

Polymorphisms in Apoptosis-Related Genes and Survival of Patients with Early-Stage Non-Small-Cell Lung Cancer

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ABSTRACT

Purpose. This study was conducted to determine the association between single-nucleotide polymorphisms (SNPs) in apoptosis-related genes and survival outcomes of patients with early-stage non-small-cell lung cancer (NSCLC).

Methods. Three hundred ten consecutive patients with surgically resected NSCLC were enrolled. Twenty-five SNPs in 17 apoptosis-related genes were genotyped by a sequenome mass spectrometry-based genotyping assay. The genotype associations with overall survival (OS) and disease-free survival (DFS) were analyzed.

Results. Three SNPs (*TNFRSF10B* rs1047266, *TNFRSF1A* rs4149570, and *PPP1R13L* rs1005165) were significantly associated with survival outcomes on multivariate analysis. When the three SNPs were combined, OS and DFS were decreased as the number of bad genotypes increased (P_{trend} for OS and DFS = 7×10^{-5} and 1×10^{-4} , respectively). Patients with one bad genotype, and patients with two or three bad genotypes had significantly worse OS and DFS compared with those with no bad genotypes [adjusted hazard ratio (aHR) for OS = 2.27,

95% confidence interval (CI) = 1.22–4.21, $P = 0.01$, aHR for DFS = 1.74, 95% CI = 1.08–2.81, $P = 0.02$; aHR for OS = 4.11, 95% CI = 2.03–8.29, $P = 8 \times 10^{-5}$; and aHR for DFS = 2.89, 95% CI = 1.64–5.11, $P = 3 \times 10^{-4}$, respectively].

Conclusion. Three SNPs in apoptosis-related genes were identified as possible prognostic markers of survival in patients with early-stage NSCLC. The SNPs, and particularly their combined genotypes, can be used to identify patients at high risk for poor disease outcome.

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer death in the developed world, with less than 15% of patients surviving beyond 5 years.¹ The high mortality rate of NSCLC is related in part to the high proportion of patients who present with unresectable tumor. Moreover, even among patients with resectable-stage NSCLC, a large proportion of the patients still die from disease recurrence. Although pathologic staging is the most accurate predictor for operable NSCLC, patients with the same pathologic stage of disease display marked variability in recurrence and survival.² Therefore, intensive research is currently in progress to identify prognostic biomarkers that would allow more precise identification of those patients with highest or lowest risk of relapse following surgery.³ This may help to select a group of patients for adjuvant therapy, with either conventional cytotoxic chemotherapy or novel targeted therapeutic agents.

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First Received: 23 December 2009;

Published Online: 27 April 2010

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Apoptosis is a genetically controlled cell suicide mechanism that enables multicellular organisms to regulate cell number in tissues and to eliminate unnecessary or damaged cells.⁴ It is assumed that a decreased capacity to eliminate DNA alterations may facilitate the accumulation of somatic mutations, and thereby contribute to tumor initiation, progression, and metastasis.^{5–7} Several studies have shown considerable interindividual variation in apoptotic capacity, and such a variation is largely attributed to an individual's genetic constitution.^{8,9} In agreement with these observations, many studies have demonstrated that several polymorphisms in apoptosis-related genes affect either the expression or the activities of enzymes, and therefore they are associated with the risk of various human cancers, including lung cancer.^{10–13} Accordingly, it is reasonable to suggest that an alteration in apoptotic capacity related to polymorphisms of apoptosis-related genes could affect prognosis of patients with surgically resected NSCLC. Therefore, this study analyzed the relationship between potentially functional polymorphisms of apoptosis-related genes and survival outcome in patients with early-stage NSCLC.

PATIENTS AND METHODS

Patients

This study included all patients ($n = 310$) with pathologic stages I, II or IIIA (microinvasive N2) NSCLC who underwent curative surgical resection at the Kyungpook National University Hospital (Daegu, Korea) between September 1998 and August 2006. All patients included were ethnic Korean. Patients who received chemotherapy or radiotherapy prior to surgery were excluded to avoid the effects on DNA. All tissues were obtained at time of surgery and then rapidly frozen in liquid nitrogen and stored at -80°C until relevant bioassays were conducted. Written informed consent was obtained from all patients prior to surgery, and this study was approved by the Institutional Review Board of the Kyungpook National University Hospital.

Polymorphism Selection and Genotyping

Due to the high number of single-nucleotide polymorphisms (SNPs) in the human genome, the initial challenge was the efficient selection of potentially functional SNPs. Thus, a prioritizing strategy was created using public databases that provide diverse information on the potential phenotypic risks of SNPs. First, candidate genes involved in apoptosis and related information were collected from web-based databases that included information on the

biologic pathway and potential biologic effects of SNPs. Next, SNPs with minor allele frequencies <0.10 were excluded based on the allele frequencies recorded for East Asian populations obtained from FASTSNP. The selected SNPs were then scored according to certain phenotypic risks, and ordered according to the sum of the risk scores based on the algorithm suggested by Yuan et al.¹⁴ Finally, 25 SNPs with high risk scores were included in the present study. The selected SNPs were genotyped using a sequenome mass spectrometry-based genotype assay. Among the 25 SNPs, 2 SNPs [*tumor necrosis factor superfamily, member 10 (TNFSF10)* rs1131579 and *TNF receptor superfamily, member 10C (TNFRSF10C)* rs12549481] with genotyping call rate $<90.0\%$, and 2 SNPs [*TNFRSF1B* rs1061624 and *B-cell CLL/lymphoma2-like 11 (BCL2L11)* rs724710] with Hardy–Weinberg equilibrium (HWE) $P < 0.05$ were excluded from further analysis. The remaining 21 SNPs in 15 genes were analyzed. For quality control, the genotyping analysis was performed blind with respect to the patients. Approximately 10% of the samples were randomly selected to be genotyped again by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) or DNA sequencing.

Statistical Analysis

The distribution of genotypes was tested for HWE with the goodness-of-fit χ^2 test. Differences in the distribution of genotypes according to the clinicopathologic factors of the patients were compared using χ^2 tests for categorical variables. The linkage disequilibrium (LD) status among SNPs was examined with the program HaploView (<http://broad.mit.edu/mpg/haploview>). Haplotype frequencies were estimated based on a Bayesian algorithm using PHASE 2.1.¹⁵ Linkage disequilibrium (LD) blocks were inferred from the definition proposed by Gabriel et al.¹⁶ The primary outcomes used for this study were overall survival (OS) and disease-free survival (DFS). OS was measured from day of surgery until date of death or date of last follow-up. DFS was calculated from day of surgery until recurrence or death from any cause. The association of OS and DFS with genotypes and haplotypes was investigated using the Kaplan–Meier method and assessed using the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using multivariate Cox proportional-hazards models, with adjustment for age (≤ 64 versus >64 years), gender (male versus female), smoking status (never versus ever-smoker), pathologic stage (I versus II–IIIA), and adjuvant therapy. All statistical testing was conducted using SPSS software, version 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient Characteristics and Clinical Predictors

Clinical and pathologic characteristics of the patients and the association with OS and DFS are shown in Table 1. Among 310 patients, 114 (36.8%) died. Estimated 5-year OS and DFS for all patients was 53% (95% CI = 46–61%) and 44% (95% CI = 37–51%). Pathologic stage was significantly associated with OS and DFS on univariate analysis ($P = 6 \times 10^{-5}$ and 4×10^{-8} , respectively).

Genotype Frequencies and Effect on OS and DFS

The SNP identification numbers, genotype and minor allele frequencies, genotyping call rates, and P -values for HWE of the 21 SNPs are shown in Table 2. None of the 21 SNPs was significantly associated with patient- or tumor-related factors, such as age, gender, smoking status, pathologic stage, or adjuvant therapy (data not shown).

On multivariate analysis, three SNPs [*TNFRSF10B* (alias *death receptor 5*, *DR5* or *TNFR apoptosis-inducing*

ligand receptor 2, *TRAILR2*) rs1047266C>T (A67 V); *TNFRSF1A* (alias *TNFR1*) rs4149570G>T; and *protein phosphatase 1 regulatory subunit 13 like* (*PPP1R13L*; alias *RelA-associated inhibitor* [*RAI*]) rs1005165C>T] were independent prognostic factors (Table 3). rs1047266C>T had a significant effect on OS and DFS under a recessive model for the variant allele [adjusted HR (aHR) = 2.23, 95% CI = 1.19–4.18, $P = 0.01$; and aHR = 2.01, 95% CI = 1.11–3.65, $P = 0.02$, respectively]. rs4149570G>T had a significant effect on OS under a dominant model for the variant allele (aHR = 1.71, 95% CI = 1.08–2.71, $P = 0.02$) and borderline effect on DFS (aHR = 1.41, 95% CI = 0.97–2.05, $P = 0.08$). rs1005165C>T exhibited worse OS and DFS under a recessive model for the variant allele (aHR = 1.94, 95% CI = 1.27–2.96, $P = 0.002$; and aHR = 1.88, 95% CI = 1.30–2.75, $P = 0.001$, respectively; Table 4; Fig. 1a–c).

Combined Analysis of Multiple SNPs

We performed an exploratory analysis investigating the combined effects on OS and DFS of the three SNPs that

TABLE 1 Univariate analysis for survival by demographics, smoking status, histological type, pathologic stage, and adjuvant chemotherapy

Variables	No. of cases	Overall survival			Disease-free survival		
		No. of deaths (%) ^a	5Y-OSR (%) ^b	Log-rank P	No. of events (%) ^a	5Y-DFS ^b (%) ^b	Log-rank P
Overall	310	114 (36.8)	53		148 (47.7)	44	
Age (years)							
≤64	164	55 (33.5)	58	0.24	75 (45.7)	46	0.58
>64	146	58 (40.0)	48		73 (50.0)	42	
Gender							
Male	245	98 (40.0)	51	0.17	121 (49.4)	43	0.74
Female	65	16 (24.6)	67		27 (41.5)	51	
Smoking status							
Never	61	16 (26.2)	67	0.25	27 (44.3)	45	0.97
Ever	249	98 (39.4)	51		121 (48.6)	44	
Histological type ^c							
Squamous cell carcinoma	189	70 (37.0)	54	0.86	88 (46.6)	46	0.24
Adenocarcinoma	116	42 (36.2)	52		58 (50.0)	40	
Pathologic stage							
I	177	45 (25.4)	61	6×10^{-5}	59 (33.3)	56	4×10^{-8}
II	46	23 (50.0)	44		29 (63.0)	31	
IIIA	87	46 (52.9)	43		60 (69.0)	28	
Adjuvant treatment ^d							
Yes	62	36 (58.1)	42	0.57	42 (67.7)	27	0.75
No	71	33 (46.5)	46		47 (66.2)	31	

^a Row percentage

^b Five-year overall survival rate (OSR) and 5-year disease-free survival rate (DFS), proportion of survival derived from Kaplan–Meier analysis

^c Five large cell carcinomas were excluded from this analysis

^d In pathologic stage II + IIIA: 55 received chemotherapy, 5 cases received radiotherapy, and 2 cases received both chemotherapy and radiotherapy

TABLE 2 Genotype frequencies of 21 polymorphisms in apoptosis-related genes

Gene		Polymorphism ^a		Genotype ^b , n						
Symbol	Name	ID no.	Base (amino acid) change	1/1	1/2	2/2	CR (%)	MAF	HWE	P
<i>TNFSF10</i>	Tumor necrosis factor (TNF) superfamily, member 10	rs3136586	−489A>G	84	151	59	94.8	0.457	0.552	
		rs1131532	C>T(F275F)	72	152	68	94.2	0.493	0.480	
<i>TNFSF1</i>	TNF superfamily, member 1	rs1041981	C>A(T60N)	103	156	46	98.4	0.407	0.295	
<i>TNFRSF10B</i>	TNF receptor superfamily member 10B	rs1047266	C>T(A67V)	175	115	19	99.7	0.248	0.985	
<i>TNFRSF1A</i>	TNF receptor superfamily member 1A	rs4149570	−329G>T	96	147	56	96.5	0.433	0.984	
		rs767455	T>C(P12P)	204	99	5	99.4	0.177	0.069	
<i>TNFRSF1B</i>	TNF receptor superfamily member 1B	rs1061622	T>G(M196R)	212	75	10	95.8	0.160	0.299	
		rs3397	*215C>T	124	136	36	95.5	0.351	0.890	
<i>BID</i>	BH3 interacting domain death agonist	rs8190315	A>G(S56G)	222	58	6	92.3	0.122	0.345	
<i>BCL2</i>	B-cell CLL/lymphoma 2	rs2279115	−938C>A	112	138	47	95.8	0.391	0.680	
		rs1801018	A>G(T7T)	251	54	3	99.4	0.097	0.960	
<i>APAF1</i>	Apoptotic peptidase activating factor 1	rs2289315	−529G>A	189	93	15	95.8	0.207	0.423	
<i>BIRC5</i>	Baculoviral IAP repeat-containing 5	rs9904341	91C>G	80	151	76	99.0	0.493	0.778	
		rs2071214	A>G(T7T)	186	100	16	97.4	0.219	0.595	
<i>BRCA1</i>	Breast cancer 1, early onset	rs799917	C>T(P817L)	138	135	26	96.5	0.313	0.384	
<i>BRCA2</i>	Breast cancer 2, early onset	rs144848	T>G(N372H)	159	124	15	96.1	0.258	0.139	
<i>PPP1R13L</i>	Protein phosphatase 1 regulatory subunit 13 like	rs1005165	−7774C>T	76	149	75	96.8	0.498	0.908	
<i>TP53</i>	Tumor protein p53	rs1042522	G>C(R72P)	106	136	60	97.4	0.424	0.176	
<i>PTGS2</i>	Prostaglandin-endoperoxide synthase 2	rs5275	*427T>C	192	102	14	99.4	0.211	0.923	
<i>RIPK1</i>	Receptor-interacting serine-threonine kinase 1	rs4959774	−722A>G	176	104	21	97.1	0.243	0.301	
		rs2272990	G>A(F28F)	166	99	18	91.3	0.239	0.534	

CR call rate, MAF minor allele frequency, HWE *P*, *P* for Hardy–Weinberg equilibrium test

^a Information about polymorphisms and IDs were obtained from NCBI database (<http://www.ncbi.nlm.nih.gov/SNP>). In the reference sequence, the transcription start site was counted as +1

^b Wild-type allele is denoted by 1 and the polymorphic allele by 2

had a significant association with survival outcomes in the individual SNP analysis. The *TNFRSF10B* rs1047266 TT, *TNFRSF1A* rs4149570 GT + TT, and *PPP1R13L* rs1005165 TT genotypes were considered as bad genotypes, and then their combined effects were evaluated by grouping the patients based on the number of bad genotypes. As shown in Table 5 and Fig. 1d, OS and DFS were decreased as the number of bad genotypes increased (P_{trend} for OS and DFS = 7×10^{-5} and 1×10^{-4} , respectively). On multivariate analysis, compared with patients with no bad genotypes, the aHRs for OS and DFS for patients with one bad genotype were 2.27 (95% CI = 1.22–4.21, $P = 0.01$) and 1.74 (95% CI = 1.08–2.81, $P = 0.02$), respectively; and the aHRs for OS and DFS for patients with two or three adverse genotypes were 4.11 (95% CI = 2.03–8.29, $P = 8 \times 10^{-5}$) and 2.89 (95% CI = 1.64–5.11, $P = 3 \times 10^{-4}$), respectively. For the clinicopathologic parameters, the pathologic stage was also a significant prognostic factor in a Cox model for OS and DFS (P for OS and DFS = 3×10^{-4} and = 2×10^{-7} , respectively).

DISCUSSION

We investigated the association between SNPs in apoptosis-related genes and survival outcomes in patients with surgically resected NSCLC. In the present study, three SNPs (*TNFRSF10B* rs1047266, *TNFRSF1A* rs4149570, and *PPP1R13L* rs1005165) were significantly associated with OS and DFS of the patients. In addition, a strong combined effect of the three SNPs on each clinical end point was observed. Multivariate logistic regression analysis revealed that the combined genotypes of these three SNPs were independent prognostic factors after adjusting for clinicopathologic factors, including pathologic stage. These findings support the hypothesis that polymorphic variants of apoptosis-related genes affect clinical prognosis of cancer patients and suggest that the rs1047266, rs4149570, and rs1005165 SNPs have potential value as prognostic markers for patients with surgically resected early-stage NSCLC.

The extrinsic pathway of apoptosis (also known as the death receptor pathway), one of the two major apoptotic

TABLE 3 Univariate and multivariate analyses for overall survival and disease-free survival by polymorphisms in apoptosis-related genes

Gene symbol	Polymorphism	Log-rank <i>P</i> for OS			Log-rank <i>P</i> for DFS			<i>P</i> for OS in Cox model			<i>P</i> for DFS in Cox model		
		Referent	Dominant	Recessive	Referent	Dominant	Recessive	Referent	Dominant	Recessive	Referent	Dominant	Recessive
<i>TNFSF10</i>	rs3136586	0.52	0.29	0.93	0.65	0.43	0.82	0.63	0.45	0.71	0.32	0.54	0.13
	rs1131532	0.70	0.41	0.73	0.66	0.37	0.94	0.91	0.96	0.83	0.68	0.38	0.82
<i>TNFSF1</i>	rs1041981	0.64	0.42	0.84	0.24	0.24	0.41	0.81	0.74	0.68	0.09	0.27	0.13
	rs1047266	0.02	0.84	0.01	0.002	0.85	0.001	0.03	0.77	0.01	0.04	0.77	0.02
<i>TNFRSF10B</i>	rs4149570	0.07	0.02	0.42	0.08	0.02	0.44	0.07	0.02	0.35	0.16	0.08	0.23
	rs767455	0.48	0.78	0.27	0.41	0.68	0.25	0.67	0.97	0.39	0.48	0.65	0.24
<i>TNFRSF1B</i>	rs1061622	0.80	0.71	0.67	0.73	0.49	0.87	0.81	0.61	0.81	0.39	0.17	0.57
	rs3397	0.68	0.95	0.42	0.89	0.96	0.64	0.70	1.00	0.43	0.86	0.58	0.85
<i>BID</i>	rs8190315	0.12	0.54	0.04	0.19	0.88	0.07	0.18	0.67	0.06	0.56	0.86	0.29
	rs2279115	0.33	0.15	0.87	0.19	0.08	0.97	0.48	0.24	0.87	0.71	0.41	0.87
<i>BCL2</i>	rs1801018	0.04	0.01	0.28	0.05	0.02	0.32	0.26	0.07	0.96	0.64	0.37	0.60
	rs2289315	0.91	0.72	0.72	0.89	0.78	0.78	0.84	0.57	0.74	0.21	0.09	0.98
<i>APAF1</i>	rs9904341	0.63	0.55	0.36	0.59	0.42	0.38	0.63	0.37	0.52	0.96	0.80	0.86
	rs2071214	0.84	0.55	0.85	0.94	0.78	0.92	0.96	0.78	0.85	0.93	0.70	0.88
<i>BRCA1</i>	rs799917	0.61	0.40	0.78	0.80	0.59	0.81	0.55	0.48	0.54	0.95	1.00	0.75
	rs144848	0.82	0.82	0.62	0.94	0.77	0.92	0.79	0.78	0.60	0.86	0.59	0.99
<i>PPPIR13L</i>	rs1005165	0.02	0.49	0.01	0.03	0.37	0.01	0.01	0.65	0.002	0.004	0.44	0.001
	rs1042522	0.68	0.39	0.88	0.51	0.25	0.62	0.73	0.48	0.54	0.52	0.46	0.27
<i>TP53</i>	rs5275	0.83	0.54	0.88	0.72	0.44	0.97	0.48	0.27	0.85	0.50	0.32	0.77
	rs4959774	0.37	0.38	0.18	0.32	0.32	0.16	0.25	0.47	0.09	0.09	0.08	0.07
<i>RIPK1</i>	rs2272990	0.40	0.20	0.43	0.30	0.15	0.31	0.45	0.26	0.38	0.22	0.08	0.45

OS overall survival, DFS disease-free survival

TABLE 4 Overall survival and disease-free survival according to the rs1047266, rs4149570, and rs1005165 genotypes

Polymorphism/ genotype	No. of cases (%) ^a	Overall survival				Disease-free survival			
		No. of deaths (%) ^b	5Y-OSR (%) ^c	HR (95% CI) ^d	<i>P</i> ^d	No. of events (%) ^b	5Y-DFSR (%) ^c	HR (95% CI) ^d	<i>P</i> ^d
<i>TNFRSF10B</i> rs1047266 ^e									
CC	175 (56.6)	63 (36.0)	53	1.00		84 (48.0)	44	1.00	
CT	115 (37.2)	38 (33.0)	60	0.81 (0.53–1.23)	0.31	50 (43.5)	49	0.83 (0.58–1.19)	0.31
TT	19 (6.1)	12 (63.2)	27	2.04 (1.07–3.90)	0.03	13 (68.4)	19	1.89 (1.03–3.46)	0.04
CC + CT	290 (93.9)	101 (34.8)	55	1.00		134 (46.2)	46	1.00	
TT	19 (6.1)	12 (63.2)	27	2.23 (1.19–4.18)	0.01	13 (68.4)	19	2.01 (1.11–3.65)	0.02
<i>TNFRSF1A</i> rs4149570 ^e									
GG	96 (32.1)	24 (25.0)	68	1.00		38 (39.6)	53	1.00	
GT	147 (49.2)	60 (40.8)	49	1.69 (1.05–2.72)	0.03	73 (49.7)	42	1.35 (0.91–2.01)	0.13
TT	56 (18.7)	22 (39.3)	48	1.78 (0.99–3.23)	0.06	29 (51.8)	39	1.58 (0.95–2.60)	0.08
GG	96 (32.1)	24 (25.0)	68	1.00		38 (39.6)	53	1.00	
GT + TT	203 (67.9)	82 (40.4)	49	1.71 (1.08–2.71)	0.02	102 (50.2)	42	1.41 (0.97–2.05)	0.08
<i>PPP1R13L</i> rs1005165 ^e									
CC	76 (25.3)	24 (31.6)	53	1.00		33 (43.4)	43	1.00	
CT	149 (49.7)	50 (33.6)	63	0.89 (0.54–1.47)	0.66	67 (45.0)	54	0.96 (0.62–1.47)	0.83
TT	75 (25.0)	35 (46.7)	34	1.79 (1.04–3.09)	0.04	42 (56.0)	27	1.83 (1.14–2.93)	0.01
CC + CT	225 (75.0)	74 (32.9)	60	1.00		100 (44.4)	51	1.00	
TT	75 (25.0)	35 (46.7)	34	1.94 (1.27–2.96)	0.002	42 (56.0)	27	1.88 (1.30–2.75)	0.001

5Y-OSR 5-year overall survival rate, 5Y-DFSR 5-year disease-free survival rate, HR hazard ratio, CI confidence interval

^a Column percentage

^b Row percentage

^c Five-year survival rate, proportion of survival derived from Kaplan–Meier analysis

^d HRs, 95% CIs, and their corresponding *P*-values calculated using multivariate Cox proportional-hazard models, adjusted for age, gender, smoking status, tumor histology, pathologic stage, and adjuvant therapy

^e Genotype failures: 1 case for rs1047266, 11 cases for rs4149570, and 10 cases for rs1005165

pathways, is activated when apoptosis-inducing TNF superfamily ligands, such as TNFSF10 (alias TRAIL or Apo2L), TNFSF6 (alias FasL or CD95L), TNFSF1 (alias lymphotoxin- α or TNF- β), and TNFSF2 (alias TNF or TNF- α), bind to their cognate receptors belonging to the TNFRS, such as TNFRSF10A (alias DR4 or TRAILR1), TNFRSF10B (alias DR5 or TRAILR2), TNFRSF10C (alias decoy receptor 1 [DcR1], TRAILR3 or TRID), TNFRSF6 (alias Fas, CD95, or Apo1), TNFRSF1A (alias TNFR1), and TNFRSF1B (alias TNFR2).^{17,18} The TNF and TNFR superfamily plays crucial roles in innate and adaptive immunity and cellular proliferation, differentiation, and apoptosis, and thus deregulation of the TNF and TNFR superfamily that can elicit apoptosis results in cancer as well as immune disorders.^{17,18}

In the present study, *TNFRSF10B* (DR5) rs1047266C>T was significantly associated with poor survival outcomes. DR5 is one of the TNFSF10 (TRAIL) receptors, including DR4, DR5, DcR1, DcR2, and the soluble decoy receptor, osteoprotegerin, and initiates TRAIL-mediated apoptosis.¹⁸ DR5 is expressed in a variety of cancers, including

NSCLC, and its expression has been linked to survival outcomes in many types of cancer.^{19–22} In addition, several studies have shown that activation of DR5 by agonistic agents, such as the agonistic monoclonal antibody, apomab, induces selective tumor cell apoptosis, and thereby inhibits lung tumor growth and improves survival.^{23–25} Taken together, these observations suggest that DR5 plays an important role in development and progression of many human cancers, including NSCLC. Therefore, it is possible that functional SNP(s) in the *TNFRSF10B* gene affect expression or activity of the protein and thereby influence survival outcomes. However, whether rs1047266C>T affects protein folding or interaction sites of the protein, or whether it is in LD with a true susceptibility variant, remains to be elucidated. Because this SNP is located in the vicinity of the ligand binding domain of DR5, it may alter TRAIL binding to DR5. Two interface regions in DR5 have been shown to mediate most of the ligand–receptor interaction: the 50s loop (residues 51–65) and the 90s loop (residues 91–104).²⁶ rs1047266C>T results a substitution of a valine for alanine at codon 67 (A67V), suggesting that

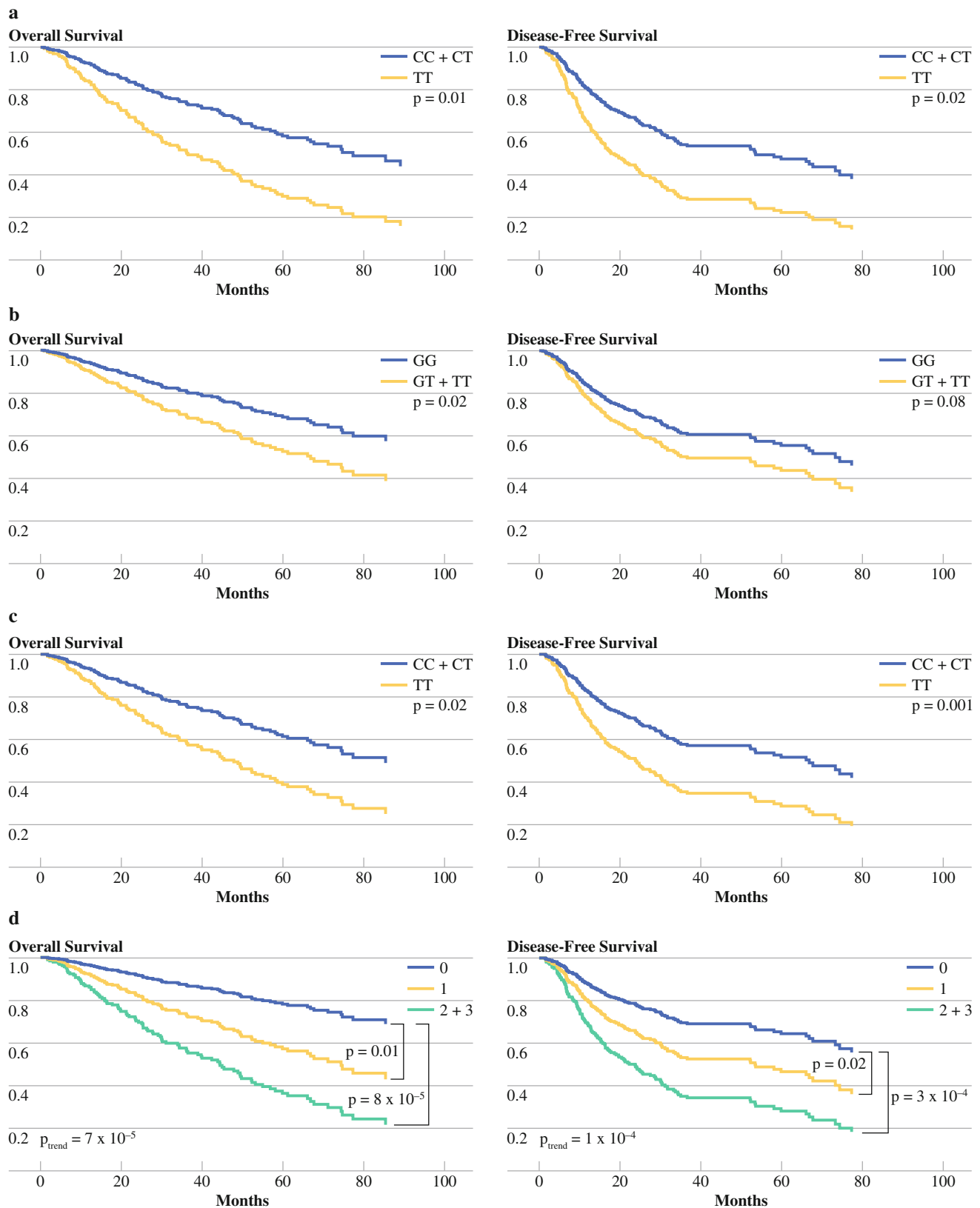


FIG. 1 Overall survival and disease-free survival curves according to TNFRSF10B rs1047266 (a), TNFRSF1A rs4149570 (b), and PPP1R13L rs1005165 (c) genotypes, and the number of bad genotypes of the three polymorphisms (d). P-values in the multivariate Cox hazard models

TABLE 5 Combined effects of TNFRSF10B rs1047266C>T, TNFRSF1A rs4149570G>T, and PPP1R13L rs1005165C>T on overall and disease-free survival

No. of bad genotypes	No. of cases (%) ^a	Overall survival					Disease-free survival				
		No. of deaths (%) ^b	5Y-OSR (%) ^c	<i>P</i> _(LR)	HR (95% CI) ^d	<i>P</i> ^d	No. of events (%) ^b	5Y-DFS (%) ^c	<i>P</i> _(LR)	HR (95% CI) ^d	<i>P</i> ^d
0	64 (21.8)	12 (18.8)	75	3.0×10^{-4}	1.00		21 (32.8)	58	0.01	1.00	
1	173 (59.0)	64 (37.0)	57		2.27 (1.22–4.21)	0.01	84 (48.6)	49		1.74 (1.08–2.81)	0.02
2 or 3 ^e	56 (19.1)	28 (50.0)	25		4.11 (2.03–8.29)	8×10^{-5}	32 (57.1)	18		2.89 (1.64–5.11)	3×10^{-4}
<i>P</i> _{trend}					7×10^{-5}					1×10^{-4}	

5Y-OSR 5-year overall survival rate, 5Y-DFS 5-year disease-free survival rate, HR hazard ratio, CI confidence interval, LR log rank

^a Column percentage

^b Row percentage

^c Five-year survival rate, proportion of survival derived from Kaplan–Meier analysis

^d HRs, 95% CIs, and their corresponding *P*-values calculated using multivariate Cox proportional hazard models, adjusted for age, gender, smoking status, tumor histology, pathologic stage, and adjuvant therapy

^e Bad genotypes: 52 cases for two bad genotypes and 4 cases for three bad genotypes

this amino acid alteration may disfavor TRAIL binding to DR5 and result in deficient apoptosis signaling. However, the association of rs1047266C>T with survival outcomes may be due to LD with other functional variant(s). Therefore, future studies on the *TNFRSF10B* sequence variants and their biologic function are needed to understand the role of *TNFRSF10B* SNPs in determining survival outcomes of NSCLC patients.

In the present study, the *TNFRSF1A* (*TNFR1*) rs4149570G>T (−329G>T, transcription start site denoted as +1) was also significantly associated with poor survival outcomes. TNFR1 is the major receptor for TNFSF2 (TNF or TNF-α), a proinflammatory cytokine produced by a wide range of immune cells, including activated macrophages and natural killer cells. It has been revealed that TNFR1 is expressed on most normal and malignant cells and initiates the majority of TNF biological activities.^{27–29} Although the effect of rs4149570G>T on prognosis of human cancer has not been studied yet, this SNP has been reported to influence TNFR1 expression and to be associated with increased risk of hepatocellular carcinoma, which is compatible with our finding of an association of the rs4149570T allele with poor survival outcomes. Therefore, one may expect that *TNFRSF1A* rs4149570G>T affects TNFR1 expression in tumors, thereby resulting in poor survival outcomes.

In the present study, *PPP1R13L* rs1005165C>T was associated with poor survival outcomes under a recessive model for the variant T allele. *PPP1R13L* encodes an inhibitor of the RelA subunit of the nuclear factor-kappa B (NF-κB), which has a central function in apoptosis regulation.³⁰ Thus, the product of *PPP1R13L* may block NF-κB activity, forcing apoptosis of damaged or unwanted cells. Changes in *PPP1R13L* level have been shown to modify

cisplatin-induced apoptosis.³¹ Although the functional relevance of rs1005165C>T is still unknown, analysis of the potential transcription factor binding sites by the Alibaba2 program showed that rs1005165C>T leads to alteration of transcription binding sites of the promoter of the *PPP1R13L* gene.³² Therefore, it is possible that the predicted changes in the putative transcription factor binding sites due to rs1005165C>T may affect *NF-κB* expression, thereby influencing apoptosis capacity. However, because *PPP1R13L* is located at 19q13.2-3, which contains several genes involved in DNA repair, including *excision repair cross complementing group 1* (*ERCC1*), *CD3ε epsilon associated protein* (*CD3EAP*; alias, antisense to *ERCC1*, *ASE1*), and *ERCC2* (alias, *xeroderma pigmentosum group D*), the association between rs1005165C>T and survival outcomes may be secondary to LD with other functional variant(s) in either the *PPP1R13L* gene or neighboring genes, including *ERCC1*, *CD3EAP*, and *ERCC2*, rather than a direct effect of rs1005165C>T.³³ In order to identify the variant(s) that may be in strong LD with rs1005165C>T that could be responsible for the association with survival outcomes, we screened and estimated the LDs of all the common variants (with MAF ≥ 0.01) of *ERCC1*, *CD3EAP*, *PPP1R13L*, and *ERCC2* genes, including SNPs previously reported to be associated with susceptibility or prognosis of lung cancer [rs11615T>C (N118N in *ERCC1*), rs3212986C>A (3'UTR in *ERCC1* or Q504K in *CD3EAP*), rs967591G>A (at −21 in *CD3EAP*), rs1799793G>A (D312N in *ERCC2*), and rs13181A>C (K751Q in *ERCC2*)] using the HapMap JPT data.^{33–35} Notably, we found that rs1005165C>T is located within a large haplotype block (Fig. 2). Therefore, it is possible that the functional variant(s) in strong LD with rs1005165C>T may be the causal variant(s) for the association of this SNP with survival outcomes.

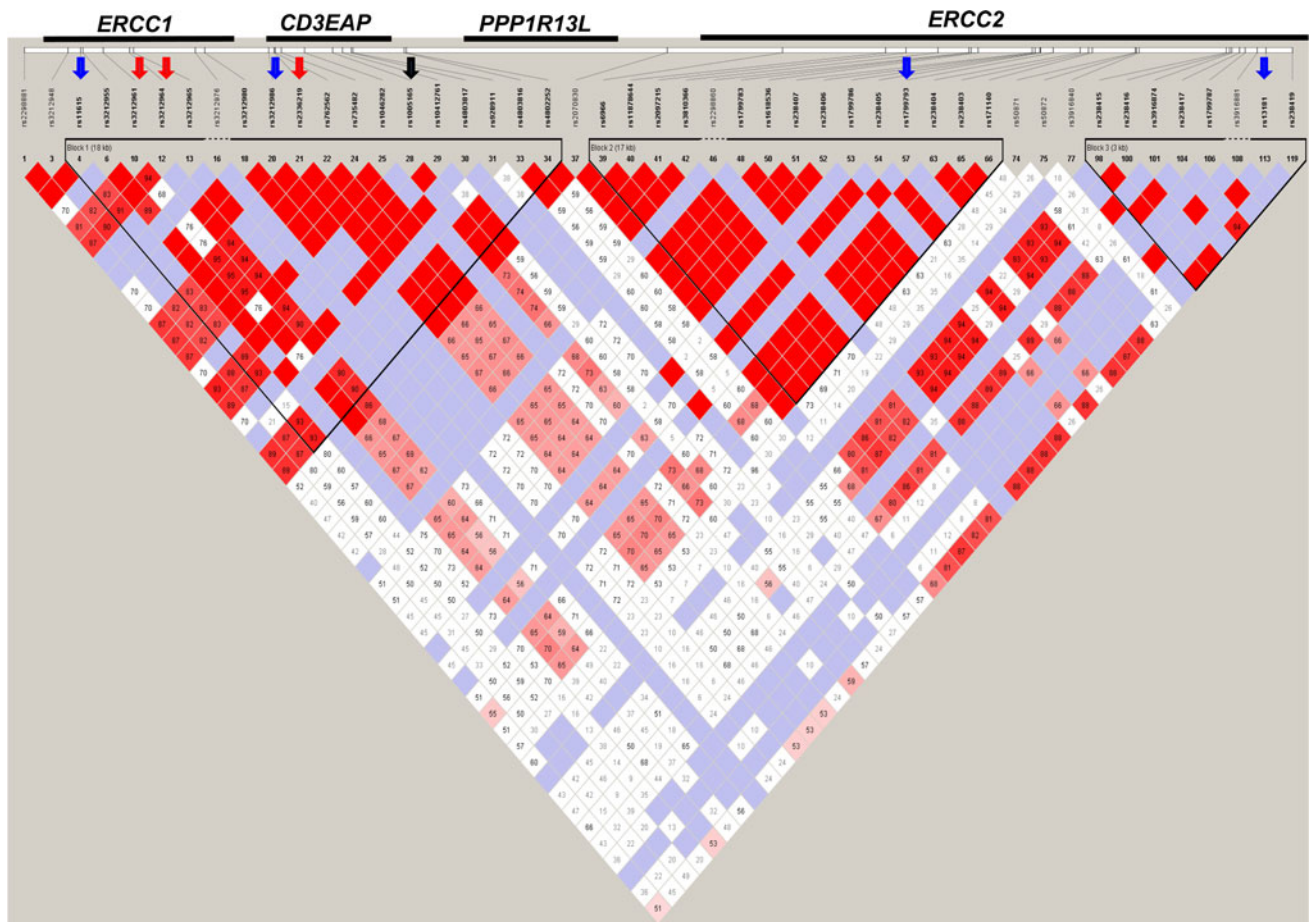


FIG. 2 Reconstructed linkage disequilibrium (LD) plot using SNPs with minor allele frequency $\geq 10\%$ in ERCC1, CD3EAP, PPP1R13L, and ERCC2 genes from HapMap JPT data. Haplotype blocks are constructed by the confidence interval method proposed by Gabriel et al.¹⁶ The black boxes indicate strong LD (confidence interval for strong LD: upper 0.98, low 0.7; fraction of strong LD in informative comparisons must be at least 0.95). White boxes indicate strong

recombination (upper confidence interval maximum 0.9) and gray boxes indicate noninformative findings. Triangles indicate haplotype blocks. Numbers in the squares are $|D'|$ ($\times 100$) values. Vertical bold arrow indicates rs1005165. Black bold boxes indicate polymorphisms which are strong LD with rs1005165, and blue bold boxes indicate polymorphisms which have been reported to be associated with lung cancer

Another interesting finding of the present study was that combined analysis of multiple SNPs had a much higher potential value in unraveling polymorphisms of prognostic importance. Although three SNPs were associated with survival outcomes in individual SNP analysis, considering the borderline CIs and multiple comparisons, the impact of any individual SNP on survival outcomes would be minimal. However, when these three SNPs were combined, OS and DFS were decreased as the number of bad genotypes increased (P_{trend} for OS and DFS = 7×10^{-5} and 1×10^{-4} , respectively). The HRs for OS and DFS of patients carrying two or three bad genotypes were 4.11 and 2.89, respectively, compared with those carrying no bad genotypes ($P = 8 \times 10^{-5}$ and 3×10^{-4} , respectively). These findings provide evidence that a pathway-based multigenic approach combining multiple polymorphisms gives more precise delineation of survival outcomes over a single candidate gene approach.

A number of limitations in the present study need to be addressed. Most importantly, our study lacked a testing (also called validating) cohort study to verify the study results. Validation of genotype–phenotype association studies requires replication using an independent data set, which would reduce the false-positive findings from the genetic association study.^{36,37} Therefore, our findings of significant associations of the three SNPs with survival outcomes should be confirmed in an independent sample. This study lacked the related functional assays, which limited our inquiry into the biologic mechanism of the observed associations between the SNPs and survival outcomes. Another limitation was the modest sample size, which enables the identification of variants that exert a relatively large effect on survival outcomes but does not have sufficient statistical power for detection of variants that had a small effect on survival outcomes; therefore, there might be type II errors. In addition, considering the

borderline CI and the multiple-comparison issue, we cannot rule out that the association analysis of individual SNP may have a type I error. Therefore, additional studies with larger sample sizes are required to confirm our findings.

Our goal was to identify prognostic markers for patients with surgically resected early-stage NSCLC. The results from this study demonstrated that three SNPs (*TNFRSF10B* rs1047266, *TNFRSF1A* rs4149570, and *PPP1R13L* rs1005165) were associated with patient survival, and survival decreased with the number of bad genotypes, suggesting the possibility of using these SNPs as prognostic factors. Larger studies are required to confirm the effects of the three SNPs in other patient populations, and resequencing neighboring regions along with functional assessment will confirm the role of these SNPs in NSCLC behavior.

ACKNOWLEDGMENT This study was supported by the National R&D Program for Cancer Control Ministry of Health & Welfare (0720550-2) and the Regional Technology Innovation Program of the Ministry of Commerce, Industry and Energy (RTI04-01-01) of Republic of Korea.

DISCLOSURES The authors indicated no commercial interest.

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