

# NOTCH1 Mutations Predict Superior Outcomes of Immune Checkpoint Blockade in Non-Small Cell Lung Cancer

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**Background:** *NOTCH1* is frequently mutated in non-small cell lung cancer (NSCLC), and also is a poor therapeutic target. It is of clinical importance to investigate the effects of *NOTCH1* mutations on anti-tumor immunity and response to immune checkpoint blockade (ICB).

**Methods:** An observational study with targeted sequencing in 963 NSCLC patients at our center were performed (FUSCC cohort). Data of the Cancer Genome Atlas Pan-Lung Cancer study (TCGA cohort) were analyzed, and gene set enrichment analysis (GSEA) was performed. The Samstein et al cohort included 350 patients with advanced NSCLC undergoing genomic profiling with the MSK-IMPACT assay, and receiving at least one dose of ICB therapy.

**Results:** *NOTCH1* mutations were more common in smokers and patients with squamous-cell carcinoma (SCC) (all *P* value <0.05). For patients who did not receive ICB therapy (TCGA cohort), the overall survival (OS) of *NOTCH1*-mutant and -WT patients were comparable (log-rank *P* = 0.72), while for patients who received ICB therapy in the Samstein et al cohort, *NOTCH1*-mutant patients had significantly superior OS than WT patients (log-rank *P* = 0.041). On multivariate Cox analysis, the predictive value of *NOTCH1* mutations reached marginal statistical significance (HR, 0.42; 95% CI, 0.17–1.04; *P* = 0.059). The median of TMB for *NOTCH1*-mutant tumors was significantly higher than that for *NOTCH1*-WT tumors, and GSEA revealed that *NOTCH1* mutations manifested various defects in the repair of DNA damage. *NOTCH1*-mutant tumors displayed an inflamed tumor microenvironment (TME), manifesting as increased PD-L1 expression and tumor-infiltrating CD8<sup>+</sup> T cells.

**Conclusion:** *NOTCH1* mutations define a molecular subtype of NSCLC, which are more common in smokers and patients with SCC, are characterized with higher TMB, inflamed TME, and display improved survival of ICB therapy for NSCLC patients.

**Keywords:** *NOTCH1*, non-small cell lung cancer, immunotherapy, survival, tumor mutational burden, tumor microenvironment

## Introduction

Lung cancer remains the leading cause of cancer-related mortality worldwide, and the majority are non-small-cell lung cancer (NSCLC).<sup>1,2</sup> Genetic variation is a typical feature of NSCLC that drives cancer initiation and progression.<sup>3</sup> Understanding the role of mutated genes in NSCLC is the basis of the development of novel treatment modalities, and precision treatment.<sup>4</sup> Mammals have four Notch paralogue genes (*NOTCH1*, *NOTCH 2*, *NOTCH 3*, and *NOTCH 4*) and five membrane-bound ligand genes (*Delta-like 1*, *Delta-like 3*, *Delta-like 4*, *Jagged-1*, and *Jagged-2*).<sup>5</sup> The Notch signaling is a highly conserved cell-cell interaction mechanism a diverse, cell context-specific, signaling output,<sup>6</sup> and plays vital roles in cancer-related functions such as proliferation, tumor angiogenesis, stemness maintenance and epithelial-to-mesenchymal transition.<sup>7,8</sup> In 2004, Dr. Weng et al identified frequent *NOTCH1* mutations in heterodimerization domain and/or the C-terminal PEST

domain among about half of patients with primary T-cell acute lymphoblastic leukemias, which were considered to be oncogenic drivers.<sup>9</sup> On the other hand, *NOTCH1* can also act as a tumor suppressor gene in a variety of malignancies, including NSCLC, myeloid leukaemia, and head and neck squamous cell carcinoma.<sup>10–12</sup> In this setting, the mutations generally result in the functional inactivation of *NOTCH1* gene, making it a poor therapeutic target so far.

Immunotherapy has dramatically impacted the current landscape of cancer treatment. Immune checkpoint blockade (ICB) targeting the programmed cell death (ligand) 1 [PD-(L)1] has been the standard therapy as first-line and second-line treatment in patients with advanced NSCLC, whether as monotherapy or in combination with chemotherapy.<sup>13,14</sup> Despite the improved survival of ICB for NSCLC patients, only about 20%-30% of patients could benefit from the treatment, which necessitates further investigation into the biomarkers to identify the patients who are more likely to respond to ICB.<sup>13</sup> To date, PD-L1 expression and tumor mutational burden (TMB) are two critical biomarkers which have been validated. PD-L1 expression has been approved by Food and Drug Administration (FDA) as a biomarkers for NSCLC,<sup>13,15,16</sup> however, it has intrinsic limitations and remains imperfect.<sup>17,18</sup> This raises the question of whether there are some other features simultaneously affecting factors to serve as a powerful predictor for therapeutic outcomes.<sup>19</sup> Previous studies have revealed the interplay of tumor genetic variations and immune response, and demonstrated that mutations of oncogenic genes and tumor suppressor genes, such as EGFR, KRAS, and P53, could reprogram the tumor microenvironment (TME) and thus affect the response to immunotherapy.<sup>20–22</sup>

In order to summarize the characteristics of NSCLC patients harboring *NOTCH1* mutations, and systematically address the influence of *NOTCH1* mutations on the anti-tumor immunity to NSCLC, we performed integrative analyses of clinical and genomic data from our center and public databases. We found that *NOTCH1* mutations were associated with higher TMB, inflamed microenvironment, and improved survival of ICB therapy for NSCLC patients, implying the potential of *NOTCH1* as a therapeutic target or predictive marker for NSCLC.

## Patients and Methods

### Ethics Statement

This study was approved by the institutional review board of FUSCC (2008223-9). Written informed consent was exempted because of retrospective analysis. This study was conducted in accordance with the Declaration of Helsinki and patient confidentiality is guaranteed.

### Patients

Patients diagnosed with NSCLC treated at Fudan University Shanghai Cancer Center (FUSCC) from June 2018 to December 2019 were screened. Patients were included in the FUSCC cohort whose tumors underwent targeting next-generation sequencing (68-gene panel, Burning Rock Company, China), covering the whole exon regions of *NOTCH1* gene. The processes of genomic DNA sequencing and variants calling were described previously.<sup>23</sup> The demographic, clinical and pathological information, including the PD-L1 tumor proportion score (TPS), were collected from the electronic medical records.

Data of TCGA Pan-Lung Cancer study was retrieved from <https://www.cbioportal.org/> (TCGA cohort). Data of the Samstein et al cohort were retrieved from the published article.<sup>16</sup> The cohort included 350 patients with advanced NSCLC receiving at least one dose of ICB therapy. In total, 329 patients received anti-PD-1/PD-L1 monotherapy, and 21 received combination therapy with anti-CTLA-4. Patients underwent genomic profiling with the Memorial Sloan Kettering Cancer Center Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) assay as part of clinical care, identifying somatic exon variations in a predefined set of 468 cancer-related genes.

### Gene Set Enrichment Analysis

For gene set enrichment analysis (GSEA),<sup>24</sup> the javaGSEA Desktop Application (GSEA 4.0.1) was downloaded from <http://software.broadinstitute.org/gsea/index.jsp>. GSEA was used to associate the gene signature with the *NOTCH1* mutation status (*NOTCH1*-mut vs *NOTCH1*-wt). The normalized enrichment score (NES) is the primary statistic for examining gene set enrichment results. Nominal p-values were calculated empirically using 1000 random phenotype

label permutations, and multiple testing correction was applied to generate False Discovery Rate (FDR) -adjusted P values. A gene set with an FDR cutoff  $<0.25$  and nominal p-value  $<0.05$  was considered to be significantly enriched in genes.

## Statistical Analysis

The demographic and clinicopathological data were compared by Student's *t* test or Mann–Whitney *U*-test as appropriate. The Kaplan–Meier method was applied to delineate the curve of overall survival (OS), and the Log-rank method was applied to evaluate the significance. The Cox proportional-hazards regression analysis was implemented to calculate the HR of OS in both univariate and multivariate analyses, and Backward Stepwise (Wald) method was used in multivariate analysis. Patients with specific missing clinicopathological data were not included in relevant specific clinicopathological characteristic analyses. The statistical analyses mentioned above were performed using GraphPad Prism 7 or IBM SPSS Statistics 22. Level of significance was set as  $P < 0.05$  (two-tailed) ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ , and  $****P < 0.0001$ ).

## Results

### *NOTCH1* Mutations Were More Common in Smokers and Patients with Squamous Cell Carcinoma

To investigate the clinical and pathologic characteristics of *NOTCH1*-mutant NSCLC, medical record information of 963 patients were collected, whose tumors underwent targeted next-generation sequencing at FUSCC from June 2018 to December 2019. Among them, *NOTCH1* mutations were detected in tumors of 55 patients, accounting for 5.7%. As shown in Table 1, patients harboring *NOTCH1* mutation displayed distinct clinical and pathologic characteristics. In the

**Table 1** Baseline Clinicopathologic Characteristics of NSCLC Patients in the FUSCC Cohort, TCGA Cohort, and Samstein et al Cohort

	FUSCC Cohort			TCGA Cohort			Samstein et al Cohort		
	<i>NOTCH1</i>		<i>P</i> value	<i>NOTCH1</i> <sup>†</sup>		<i>P</i> value	<i>NOTCH1</i>		<i>P</i> value
	Mutant	WT		Mutant	WT		Mutant	WT	
Gender			<b>0.014</b>			0.41			0.51
Female	22(40.0)	517 (56.9)		28 (35.9)	440 (41.4)		6(42.9)	174 (51.8)	
Male	33 (60.0)	391 (43.1)		50 (64.1)	623 (58.6)		8(57.8)	162 (48.2)	
Age			<b>0.04</b>			0.11			0.69
<65 year	29 (52.7)	602 (66.3)		19 (29.7)	355 (39.8)		5(35.7)	138 (41.1)	
≥65 year	26 (47.3)	306 (33.7)		45 (70.3)	536 (60.2)		9(64.3)	198 (58.9)	
Smoking			<b>0.04</b>			<b>0.023</b>			–
Never	25 (45.5)	602 (66.3)		3(4.1)	108 (10.7)		–	–	
Former	22 (40.0)	194 (21.4)		44 (59.5)	661 (65.3)		–	–	
Current	8(14.5)	112 (12.3)		27 (36.5)	244 (24.1)		–	–	
Pathology			<b>&lt;0.001</b>			<b>0.033</b>			<b>0.005<sup>#</sup></b>
ADC	39 (70.9)	835 (92.0)		36 (46.2)	624 (58.5)		6(42.9)	265 (78.9)	
SCC	9(16.4)	41 (4.5)		42 (53.8)	442 (41.5)		5(35.7)	40 (11.9)	
Other	7(12.7)	32 (3.5)		–	–		3(21.4)	31 (9.2)	
Stage			0.38 <sup>#</sup>			0.64 <sup>#</sup>			–
0/I	38 (69.1)	676 (74.4)		44 (57.1)	531 (51.5)		–	–	
II/III	16 (29.1)	226 (24.9)		31 (40.3)	465 (45.1)		–	–	
IV	1(1.8)	6(0.7)		2(2.6)	36 (3.5)		14	336	

**Notes:** <sup>#</sup>Fisher's exact test. <sup>†</sup>Numbers may not sum to the total because of missing data. Significant results are in bold.

FUSCC cohort, *NOTCH1* mutations were more common in male patients at least 65 years old, smokers and patients with squamous-cell carcinoma (SCC) (all  $P$  value  $<0.05$ ).

We further analyzed the data of two publicly available cohorts of NSCLC patients, the TCGA cohort and Samstein et al<sup>16</sup> cohort. In the TCGA cohort, *NOTCH1* mutations were found in 6.8% (78/1141) of patients. Former/current smokers and patients with SCC were more likely to harbor *NOTCH1* mutations (both  $P$  value  $<0.05$ ). The Samstein et al cohort collected 350 patients with advanced NSCLC treated with ICB, and *NOTCH1* mutations were found in 14 (4.0%) patients. *NOTCH1*-mutant tumors had significantly higher proportion of SCC (Fisher's exact  $P$  value = 0.005), while the information of smoking status was unavailable in this cohort. The difference of the distribution in gender and age were equivalent between *NOTCH1*-mutant and -WT patients in both cohorts. The majority of patients in the FUSCC cohort and TCGA cohort were diagnosed with early-stage disease, and the stage of *NOTCH1*-mutant and -WT patients were comparable in both cohorts. The data of three NSCLC cohorts with different context collectively shown that smokers and patients with SCC were more likely to be *NOTCH1* mutant.

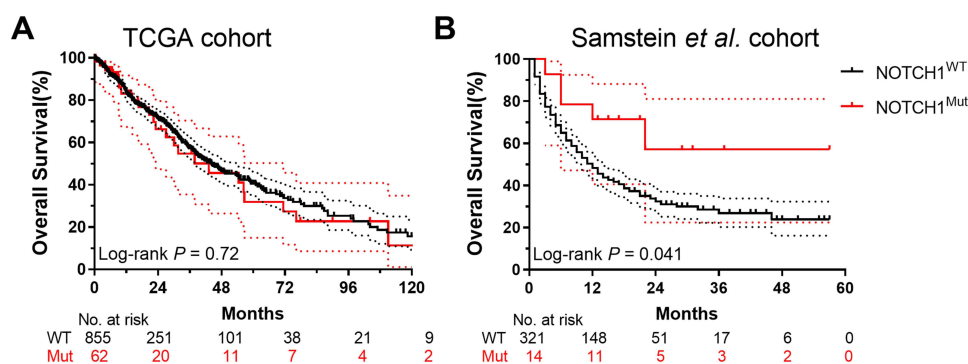
## *NOTCH1* Mutations Predicted Improved Survival of ICB Therapy for NSCLC Patients

The prognostic and predictive value of *NOTCH1* mutations for patients with NSCLC were subsequently investigated. Patients of the TCGA cohort did not receive ICB therapy. The 5-year OS of *NOTCH1*-mutant and -WT patients were 39.1% (95% confidence interval [CI], 15.0%–50.4%) and 40.5% (95% CI, 34.6%–46.3%), respectively (HR, 1.08; 95% CI, 0.69–1.68; log-rank  $P$  = 0.72; Figure 1A). This data suggested that *NOTCH1* mutant status was not a prognostic indicator for patients with NSCLC who did not receive ICB.

In the Samstein et al cohort, 329 patients were administrated with anti-PD-1/PD-L1 antibody, and 21 patients with a combination of anti-PD-1/PD-L1 antibody and anti-CTLA-4 antibody. The 3-year OS of *NOTCH1*-mutant and -WT patients were 57.1% (95% confidence interval [CI], 22.4%–81.1%) and 26.9% (95% CI, 20.3%–33.9%), respectively (HR, 0.40; 95% CI, 0.16–0.96; log-rank  $P$  = 0.041; Figure 1B).<sup>16</sup> Cox regression analyses were performed to further confirm the independent predictive value of *NOTCH1* mutation status. On univariate analysis, *NOTCH1* mutational status, TMB and drug type were predictors of survival, and were included in the multivariate analysis. On multivariate analysis, the value of *NOTCH1* mutational status reached marginal statistical significance (HR, 0.42; 95% CI, 0.17–1.04;  $P$  = 0.059; Table 2).

## *NOTCH1* Mutations Correlated With Higher TMB

The vital significance of TMB in the setting of ICB therapy has been recently demonstrated by cohort studies and clinical trials across multiple cancer types. We next sought to elucidate the influences of *NOTCH1* mutation status on the tumor mutational load. In the TCGA cohort, the median of TMB for *NOTCH1*-mutant tumors (8.9; IQR, 6.3–12.6) was significantly higher than that for *NOTCH1*-WT tumors (6.6; IQR, 3.5–10.7; Wilcoxon  $P$  < 0.001, Q value < 0.001; Figure 2A). In the Samstein et al cohort, *NOTCH1*-mutant tumors also displayed significantly higher TMB (median, 14;



**Figure 1** NSCLC patients harboring *NOTCH1* mutations could benefit from ICB therapy. **(A)** Overall survival of *NOTCH1*-WT and -mutant NSCLC patients, who did not receive ICB therapy, was comparable in the TCGA dataset. **(B)** Overall survival of *NOTCH1*-mutant subset was significantly superior than that of *NOTCH1*-WT subset among NSCLC patients receiving ICB therapy in the Samstein et al<sup>16</sup> cohort. Dot lines indicate 95% confidence intervals.

**Table 2** Univariate and Multivariate Cox Regression Analysis of NSCLC Patients Receiving ICB Therapy in the Samstein et al Cohort

	Univariate COX Regression Analysis			Multivariate COX Regression Analysis		
	HR	95% CI	P value	HR	95% CI	P value
Gender (Female v male)	0.86	0.66–1.11	0.25	–	–	–
Age ( $\geq 65$ v $< 65$ year)	1.08	0.83–1.41	0.58	–	–	–
Pathology			0.36			–
ADC	1			–	–	–
SCC	1.31	0.91–1.88	0.15	–	–	–
Other	1.04	0.64–1.66	0.89	–	–	–
<i>NOTCH1</i> (Mutant v WT)	0.40	0.16–0.96	<b>0.041</b>	0.42	0.17–1.04	<b>0.059</b>
TMB			<b>0.03</b>			<b>0.002</b>
Bottom 80%	1			1		
Top 10–20%	0.86	0.56–1.32	0.50	0.92	0.60–1.41	0.70
Top 10%	0.36	0.20–0.65	<b>0.001</b>	0.36	0.20–0.64	<b>0.001</b>
Drug type (Combo v PD-1/PD-L1)	0.64	0.46–0.90	<b>0.01</b>	0.38	0.19–0.75	<b>0.005</b>

Notes: Significant results are in bold.

IQR, 6.8–20.2) than that of *NOTCH1*-WT tumors (median, 6.9; IQR, 3.9–11.7; Wilcoxon  $P = 0.02$ , Q value = 0.073; Figure 2B).

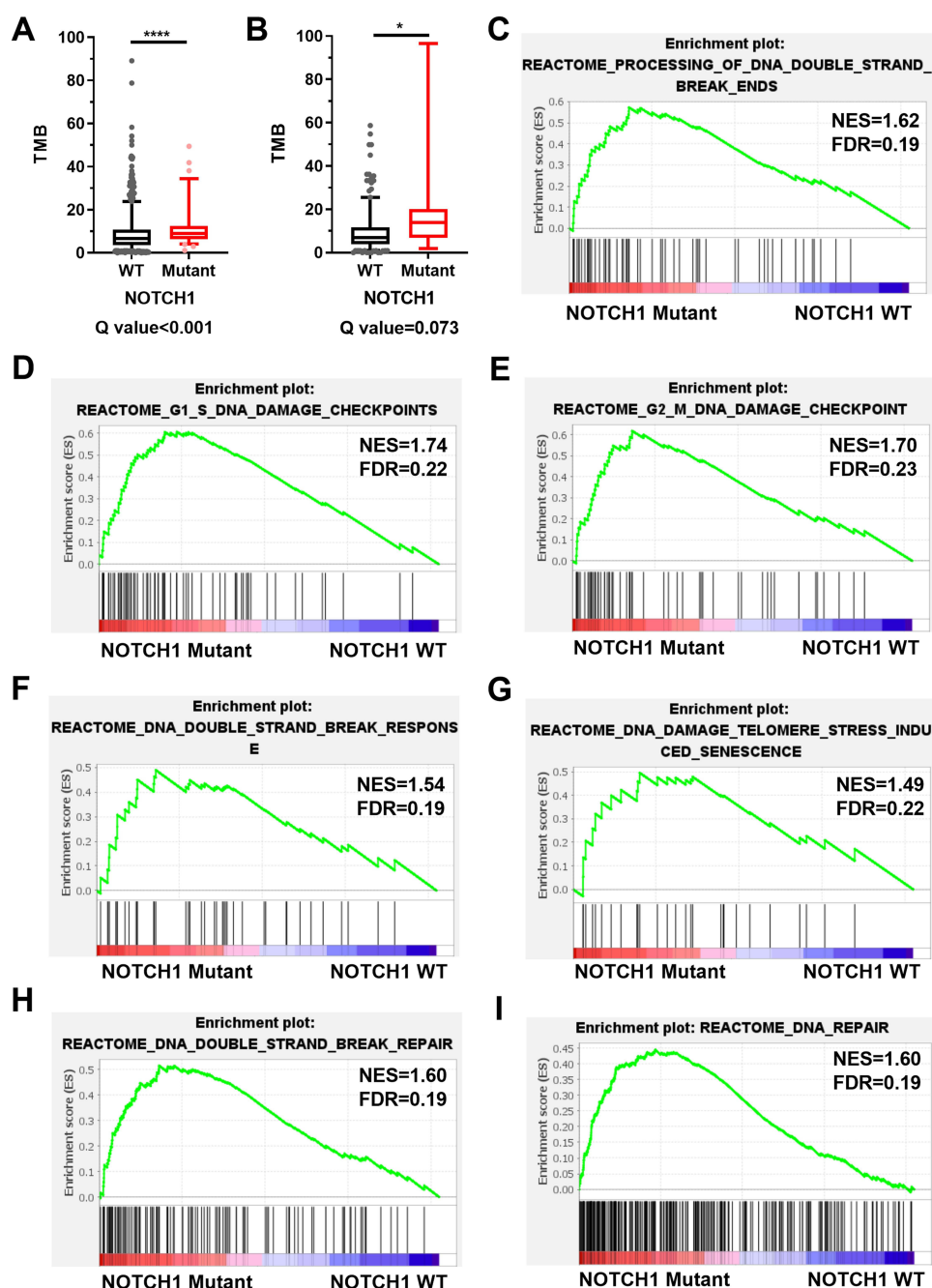
We speculated whether alterations in DNA damage and repair-related genes resulted from *NOTCH1* mutations could account for the differential tumor mutational load. GSEA revealed prominent enrichment of signatures relating to PROCESSING OF DNA DOUBLE STRAND BREAK ENDS (Figure 2C), DNA DOUBLE STRAND BREAK REPAIR (Figure 2D) and RESPONSE (Figure 2E), DNA REPAIR (Figure 2F), DNA DAMAGE TELOMERE STRESS INDUCED SENESENCE (Figure 2G), G1/S DNA DAMAGE CHECKPOINTS (Figure 2H), and G2/M DNA DAMAGE CHECKPOINTS (Figure 2I). *NOTCH1* mutations manifested various defects in the repair of DNA damage that greatly enhanced point mutation.

## *NOTCH1* Mutations Were Associated with an Inflamed Microenvironment

We subsequently investigate whether *NOTCH1* mutations and mutational load could lead to the remodeling of the immune microenvironment of NSCLC. The proportion for PD-L1 strong positive (TPS  $\geq 50\%$ ) and weak positive ( $1\% < \text{TPS} \leq 49\%$ ) of *NOTCH1*-mutant NSCLC were 19.5% and 26.8%, respectively, which were significantly higher than those of *NOTCH1*-WT tumors (strong positive, 8.0%; weak positive, 19.3%;  $P < 0.01$ , Figure 3A). The TIMER 2.0 was used to evaluate the association between *NOTCH1* mutation status and the immune cell subtypes. Notably, *NOTCH1*-mutant tumors had significantly higher level of CD8<sup>+</sup> T cell than *NOTCH1*-WT tumors by XCELL algorithm ( $P = 0.0085$ , Figure 3B) and MCP-COUNTER algorithm ( $P = 0.029$ , Figure 3C). Besides, *NOTCH1*-mutant tumors also exhibited remarkable increases in the mRNA level of CD8A in the TCGA database ( $P = 0.029$ , Figure 3D). Collectively, *NOTCH1*-mutant tumors displayed an inflamed TME, manifesting as remarkably increase of PD-L1 expression and tumor-infiltrating CD8<sup>+</sup> T cells, which had been demonstrated to be associated with benefits from ICB therapy.

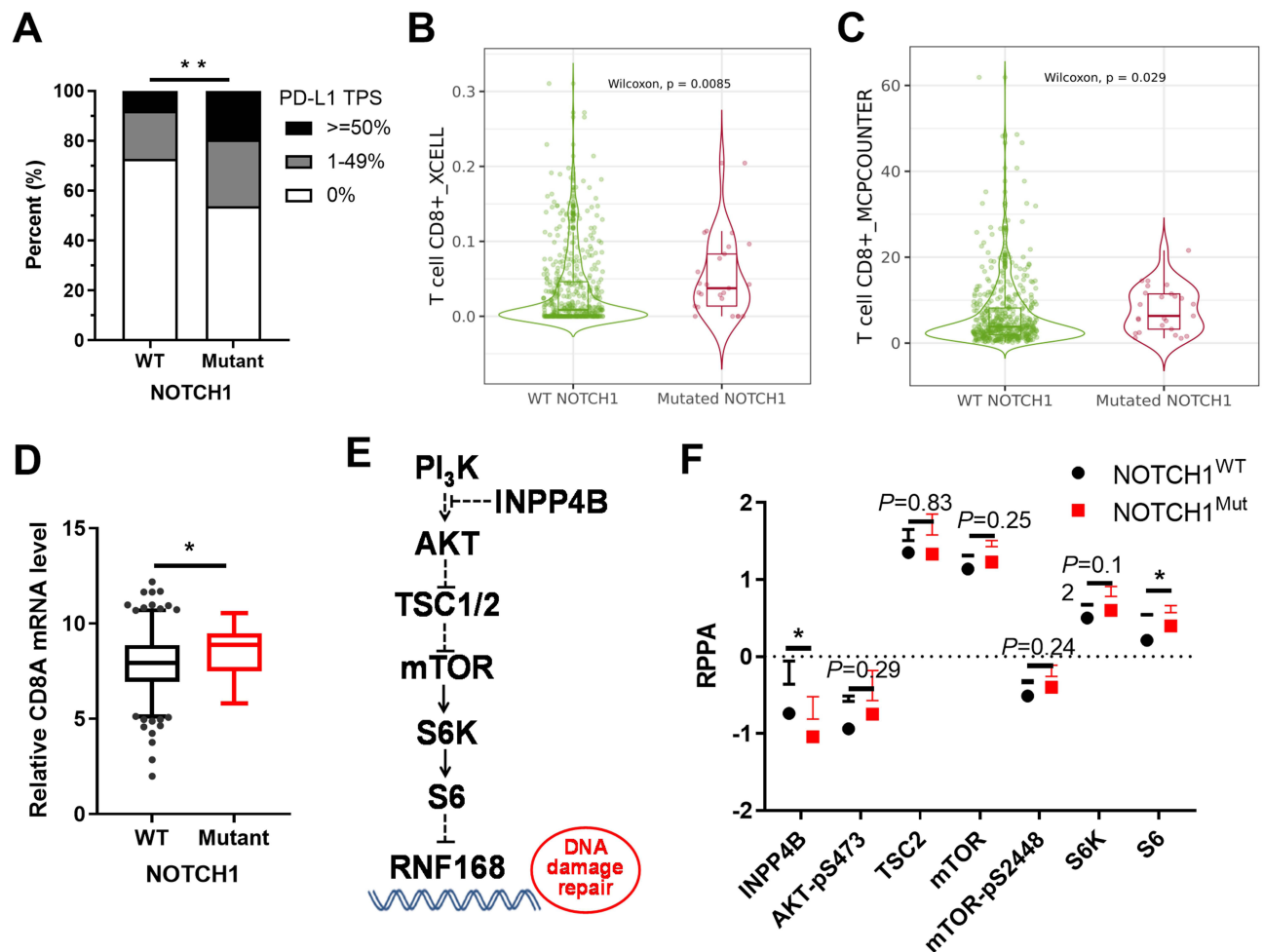
To identify the potential signaling pathways that mediated the increased mutation load and the inflamed immune microenvironment, we compared the protein levels between *NOTCH1*-mutant and -WT tumors in the TCGA database. Overall, expression levels of 208 proteins were assessed by RPPA, and the levels of eight proteins (PARP1, CDKN1B, SMAD4, PRDX1, INPP4B, S6, BCL2, CHEK2) were significantly different between two groups (all  $P < 0.05$ ). Of note, S6, the downstream protein of PI3K-AKT-mTOR signaling, was significantly activated by *NOTCH1* mutations, and INPP4B, suppressor of PI3K-AKT signaling, was significantly decreased (Figure 3E and F). Besides, other key proteins of the signaling, including AKT-pS473, mTOR, mTOR-pS2448 and S6K, were higher in the mutant group, and TSC2,





**Figure 2** Impacts of *NOTCH1* mutations on TMB and DNA damage repair-related gene signature. (**A** and **B**) The TMB of *NOTCH1*-mutant NSCLC was significantly higher than that of *NOTCH1*-WT tumors in the TCGA dataset (**A**) and Samstein et al cohort (**B**). (**C**–**I**) GESA plots showing signatures of PROCESSING OF DNA DOUBLE STRAND BREAK ENDS (**C**), DNA DOUBLE STRAND BREAK REPAIR (**D**), DNA DOUBLE STRAND BREAK RESPONSE (**E**), DNA REPAIR (**F**), DNA DAMAGE TELOMERE STRESS INDUCED SENESCENCE (**G**), G1/S DNA DAMAGE CHECKPOINTS (**H**), and G2/M DNA DAMAGE CHECKPOINTS (**I**). \* $p < 0.05$  and \*\*\* $p < 0.0001$ .

suppressor of mTOR, was lower in the mutant group, although statistical significance was not reached (Figure 3E and F). Our<sup>25</sup> and Lastwika's<sup>26</sup> previous studies demonstrated that AKT-mTOR signaling could promote the expression of PD-L1 and the infiltration of CD8<sup>+</sup> T cells in NSCLC, and Xie et al<sup>27</sup> found that hyperactivation of mTOR-S6 signaling could impair the DNA damage response via inhibiting RNF168. These findings hinted that *NOTCH1* mutations increased TMB and reprogrammed the TME probably via activating the PI<sub>3</sub>K-AKT-mTOR signaling.



**Figure 3** *NOTCH1* mutations were associated with inflamed TME. (A) *NOTCH1* mutations correlated with remarkably higher PD-L1 TPS in the FUSCC cohort. (B and C) *NOTCH1* mutations were associated with significantly higher CD8+ T cell by X-CELL (B) and by MCP-COUNTER (C). (D) The mRNA level of CD8A of *NOTCH1*-mutant NSCLC was significantly higher than that of *NOTCH1*-WT tumors in the TCGA dataset. (E) Schematic of PI3K-AKT-mTOR pathways that inhibit RNF168, result in suppression of DNA damage repair. (F) Analysis of protein levels by RPPA showed activation of AKT-mTOR signaling in *NOTCH1*-mutant NSCLC in the TCGA dataset. \* $p < 0.05$  and \*\* $p < 0.01$ .

## Discussion

The present study comprehensively analyzed three cohorts of NSCLC patients with different background, and identified *NOTCH1*-mutant NSCLC as a unique subtype with distinct clinicopathological characteristics and treatment response. Patients with *NOTCH1*-mutant NSCLC were more likely to be smokers and squamous cell carcinoma in histology. Moreover, *NOTCH1* mutations intrinsically increased tumor mutation load, promoted PD-L1 expression and facilitated T cell infiltration, and thus enhanced sensitivity to ICB therapy.

In the present study, we comprehensively analyzed the *NOTCH1* mutational status of three NSCLC cohorts with different treatment modalities and ethnic background, and revealed that the mutation rates ranged from 4% to 6.8%. Considering the relatively high prevalence of NSCLC worldwide, there would be a huge number of patients harboring *NOTCH1* mutations. Moreover, *NOTCH1* is a poor therapeutic target, and there has been no chemical inhibitors or antibodies which are proven to be clinically effective. Our analyses highlighted not only the distinct clinicopathological characteristics, but also the superior efficacy of ICB therapy of these patients.

From a biological perspective, GSEA showed prominent enrichment of signatures of DNA double-strand break response and repair, indicating the defects in repairing DNA damage of *NOTCH1*-mutant NSCLC. These defects resulted in significantly increased mutational burden and increased number of neoantigens presented on major histocompatibility

complex molecules that facilitate immune recognition as foreign, thus promote immunogenicity and render *NOTCH1*-mutant tumors susceptible to antitumor immunity.

Different from chemotherapy or molecular-targeted therapies which attack cancer cells via a single oncogenic variants or other autonomous features, immunotherapy enhances the antitumor response to eliminate cancer cells. Hence, tumor immune microenvironment is the basis of ICB therapies.<sup>28</sup> Previous studies have classified the TME as four categories according to the level of tumor infiltrating T cells and expression level of PD-L1. Of note, an inflamed TME, considered as a paradigm of adaptive resistance of tumor mediated by PD-1/PD-L1 pathway, is characterized as increased level of PD-L1 expression and T cells infiltration.<sup>29,30</sup> It has been demonstrated that an inflamed TME is associated with clinical benefits from immunotherapy.<sup>29,30</sup> The current study used a cohort of patients undergoing complete resection and TIMER 2.0 platform to reveal that *NOTCH1*-mutant NSCLC displayed an inflamed TME.

These findings have significant clinical and research implications. As the current study demonstrated *NOTCH1* mutational status as a predictor of the outcomes of NSCLC treated with ICB, more attention should be paid to the mutation status of *NOTCH1* in clinical practice and trials. Further studies should obtain the mutation status of *NOTCH1* if possible, and consider it as a potential confounding factor of survival.

The major limitation of the current study derives from the nature of retrospective design, which potentially introduce selection bias. Our study applied three NSCLC cohorts with large sample size, diagnosed with different stages, and from different ethnic backgrounds. Combinational analyses of the data from these three cohorts highlighted the distinct characteristics and treatment response of *NOTCH1*-mutant NSCLC. Additionally, other members of *NOTCH* family except *NOTCH1* were not included in the study, and we could not figure out whether mutations of other *NOTCH* genes had similar effects as *NOTCH1*. Lastly, the results mainly derived from analyses of clinicopathologic data of NSCLC patients. We would perform laboratory experiments in the future to further explore the mechanisms of *NOTCH* mutations.

In summary, the current study identifies *NOTCH1*-mutant NSCLC as a distinct clinical subtype, and reveals the potential interplay between *NOTCH1* mutations of tumor cells and tumor immune microenvironments. *NOTCH1* mutations intrinsically increase the mutational load of NSCLC cells, reprogrammed the immune microenvironment as an inflamed subtype, and thus lead to significant survival benefits among patients receiving ICB therapies.

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## Disclosure

Qingyuan Huang, Hang Cao, and Qianlan Yao are co-first authors in this study. The authors report no conflicts of interest in this work.

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