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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Genetic Variant of Notch Regulator DTX1 Predicts Survival After Lung Cancer Surgery

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ABSTRACT

Background. We evaluated the association between genetic variants in the Notch pathway and survival outcomes of patients with surgically resected NSCLC.

Methods. Sixty-four single nucleotide polymorphisms (SNPs) in the Notch pathway genes were evaluated in the discovery study (n = 354) and two sequential validation studies (n = 772 and n = 746, respectively). The association of genotype with overall survival (OS) and disease-free survival (DFS) was evaluated.

Results. Of the 64 SNPs analyzed in the discovery study, 9 were significantly associated with OS or DFS. Among them, the association remained significant only for *Deltex-1* (*DTX1*) rs1732786A>G in the first validation study. The

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second validation study confirmed again the association between *DTX1* rs1732786A>G and survival outcomes. In the combined analysis, rs1732786A>G was significantly associated with better OS and DFS (adjusted HR ·aHR· for OS, 0.75; 95% CI 0.64–0.87; $P = 0.0002$; aHR for DFS, 0.79; 95% CI 0.71–0.89; $P = 0.0001$). In vitro luciferase assay showed that the rs1732786G allele was associated with higher promoter activity compared to rs1732786A allele. Consistently, relative mRNA expression level of *DTX1* showed significant positive correlation with rs1732786 A-to-G change ($P_{trend} = 0.02$) in tumor tissues. **Conclusions.** These results suggest that *DTX1* rs1732786 is a potential prognostic factor that may have clinical utility in the management of early stage NSCLC.

The Notch signaling pathway has been implicated in critical cellular processes in development, such as cell fate determination and stem cell differentiation, as well as in tumorigenesis.[1,2] Notch signaling is activated by contacting a neighboring ligand-expressing cell and ligand binding to a receptor, which initiates proteolytic cleavage of the receptor. This cleavage releases the Notch intracellular domain (NICD), which then translocates into the nucleus where it binds to the transcription factor CBF-1/suppressor of hairless/Lag1(CSL), resulting in modulation of the expression of many target genes.[3,4] The outcome of Notch signaling activity depends on cellular contextual cues, such as interactions with the tumor microenvironment and crosstalk with other signaling pathways.[1,5]

Aberrant regulation of the Notch pathway has been implicated in the pathogenesis of many cancers.[6–8] Earlier studies discovered that deregulated expression of Notch receptors, ligands, and target genes is associated with many solid tumors, including lung cancer and hematologic malignancies.[1,9] Although its role is somewhat controversial, Notch signaling is generally suspected as having a growth-promoting function in NSCLC, as suggested by positive correlation with lymph node metastasis or higher clinical stages.[10] In contrast, it has been reported that the expression of Notch is down-regulated in lung cancer cell lines and tumor tissues, and Notch expression is associated with better survival.[11] This discrepancy may suggest that the role of Notch in lung cancer is highly dependent upon cellular context and may be associated with disease subtypes or specific genetic changes.

Several studies have investigated the association of Notch pathway genetic polymorphisms with risk or prognosis in various types of cancer, including lung cancer.[12–15] Recently, Xu et al.[15] reported genetic variants in the Notch pathway could predict overall survival in 1185 Caucasian NSCLC patients who were identified by the PLCO cancer screening trial, amongst whom 655 had a potentially curable disease at stage I–IIIA. Prognostic biomarkers that allow more precise prognostication of patients after surgery would greatly help to guide adjuvant therapy and postoperative follow-up in NSCLC. The present study involved three independent sets of patients, comprising a total of 1872 early-stage NSCLC patients from seven major institutions in Korea. We evaluated and validated the associations between potentially functional variants in the Notch pathway and the prognosis of surgically treated NSCLC. We also performed in vitro assays and mRNA expression analysis using lung tumor tissue specimens to explore biological relevance of the association.

MATERIALS AND METHODS

Patients

The discovery set included 354 patients who was diagnosed with pathologic stage I, II, or IIIA NSCLC and underwent curative surgical resection at the Kyungpook National University Hospital (KNUH) between December 1997 and January 2010. The validation set comprised 772 patients from Seoul National University Bundang Hospital ($n = 428$) and Chonnam National University Hospital ($n = 344$). The second validation set consisted of 746 patients from National Cancer Center of Korea ($n = 273$), Seoul National University Bundang Hospital ($n = 168$), Keimyung University Dongsan Medical Center ($n = 142$), Asan Medical Center ($n = 90$), and Pusan National University Hospital ($n = 73$). All of the patients were ethnic Koreans, and patients who received chemotherapy or radiotherapy before surgery were excluded. The pathologic staging of the tumors was determined according to the International System for Staging Lung Cancer.[16] Written, informed consent was obtained from all patients before surgery, and this study was approved by the institutional review boards of each participating institution.

Polymorphism Selection and Genotyping

We identified 28 candidate genes involved in the Notch pathway by searching the database from SABiosciences (http://www.sabiosciences.com/rt-pcr-product/HTML/PAHS-059Z.html). A total of 252 potentially functional single nucleotide polymorphisms (SNPs) were selected from these genes, with minor allele frequency ≥ 0.1 by the HapMap JPT data by screening the public SNP database (http://www.ncbi.nlm.nih.gov/SNP). Using the FuncPred utility for functional SNP prediction and the TagSNP utility for linkage disequilibrium (LD) tag SNP selection in the SNPinfo web server (https://snpinfo.niehs.nih.gov/), 79 potentially functional SNPs were selected for genotyping

after excluding those in LD ($r^2 \geq 0.8$). Among 79 SNPs, 15 SNPs with a call rate $< 95\%$ or with a P value for Hardy–Weinberg equilibrium (HWE) < 0.05 were excluded from further analysis. Finally, the remaining 64 SNPs in the Notch pathway genes were analyzed for the association study (Supplementary Table 1). For validation, we selected and genotyped nine SNPs that were associated with overall (OS) or disease-free survival (DFS) at $P < 0.05$ in the discovery set, using SEQUENOM's MassARRAY® iPLEX assay (SEQUENOM Inc., San Diego, CA).

Promoter-Luciferase Constructs and Luciferase Assay

Because *DTX1* rs1732786A>G was consistently associated with survival outcome of patients in the discovery and validation sets, we used a luciferase assay to investigate whether the rs1732786A>G (−16 from transcription start site) of *DTX1* modulates the promoter activity of the gene. A 618 bp fragment including rs1732786A>G was synthesized by PCR using genomic DNA from a heterozygotic donor. A forward primer with a *KpnI* restriction site (5′-GGGGTACCGACGCAGTTGGGAGTGCAAA-3′) and a reverse primer with an *XhoI* restriction site (5′-CCGCTCGAGCGTTCTCAATGTGGTGGCAC-3′) were used. The PCR products were cloned into the *Kpn*I/*Xho*I site of the pGL3-basic vector (Promega, Madison, WI), resulting in pGL3-Basic-*DTX1* constructs containing either the rs1732786 A or G allele. The sequence of all of the clones was verified by DNA sequencing. The NSCLC cell lines (H1299, H1703, and A549) were purchased from the Korean Cell Line Bank (KCLB), Seoul, Korea, and authenticated by the KCLB using short tandem-repeat DNA fingerprinting. The cells were transfected with pRL-SV40 vector (Promega) and pGL3-basic vector using Effectene® transfection reagent (Qiagen, Hilden, Germany). The cells were harvested 24 h after transfection and lysates were prepared using the Dual-Luciferase® Reporter Assay System (Promega). Luciferase activity was measured using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT). The results were normalized to pRL-SV40 *Renilla* luciferase activity. All experiments were performed twice in octuplicate.

Quantitative Reverse Transcription–Polymerase Chain Reaction

Quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed to determine *DTX1* and *HES1* mRNA expression using 133 pairs of tumor and corresponding normal lung tissues. Total RNA was isolated from the fresh frozen tumors and paired nonmalignant lung tissues of 154 NSCLC patients who underwent surgery in Kyungpook National University Chilgok Hospital between September 2011 and August 2014 using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed using the QuantiTect reverse transcription kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Real time-PCR was performed for each gene and for beta-actin using QuantiFast SYBR Green PCR Master Mix (QIAGEN) in a LightCycler 480 (Roche Applied Science, Mannheim, Germany) with the following primers : *DTX1* forward, 5′-AGTTCACCGCAAGAGGATTC-3′; *DTX1* reverse, 5′-GTGCCGATAGTGAAGATGAGTC-3′; *HES1* forward, 5′-AACGCAGTGTCACCTTCC-3′; *HES1* reverse, 5′-TCAAGTTCCTGTTTAGAGTCCG-3′; beta-actin forward, 5′-TTGTTACAGGAAGTCCCTTGCC-3′; beta-actin reverse, 5′-ATGCTATCACCTCCCCTGTGT-3′. The relative mRNA expression was normalized using the beta-actin expression and then calculated using the $2^{-\Delta\Delta CT}$ method.[17]

Data Analysis

Differences in the distribution of genotypes according to clinicopathologic factors of the patients were examined using χ^2 tests. Hardy–Weinberg equilibrium was assessed using a goodness-of-fit χ^2 test with 1 degree of freedom. Overall survival (OS) was measured from the date of surgery to the date of death from any cause or the last follow-up. Disease-free survival (DFS) duration was calculated from the date of surgery until first evidence of disease recurrence or last date of follow-up for patients who were free of disease. Kaplan–Meier method and log-rank tests were used to analyze the differences in OS and DFS across different genotypes. Cox proportional hazards regression model was used for the multivariate survival analyses. The hazard ratio (HR) and 95% confidence interval (CI) were estimated. Statistical analyses were performed using statistics software (SAS, version 9.4, SAS Institute, Cary, NC).

RESULTS

The baseline clinical and pathological characteristics of the patients and the impact of these characteristics on OS/DFS in the discovery and the two validation sets are shown in Supplementary Table 2. In the combined cohort (n = 1872), age, sex, smoking status, pack-years of smoking, tumor histology, and pathologic stage were significantly associated with OS and DFS (Table 1).

Among the 64 SNPs analyzed in the discovery set (n = 354), 9 SNPs were significantly associated with OS or DFS (Supplementary Table 1). We evaluated these nine SNPs in an independent validation analysis (validation cohort I, n = 772), and the association was consistently observed only for the *DTX1* rs1732786A>G

TABLE 1 Univariate analysis for survival outcomes by clinicopathological features in the combined cohort

Variables	Overall survival				Disease free survival		
	No. of cases	No. of death (%)[a]	5Y-OSR (%)[b]	Log-Rank *P*	No. of death (%)[a]	5Y-DFSR (%)[b]	Log-Rank *P*
Overall	1872	407 (21.7)	70		679 (63.7)	55	
Age (years)							
< 65	925	172 (18.6)	76	8.9×10^{-7}	332 (36.8)	60	2.9×10^{-3}
≥ 65	947	235 (24.8)	64		357 (37.7)	50	
Gender							
Male	1277	332 (26.0)	66	1.1×10^{-10}	505 (39.5)	52	2.0×10^{-5}
Female	595	75 (12.6)	81		174 (29.2)	63	
Smoking status							
Ever	1246	325 (26.1)	65	2.2×10^{-10}	493 (39.6)	52	3.9×10^{-5}
Never	626	82 (13.1)	81		186 (29.7)	62	
Pack-years[c]							
< 40	1424	263 (18.5)	74	7.0×10^{-10}	214 (34.6)	56	4.0×10^{-3}
≥ 40	448	144 (22.1)	59		279 (44.5)	48	
Histological type							
SCC	707	189 (26.7)	65	4.8×10^{-8}	279 (39.5)	53	2.2×10^{-3}
AC	1081	188 (17.4)	75		1081 (33.3)	58	
LCC	84	30 (35.7)	58		40 (47.6)	46	
Pathologic stage							
I	1110	153 (13.8)	82	1.2×10^{-38}	282 (25.6)	69	3.4×10^{-54}
II–IIIA	762	254 (23.3)	51		397 (52.1)	32	
Adjuvant therapy[d]							
No	309	112 (36.2)	49	0.70	163 (52.7)	36	0.44
Yes	453	142 (31.3)	52		234 (51.7)	29	

SCC, squamous cell carcinoma; AC, adenocarcinoma; LCC, large cell carcinoma

[a]Row percentage

[b]Five year-overall survival rate (5Y-OSR) and Five year-disease free survival rate (5Y-DFSR), proportion of survival derived from Kaplan–Meier analysis

[c]In ever-smokers

[d]In stages II–IIIA

(Supplementary Table 3). We further evaluated *DTX1* rs1732786A>G in the second validation analysis (validation cohort II, $n = 746$) and confirmed the significant association between the SNP and the prognosis of patients (Table 2). In the combined analysis, rs1732786A>G was significantly associated with better OS and DFS (adjusted HR ·aHR· for OS = 0.75, 95% CI 0.64–0.87, $P = 2 \times 10^{-4}$; aHR for DFS = 0.79, 95% CI 0.70–0.88, $P = 5 \times 10^{-5}$; under a codominant model; Table 2; Fig. 1). The association between *DTX1* rs1732786A>G and survival outcomes was further evaluated after categorizing the patients by age, gender, smoking status, histologic type, pathologic stage, and adjuvant therapy. The significant association was consistent across most of the subgroups except for females and stage I disease (Supplementary Table 4). There was no differential effect of the SNP on survival outcomes between the subgroups of each variable based on a homogeneity test (*P* values for the homogeneity test > 0.05 for all comparisons, Supplementary Table 4). The SNP was not significantly associated with patient- or tumor-related factors, such as age, gender, smoking status, histological type, pathologic stage, or adjuvant therapy (Supplementary Table 5).

To verify the functional relevance of the rs1732786A>G in the promoter region of *DTX1*, we investigated whether rs1732786A>G (-16 from transcription start site) modulates the promoter activity of the *DTX1* gene. *In vitro* promoter assays showed that the rs1732786G allele had significantly higher promoter activity than the rs1732786A allele ($P < 0.01$ for all comparisons; Fig. 2). These results suggest that the rs1732786G allele is associated with higher expression of *DTX1* compared with the rs1732786A

TABLE 2 Overall survival and disease free survival according to *DTX1* rs1732786A>G genotypes in the discovery, validation I and II, and combined cohort

Cohort	Genotypes	OS					DFS			
		No. of cases (%)[a]	No. of death (%)[b]	5Y-OSR (%)[c]	HR (95% CI)[d]	P^d	No. of events (%)[b]	5Y-DFSR (%)[c]	HR (95% CI)[d]	P^d
Discovery	AA	141 (40.6)	50 (35.5)	53	1.00		70 (49.6)	39	1.00	
	AG	150 (43.2)	50 (33.3)	53	0.83 (0.56–1.24)	0.37	67 (44.7)	47	0.85 (0.60-1.19)	0.34
	GG	56 (16.1)	13 (23.2)	67	0.48 (0.26–0.88)	0.02	24 (42.9)	49	0.70 (0.44–1.13)	0.14
	Dominant	206 (59.4)	63 (30.6)	57	0.72 (0.49–1.06)	0.09	91 (44.1)	47	0.81 (0.59–1.11)	0.18
	Recessive	291 (83.9)	100 (34.4)	53	0.53 (0.29–0.94)	0.03	137 (47.1)	44	0.77 (0.50–1.19)	0.24
	Codominant				0.73 (0.56–0.95)	0.02			0.84 (0.67–1.05)	0.13
Validation I	AA	296 (39.2)	84 (28.4)	60	1.00		140 (47.3)	41	1.00	
	AG	360 (47.6)	85 (25.6)	68	0.75 (0.56–1.02)	0.07	156 (43.3)	47	0.81 (0.65–1.02)	0.07
	GG	100 (13.2)	19 (29.0)	70	0.61 (0.37–1.01)	0.06	35 (45.0)	56	0.62 (0.43–0.91)	0.01
	Dominant	460 (60.8)	104 (22.6)	69	0.72 (0.54–0.97)	0.03	191 (41.5)	49	0.77 (0.62–0.96)	0.02
	Recessive	656 (86.8)	169 (25.8)	65	0.72 (0.44–1.15)	0.17	296 (45.1)	44	0.70 (0.49–1.00)	0.05
	Codominant				0.77 (0.62–0.96)	0.02			0.80 (0.68–0.94)	0.01
Validation II	AA	298 (41.0)	48 (16.1)	80	1.00		82 (27.5)	65	1.00	
	AG	327 (45.0)	41 (12.5)	83	0.62 (0.41–0.96)	0.03	74 (22.4)	72	0.63 (0.46–0.87)	0.01
	GG	102 (14.0)	9 (9.2)	86	0.51 (0.25–1.03)	0.06	17 (16.7)	77	0.52 (0.31–0.88)	0.01
	Dominant	429 (59.0)	50 (11.7)	84	0.60 (0.40–0.90)	0.01	91 (11.2)	73	0.61 (0.45–0.82)	0.001
	Recessive	625 (86.0)	89 (14.2)	81	0.64 (0.32–1.28)	0.21	156 (25.0)	69	0.67 (0.40–1.10)	0.11
	Codominant				0.67 (0.49–0.93)	0.01			0.70 (0.55–0.88)	0.003
Combined	AA	735 (40.0)	182 (24.8)	67	1.00		292 (39.8)	51	1.00	
	AG	837 (46.0)	176 (21.0)	72	0.74 (0.60–0.92)	0.005	297 (35.6)	57	0.78 (0.66–0.91)	0.002
	GG	258 (14.0)	41 (15.9)	76	0.56 (0.40–0.80)	0.001	76 (29.5)	62	0.63 (0.48–0.80)	0.003
	Dominant	1095 (60.0)	217 (19.2)	73	0.70 (0.57–0.86)	5×10^{-4}	373 (34.1)	58	0.75 (0.63–0.86)	1×10^{-4}
	Recessive	1527 (86.0)	358 (22.8)	70	0.66 (0.48–0.91)	0.011	589 (37.5)	54	0.72 (0.56–0.90)	0.006
	Codominant				0.75 (0.64–0.87)	2×10^{-4}			0.79 (0.70–0.88)	5×10^{-5}

OS, overall survival; DFS, disease free survival

[a]Column percentage

[b]Row percentage

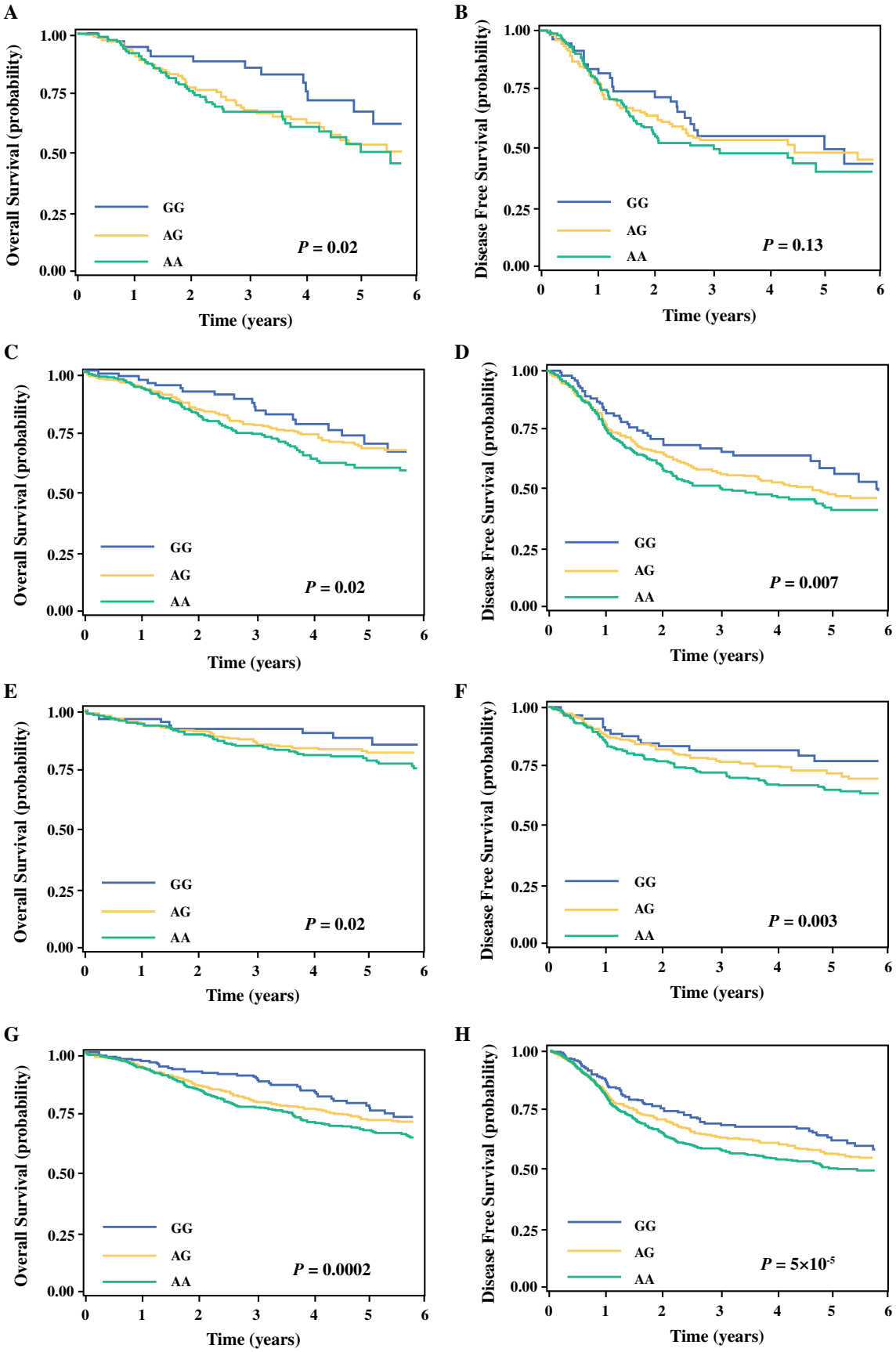
[c]Five year-overall survival rate (5Y-OSR) and five year-disease free survival rate (5Y-DFSR), proportion of survival derived from Kaplan–Meier analysis

[d]Hazard ratios (HRs), 95% confidence intervals (CIs) and corresponding *P* values were calculated using multivariate Cox proportional hazard models, adjusted for age, gender, smoking status, tumor histology, pathologic stage and adjuvant therapy

allele. Next, we determined *DTX1* mRNA expression in tumors and paired non-malignant lung tissues. The relative expression level of *DTX1* was significantly lower in tumors than in nonmalignant lung tissues ($P = 0.003$; Fig. 3a). As shown in Fig. 3b, the *DTX1* mRNA expression in tumor tissues (genotype distribution: AA 47, AG 61, and GG 25) showed significant positive correlation with A-to-G change ($P_{trend} = 0.02$). We further evaluated whether *DTX1* rs1732786A>G affects the expression of *HES1*, because DTX1 has been reported to repress the expression of the Notch target genes, and *HES1* is one of the most studied Notch target genes.[18] The relative expression level of *HES1* was significantly higher in tumors than in nonmalignant lung tissues ($P = 0.02$; Fig. 3c). As expected, *HES1* mRNA expression was negatively correlated with *DTX1* rs1732786 A-to-G change ($P_{trend} = 0.02$; Fig. 3d). Notably, higher *DTX1* mRNA expression was associated with significantly better OS and DFS compared with lower expression ($P = 0.03$ for OS and $P = 0.002$ for DFS; Fig. 3e, f). We evaluated protein expression of DTX1 using NSCLC cell lines with various levels of *DTX1* mRNA expression and observed a strong correlation between mRNA and protein expression of DTX1 (Pearson $r = 0.995$, $P = 0.005$; Supplementary Fig. 1).

DISCUSSION

We investigated the association between potentially functional variants in the Notch pathway and the survival of patients with early stage NSCLC. Among 64 SNPs in 28 candidate genes, *DTX1* rs1732786A>G was replicated across three independent cohorts comprising 1872 patients.


A
Overall Survival (probability)
1.00
0.75
0.50
0.25
0.00
0 1 2 3 4 5 6
Time (years)
GG
AG
AA
P = 0.02
B
Disease Free Survival (probability)
1.00
0.75
0.50
0.25
0.00
0 1 2 3 4 5 6
Time (years)
GG
AG
AA
P = 0.13
C
Overall Survival (probability)
1.00
0.75
0.50
0.25
0.00
0 1 2 3 4 5 6
Time (years)
GG
AG
AA
P = 0.02
D
Disease Free Survival (probability)
1.00
0.75
0.50
0.25
0.00
0 1 2 3 4 5 6
Time (years)
GG
AG
AA
P = 0.007
E
Overall Survival (probability)
1.00
0.75
0.50
0.25
0.00
0 1 2 3 4 5 6
Time (years)
GG
AG
AA
P = 0.02
F
Disease Free Survival (probability)
1.00
0.75
0.50
0.25
0.00
0 1 2 3 4 5 6
Time (years)
GG
AG
AA
P = 0.003
G
Overall Survival (probability)
1.00
0.75
0.50
0.25
0.00
0 1 2 3 4 5 6
Time (years)
GG
AG
AA
P = 0.0002
H
Disease Free Survival (probability)
1.00
0.75
0.50
0.25
0.00
0 1 2 3 4 5 6
Time (years)
GG
AG
AA
P = 5×10-5

◀**FIG. 1** Kaplan–Meier plots of overall and disease free survival in the discovery set (**a**, **b**), in the validation I set (**c**, **d**), in the validation II set (**e**, **f**), and in the combined set (**g**, **h**) according to *DTX1* rs1732786A>G genotypes under the codominant model. *P* values, multivariate Cox proportional hazard model

Multivariate analysis revealed that this variant is an independent prognostic factor. In addition, this study provides functional evidence for the role of *DTX1* rs1732786A>G. These findings suggest that *DTX1* rs1732786A>G could be a useful prognostic marker for guiding the management of patients in early stage NSCLC after surgery.

Notch signaling is implicated in carcinogenesis because of its role in regulating multiple steps of cancer development and progression, including cell growth, apoptosis, migration, and invasion.[19] Notch signaling is thought to have an oncogenic role in NSCLC.[20,21] Previous studies showed that NSCLC tissues had elevated Notch expression, and the level of Notch expression was positively correlated with disease progression, metastasis, and poorer patient survival.[20,22–26] DTX1, a transcriptional target of Notch itself, is known to be one of the major regulators of the Notch pathway.[18,27,28] DTX1 is an E3 ubiquitin ligase that mediates the degradation of the intracellular Notch receptor through a ubiquitination-dependent pathway, thereby repressing the expression of Notch target genes.[18,28] It also was shown that DTX1 negatively regulates Notch signaling by inhibiting coactivator recruitment.[27] In the present study, *DTX1* rs1732786A>G was associated with better survival outcomes. The variant was associated with increased promoter activity and mRNA expression of *DTX1*. Given the generally accepted oncogenic function of Notch signaling in cancer development and progression, increased expression of Notch regulator DTX1 may lead to decreased Notch signaling activity and in turn to a reduction in malignant phenotypes, such as disease progression, metastasis, and poor prognosis. In line with our expectations, higher tumor *DTX1* mRNA expression was correlated with better survival of patients. Therefore, better prognosis among patients with the variant allele is biologically plausible. In addition, as suggested by the negative regulator role of DTX1 in Notch signaling, *DTX1* rs1732786A>G was negatively correlated with *HES1* mRNA expression. Our results suggest that DTX1 has a tumor suppressor function in early stage NSCLC. However, because the role of Notch signaling in NSCLC and the precise biological mechanism of DTX1-mediated regulation of the Notch pathway is still unclear, further studies are needed to understand the mechanism of association between this SNP and the survival of NSCLC patients.

The purpose of our three-stage study design was to validate positive findings from the discovery study to support the contention that the genotype-survival association is valid. By replicating the positive association

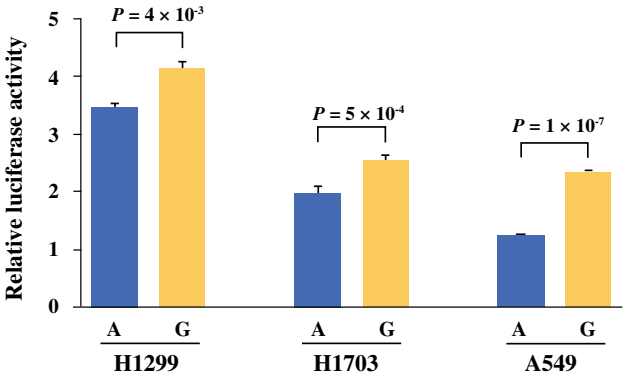


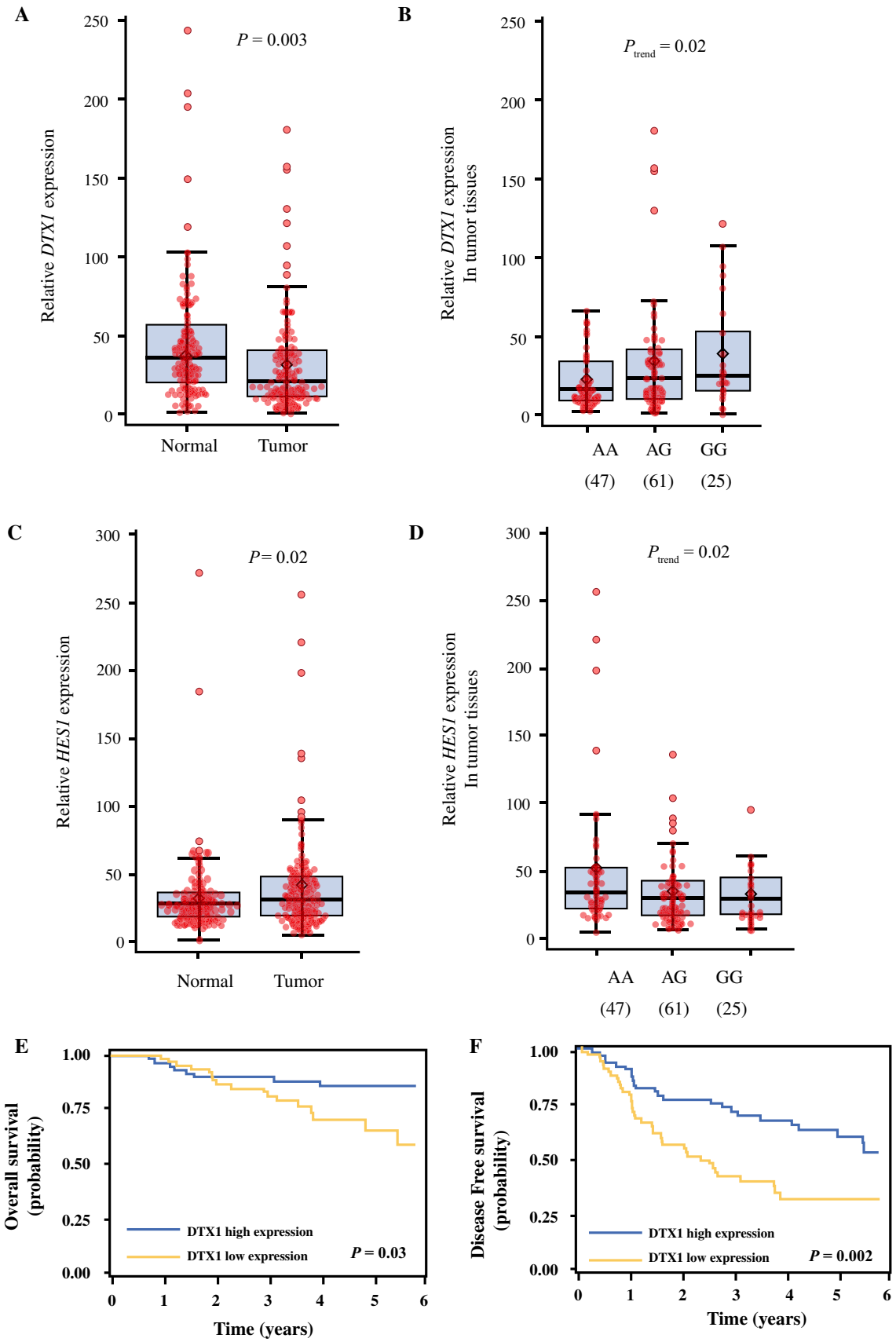
FIG. 2 Functional analysis of the rs1732786A>G in the *DTX1* promoter region (−16 from transcription start site) using luciferase assay. The NSCLC cell lines (H1299, H1703 and A549) were transfected with pGL3-Basic-*DTX1* constructs containing either rs1732786 A or G allele and pRL-SV40 vector. Each bar represents mean ± SEM of luciferase activity normalized to pRL-SV40 *Renilla* luciferase activity. All experiments were performed twice in octuplicate. *P* values, Student's *t* test

between *DTX1* rs1732786A>G and survival outcomes, we could reduce the possibility of false positive finding and support the plausibility of the SNP as a prognostic factor in early stage NSCLC. Xu et al. recently reported that *DTX1* rs1732793G>A was significantly associated with a poor OS in NSCLC.[15] In our discovery study, *DTX1* rs2264886, which was in complete LD with rs1732793, was not significantly correlated with survival outcomes. This discrepant result may be attributable to the difference in ethnicity between the two study populations. However, further study is warranted to validate rs1732793 as a prognostic factor in NSCLC.

There were several limitations to this study. First, this study included only a Korean patient population. Therefore, the results may not be generalizable for other ethnic groups, although the results were replicated in independent Korean patient cohorts. Second, only 453 of 752 stage II/IIIA patients received adjuvant therapies, indicating that approximately 40% of the patients were not treated by the current standard-of-care. However, most patients from participating institutions received surgery between late 1990s and late 2000s, and adjuvant chemotherapy was not performed as a standard postsurgical management for NSCLC in Korea until the later part of the enrollment period. Cox proportional hazards regression model was used for multivariate survival analyses with adjustment for adjuvant therapy as well as other clinical factors.

CONCLUSIONS

Our study suggests that genetic variation in the Notch regulator DTX1 is a potential predictor of survival in NSCLC and is worth evaluating for its clinical utility in


A
P = 0.003
Relative DTX1 expression
250
200
150
100
50
0
Normal
Tumor
B
P trend = 0.02
Relative DTX1 expression
In tumor tissues
AA
AG
GG
(47)
(61)
(25)
C
P= 0.02
Relative HES1 expression
300
250
200
150
100
50
0
Normal
Tumor
D
P trend = 0.02
Relative HES1 expression
In tumor tissues
AA
AG
GG
(47)
(61)
(25)
E
Overall survival
(probability)
1.00
0.75
0.50
0.25
0.00
DTX1 high expression
DTX1 low expression
P = 0.03
0
1
2
3
4
5
6
Time (years)
F
Disease Free survival
(probability)
1.00
0.75
0.50
0.25
0.00
DTX1 high expression
DTX1 low expression
P = 0.002
0
1
2
3
4
5
6
Time (years)

◀**FIG. 3** *DTX1* and *HES1* mRNA expression level by *DTX1* rs1732786A>G genotypes and Kaplan–Meier plots of overall and disease free survival according to *DTX1* expression. **a** *DTX1* mRNA expression levels were significantly down-regulated in tumor tissues than non-malignant lung tissues ($P = 0.003$). *P* value, Student's *t* test. **b** *DTX1* expression level showed a significant positive correlation with A-to-G change ($P_{trend} = 0.02$). **c** *HES1* mRNA expression level was significantly higher in tumors than in non-malignant lung tissues ($P = 0.02$). *P* value, Student's *t* test. **d** *HES1* mRNA expression was negatively correlated with A-to-G change ($P_{trend} = 0.02$). The horizontal lines within the boxes represent the median values; the upper and lower boundaries of the boxes represent 75th and 25th percentiles, respectively; the upper and lower bars indicate the largest and smallest observed values, respectively, except outliers. **e**, **f** Higher *DTX1* mRNA expression was associated with significant better OS ($P = 0.03$) and DFS ($P = 0.002$) compared with lower *DTX1* mRNA expression. *P* values, log-rank test

guiding adjuvant therapy and postsurgical follow-up. Further studies are required to confirm the impact of this SNP in a larger population with diverse ethnicity and to understand the biological function of DTX1 in the development and progression of lung cancer.

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DISCLOSURES The authors declare no conflict of interests.

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