in the predictive markers for surgery-only (A and B) and predictive markers for surgery plus chemotherapy (C and D). UFG: number of unfavorable genotypes as depicted in Table IV.

known as *B*-catenin, HR = 3.74, 95% CI: 2.23–6.29, $P = 6.02 \times 10^{-7}$, $q = 1.43 \times 10^{-4}$), rs10898563 of *FZD4* (HR = 2.29, 95% CI: 1.60–3.28, $P = 5.19 \times 10^{-6}$, $q = 3.46 \times 10^{-4}$) and two SNPs in *WNT5A*, rs503022 and rs629537, which were highly linked ($r^2 = 0.99$; HR = 4.34, 95% CI: 2.30–8.18, $P = 5.82 \times 10^{-5}$, $q = 3.46 \times 10^{-4}$). Other SNPs that were significantly correlated with survival include rs11658976 of *WNT3* (HR = 1.89; 95% CI: 1.31–2.73; $P = 6.43 \times 10^{-4}$), rs3765351 of *WNT4* (HR = 1.82, 95% CI: 1.28–2.59, $P = 9.24 \times 10^{-4}$) and another SNP of *FZD4*, rs713065 (HR = 0.63, 95% CI: 0.47–0.85, $P = 0.0022$).

SNPs associated with survival stratified by treatment

Since survival of early-stage NSCLC patients might be affected by the type of treatment administered, we also performed single SNP analysis stratified by treatment regimen (Supplementary Table S6, available at *Carcinogenesis* Online). Four SNPs were associated with overall survival in patients treated with surgery-only and four SNPs were associated with survival in surgery plus chemotherapy-treated patients ($q < 0.1$). Genetic variants of the two *FZD4* SNPs, rs10898563 and rs713065, were associated with 3.2-fold increased death risk (HR = 3.2, 95% CI: 2.08–4.92) and 55% decreased death risk (HR = 0.45, 95% CI: 0.31–0.65), respectively, in surgery-only group. As shown previously, rs10898563 also correlated with recurrence and survival in the overall curative therapy group. Other significant SNPs associated with survival in surgery-only treated patients were rs4135385 of *CTNNB1* (HR = 3.34, 95% CI: 1.75–6.39) and rs75264 of *WNT4* (HR = 3.04, 95% CI: 1.64–5.62). Additional SNPs associated with survival in surgery- and chemotherapy-treated patients included a SNP in *APC2* (rs4807928), two SNPs in *WNT5A* (rs590386 and rs508407), a SNP in *AXIN1*

(rs11645554). For validation, we also tested the top three SNPs ($q < 0.01$) associated with survival (Supplementary Table S5, available at *Carcinogenesis* Online), including rs10898563 of *FZD4* and rs4135358 of *CTNNB1*, in the Mayo Clinic surgery-only treated patients; however, none of the SNPs were replicated in this population (data not shown).

Discussion

The Wnt signaling pathway is one of the critical signaling pathways involved in stem cell maintenance and in embryonic development which has also been significantly implicated in tumorigenesis, tumor progression and metastasis in many malignancies including lung cancer (19–22). Several recent independent studies have reported alterations of the Wnt pathway in tumors were associated with poor clinical outcomes in NSCLC (23–26). However, the influence of germline Wnt pathway genetic polymorphisms on disease recurrence and survival of early-stage NSCLC patients treated with curative therapy has not been previously reported.

In this hospital-based study, we observed significant associations between multiple SNPs within the Wnt pathway and clinical outcomes including both recurrence and survival in patients with early-stage NSCLC. There were 43 SNPs associated with recurrence risk and 49 SNPs associated with overall survival at $P < 0.05$ in individual SNP analysis; 11 of these were associated with both recurrence and overall survival. Due to the relatively large number of SNPs tested, we limited the following discussion to those with at least borderline significance (defined as q value < 0.1) for either survival or recurrence after multiple comparison test was performed.

Table V. Individual SNPs associated with survival in NSCLC patients treated with curative therapy ($q < 0.1$)

SNP	Gene	Chr	SNP location	Allelic change	MAF	Overall			
						Best model	HR (95% CI) ^a	P value	q value
rs4135385	CTNNB1	3	Intron	A>G	0.25	Recessive	3.74 (2.23–6.29)	6.02×10^{-7}	<u>1.43×10^{-4}</u>
rs10898563	FZD4	11	3' UTR	A>G	0.35	Recessive	2.29 (1.60–3.28)	5.19×10^{-6}	<u>3.46×10^{-4}</u>
rs503022	WNT5A	3	3' flanking	C>A	0.16	Recessive	4.34 (2.30–8.18)	5.82×10^{-6}	<u>3.46×10^{-4}</u>
rs629537 ^b	WNT5A	3	3' flanking	G>A	0.16	Recessive	4.34 (2.30–8.18)	5.82×10^{-6}	<u>3.46×10^{-4}</u>
rs11658976	WNT3	17	Intron	A>G	0.40	Recessive	1.89 (1.31–2.73)	6.43×10^{-4}	<u>0.0306</u>
rs3765351	WNT4	1	3' UTR	A>G	0.44	Recessive	1.82 (1.28–2.59)	9.24×10^{-4}	<u>0.0366</u>
rs713065	FZD4	11	3' UTR	A>G	0.37	Dominant	0.63 (0.47–0.85)	0.0022	<u>0.0752</u>

Underlined bold numbers denote significant association at $q < 0.1$. MAF, minor allele frequency; UTR, untranslated region.

^aAdjusted for sex, age, race, clinical stage, pack year tobacco history and treatment regimen.

^bShowed linkage with rs503022, $r^2 = 0.99$.

For individual SNP analysis, rs2536182 near the *WNT16* gene has the strongest association with recurrence. This SNP was also replicated in the Mayo Clinic group for its association with recurrence-free survival among patients treated with surgery-only curative therapy. *WNT16* has been implicated in acute lymphoblastic leukemia and in chronic lymphocytic leukemia, with increased expression or activity being associated with proliferation and tumorigenesis and decreased expression associated with apoptosis (27–29). In addition, upregulation of a *WNT16* isoform (*WNT16B*) was found in basal cell carcinoma and could enhance keratinocyte proliferation (30). However, the involvement of *Wnt16* in NSCLC has not been previously reported. It is possible that this 3' flanking SNP could affect the expression of different *WNT16* isoforms in the lungs or is tagging for another causal SNP. Alternatively, this SNP which resides in the last intron of a nearby gene *FAM3C* could affect the gene's expression or activity. Further functional characterization may be necessary to dissect the mechanistic basis for its association with NSCLC recurrence. Another SNP, rs10898563 of *FZD4*, was most significantly associated with recurrence and survival in patients treated with surgery-only but not in patients treated with surgery and chemotherapy. Patients carrying the variant allele of this SNP had 2- to 3-fold increased recurrence and death risk compared with those of the wild-type homozygous genotype. Interestingly, stratified analysis by treatment showed opposite effects on risk suggesting its potential role as predictive marker for both recurrence and survival. *FZD4* encodes a member of the seven transmembrane receptors for the Wnt signaling ligands. *FZD4* expression has been linked to aberrant Wnt signaling in the pathogenesis of acute myelogenous leukemia (31) and prostate cancer oncogenesis involving epithelial-to-mesenchymal transition (EMT) (32,33). It is possible that increased expression of *FZD4* may predispose tumor cells toward EMT facilitating anchorage independence and tumor progression. The functional basis for the association of this gene with clinical outcome in lung cancer requires further investigation.

An intronic variant (rs4135385) located in the *CTNNB1* gene showed the strongest association with overall survival in NSCLC, but less significant association with recurrence risk. This association was also highly significant in the subgroup analysis in surgery-only patients but not in patients treated with surgery and chemotherapy (Table V). *CTNNB1* is the gene that encodes beta-catenin, a dual function protein involved in the coordination of cell adhesion and gene transcription. This well-studied gene has been implicated in the carcinogenesis and progression of numerous malignancies (34,35). Mutations of this gene, although rare, have been reported in lung cancers (26). The value of beta-catenin as prognostic factor for NSCLC is controversial; a recent meta-analysis showed reduced expression of beta-catenin in tumors correlated with poor prognosis (36). Nevertheless, genetic variants of *CTNNB1*, including rs4135385, have been associated with the prognosis of gastric and advanced NSCLC in Chinese and Caucasian populations (37,38).

Other SNPs that are associated with overall survival in NSCLC patients involved genes of *WNT5A* (rs503022 and rs629537), *WNT3* (rs11658976) and *WNT4* (rs3765351). rs503022 and rs629537 are two linked SNPs located in the 3' far end of *WNT5A*. The association of these SNPs with survival was seen in patients treated with surgery and chemotherapy but not surgery-only patients (Table V). *Wnt5a* signals via the non-canonical pathway to promote cellular movement and has been shown to activate calcium-dependent pathway which results in stimulation of cancer invasion and EMT (39,40). *Wnt5a* can also either inhibit or activate the canonical B-catenin pathway and in this way can be either oncogenic or act as a tumor suppressor depending on the cellular context and cancer sites (41–44). *WNT3* gene acts in the canonical WNT-beta-catenin signaling pathway and is implicated in some types of human cancers. *WNT3* was overexpressed in hepatocellular carcinoma as compared with normal surrounding liver tissues (45) and also highly expressed in mantle cell lymphoma (46). *WNT4* is a non-canonical Wnt mainly expressed in epithelial cells. It is involved in the alteration of cell adhesion, motility and invasion associated with EMT (47). *WNT4* expression is altered in several cancers (47–49) and might act as a potential target of Ras oncogenic signaling (50). Further research is necessary to determine the involvement of the Wnt pathway genes in the clinical outcome of NSCLC patients and whether any of the significant SNPs have functional activity in lung cancer cells or are linked to other functional SNPs in the Wnt pathway.

Patients diagnosed with NSCLC have a high incidence of smoking-related comorbid illnesses. Therefore, SNPs associated with survival without association with recurrence may be markers for mortality risk due to comorbidities. Because the median time to recurrence and relapse was >5 years in this study, it is possible that our follow-up time was not long enough to detect the association of some SNPs with recurrence and/or survival for early-stage NSCLC patients.

We have included a second independent population from the Mayo Clinic for replication of the top SNPs associated with recurrence and survival. Although both Mayo Clinic and MD Anderson Cancer Center are considered specialized tertiary medical centers, it is likely that heterogeneity between the two patient populations exists as shown by the discrepancies in clinical stage, median survival and follow-up times and in proportion of patients with recurrent disease, which may contribute to the failure of replicating some of the significant SNPs from the discovery group. Due to limiting resource, we only aimed to replicate the top SNPs associated with recurrence and survival. Hence, other significant variants might have been missed. For obvious reason, we could not validate markers for surgery and chemotherapy since the Mayo Clinic group consisted of mostly surgery-only patients.

We did not perform stratified analysis for ethnicity, smoking, disease stage and histology, due to the limited numbers in some categories and the fact that some minor constituents consisted of only a small proportion of the total population studied (e.g. non-smokers). Moreover, it is possible that some of the significant associations

might be attained by chance due to multiple testing. Nevertheless, we have limited our analysis to white subjects and performed adjustment for multiple comparisons to minimize population heterogeneity and false discovery, respectively. Since different chemotherapeutic regimens in different time periods (Supplementary Table S7, available at *Carcinogenesis* Online) might cause differential effects on NSCLC recurrence and survival, we also adjusted by types of chemotherapy agents and obtained mostly similar results (Supplementary Tables S8 and S9, available at *Carcinogenesis* Online, respectively). Further studies in stratified populations and validation in larger independent populations might reveal more specific associations and reduce potential confounder effects.

There are several strengths of our study. First, we have examined a relatively large patient population with early-stage (I or II) non-small lung cancer, whose clinical information and follow-up data were detailed and complete with replication of the top association with recurrence in an independent population. Second, we have utilized a targeted pathway-based approach which covered most of the major genes in the Wnt signaling pathway. Both tagging and potentially functional SNPs were selected from these genes for greater sensitivity and specificity of detecting association with clinical outcome. Third, we have identified sets of predictive markers that were specific and prognostic for recurrence in patients treated with two different curative therapies, which may be applied clinically to identify high-risk population for poor treatment outcome.

Overall, this is the first report showing the association of the genetic variants in the Wnt signaling pathway with clinical outcome for NSCLC. Future epidemiological and functional studies are warranted to confirm these findings for translational application and to uncover the mechanistic basis of the Wnt pathway genes in affecting cancer progression and mortality.

Supplementary material

Supplementary Tables S1–S9 can be found at <http://carcin.oxford-journals.org/>

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