NOTCH1 Mutation and Survival Analysis of Tislelizumab in Advanced or Metastatic Esophageal Squamous Cell Carcinoma: A Biomarker Analysis From the Randomized, Phase III, RATIONALE-302 Trial

Zhihao Lu, MD[1]; Wenting Du, PhD[2]; Xi Jiao, MD[1]; Yanni Wang, PhD[1]; Jingwen Shi, PhD[3]; Yang Shi, PhD[3]; Yongqian Shu, MD[4]; Zuoxing Niu, MD[5]; Hiroki Hara, MD[6]; Jun Wu, MD[7]; Chih-Hung Hsu, MD[8]; Eric Van Cutsem, MD[9]; Malcolm V. Brock, MD[10]; Zhang Zhang, PhD[11]; Ningning Ding, PhD[12]; Yun Zhang, PhD[3]; Zhirong Shen, PhD[3]; and Lin Shen, MD[1,13]

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ABSTRACT

PURPOSE Although multiple agents targeting PD-1 have been approved as second-line treatment for esophageal squamous cell carcinoma (ESCC), only a fraction of patients derive long-term survival. Hence, reliable predictive biomarkers are urgently needed.

METHODS Comprehensive tumor genomic profiling and transcriptome sequencing were performed on samples from the RATIONALE-302 study. We also conducted single-cell RNA sequencing analysis on *Notch1* knockdown ESCC murine models to further explore the potential molecular mechanisms underlying anti–PD-1 benefit.

RESULTS We identified *NOTCH1* mutation as a potential predictive biomarker for longer overall survival (OS) with tislelizumab versus chemotherapy (18.4 months *v* 5.3 months; hazard ratio, 0.35 [95% CI, 0.17 to 0.71]). At the transcriptional level, type I IFN (IFN-I)/toll-like receptor expression signatures were positively associated with OS benefit of tislelizumab, whereas B-cell and neutrophil signatures predicted unfavorable OS. Exploratory analyses showed that the presence of *NOTCH1* mutation correlated with enrichment of IFN-I signatures and reduced infiltration of B cells and neutrophils. In murine models, comparative single-cell transcriptome analyses further revealed that *Notch1* deficiency facilitated a more immunologically activated tumor microenvironment which potentiated anti–PD-1 treatment.

CONCLUSION Our data provide novel insights for anti–PD-1 treatment selection using *NOTCH1* mutations and may provide a rationale for combination therapy in ESCC.

ACCOMPANYING CONTENT

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INTRODUCTION

Multiple agents targeting PD-1 have shown significant clinical benefits over chemotherapy in second-line esophageal squamous cell carcinoma (ESCC),[1-5] making immune checkpoint inhibitors (ICIs) the backbone of treatment in metastatic or advanced ESCC. Nevertheless, the survival benefit of ICIs may be heterogeneous, and only a fraction of patients with ESCC derive long-term survival from ICIs.[6-11] Therefore, there is an urgent need to identify reliable predictive biomarkers to guide patient selection, understand response variability and resistance mechanisms, and discover novel targets for ICIs in ESCC.

PD-L1 expression on tumor cells and/or immune cells may associate with better clinical benefit from ICIs[12]; however, its predictive value in ESCC is limited because of heterogeneous and dynamic expression patterns and patient benefit regardless of PD-L1 status.[1-5,13] Tumor mutational burden (TMB) status represents another predictive biomarker for ICI response, but its predictive role for overall survival (OS) in ESCC has been determined only using appropriate algorithms and cutoffs.[14-16] Other biomarkers (eg, T-cell receptor clonality, circulating cell-free DNA, human leukocyte antigen genotype, specific immune cell phenotype, and molecular classification on the basis of multiomics)[5,14,17] have been proposed, but most have not been prospectively

CONTEXT

Key Objective

Reliable predictive biomarkers of response to anti–PD-1 antibodies in esophageal squamous cell carcinoma (ESCC) are urgently needed. Next-generation sequencing analysis of samples from the RATIONALE-302 study was performed to identify genomic and transcriptomic biomarkers potentially linked to clinical outcomes with tislelizumab versus chemotherapy in ESCC.

Knowledge Generated

Patients with *NOTCH1* mutation had a greater survival benefit with tislelizumab treatment over chemotherapy, compared with patients with wild-type (WT) *NOTCH1* (mutation hazard ratio [HR], 0.35; WT HR, 0.81; interaction $P = .0372$). In both patient samples and murine models, the presence of tumor-intrinsic *NOTCH1* alterations facilitated a more immunologically activated tumor microenvironment.

Relevance *(A.H. Ko)*

NOTCH1 mutation appears to be a promising predictive biomarker for benefit from anti–PD-1 antibody therapy in esophageal squamous cell carcinoma. This patient population should ideally undergo next generation sequencing to help guide treatment decision-making.*

*Relevance section written by *JCO* Associate Editor Andrew H. Ko, MD, FASCO.

validated as predictive markers or are not clinically feasible to implement during routine care.

RATIONALE-302 study demonstrated improved OS and safety from tislelizumab versus investigator-chosen chemotherapy (ICC) as second-line treatment for advanced unresectable or metastatic ESCC.[4] This led to regulatory approvals in the United States, China, and the EU. We performed post hoc biomarker analyses using next-generation sequencing to uncover genomic and transcriptomic markers potentially linked to clinical outcomes with tislelizumab monotherapy versus ICC. We also explored the molecular mechanisms underlying ICI survival benefits and potential synergetic targets for ICI treatment.

METHODS

In the open-label, phase III RATIONALE-302 (ClinicalTrials.gov identifier: NCT03430843) study, 512 patients with advanced or metastatic ESCC, whose tumor progressed after first-line systemic treatment, were randomly assigned (1:1) to intravenous tislelizumab 200 mg once every 3 weeks or ICC (paclitaxel, docetaxel, or irinotecan; n = 256 for each arm). The primary end point was OS. The efficacy data cutoff was 1 December 2020. Biomarker analyses were performed on formalin-fixed, paraffin-embedded tumor tissues collected at screening. Protocol was approved by the relevant institutional review board/independent ethics committee for each site. The study was performed in accordance with the International Conference on Harmonisation Good Clinical Practice Guideline, the principles of the Declaration of Helsinki, and local laws and regulations. All patients provided written informed consent before participation.

RESULTS

Genomic Characteristics of the RATIONALE-302 ESCC Cohort

Baseline tumor samples were collected from RATIONALE-302 and were tested sequentially for PD-L1 immunohistochemistry (IHC), gene expression profiling (GEP), and mutation profiling (Fig 1A). Baseline characteristics and clinical efficacy in the biomarker-evaluable populations were generally comparable with those in the intent-to-treat population (Table 1). To identify novel biomarkers for predicting OS, a Cox proportional hazards model was adopted to screen genomic or transcriptomic features associated with the efficacy of tislelizumab and ICC (Fig 1B). Molecular mechanisms underlying ICI survival benefits were also explored in preclinical models (Fig 1B).

Most frequently altered genes detected in the RATIONALE-302 cohort were *TP53* (94%), *CCND1* (52%), *FGF3/4/19* (50%/50%/49%), *CDKN2A* (31%), *PIK3CA* (29%), *KMT2D* (28%), *NFE2L2* (26%), *CDKN2B* (22%), *NOTCH1* (22%), and *TP63* (22%; Fig 2A). These genes primarily fall into five gene ontology categories: cell cycle, cell differentiation, RTK/RAS/PI3K pathways, chromatin remodeling, and the nuclear factor erythroid 2–related factor 2 pathway, consistent with previous reports.[18] For each category, the association of each mutated gene (altered in ≥10% of patients) with OS in each treatment arm is shown in Figure 2B. Among these,

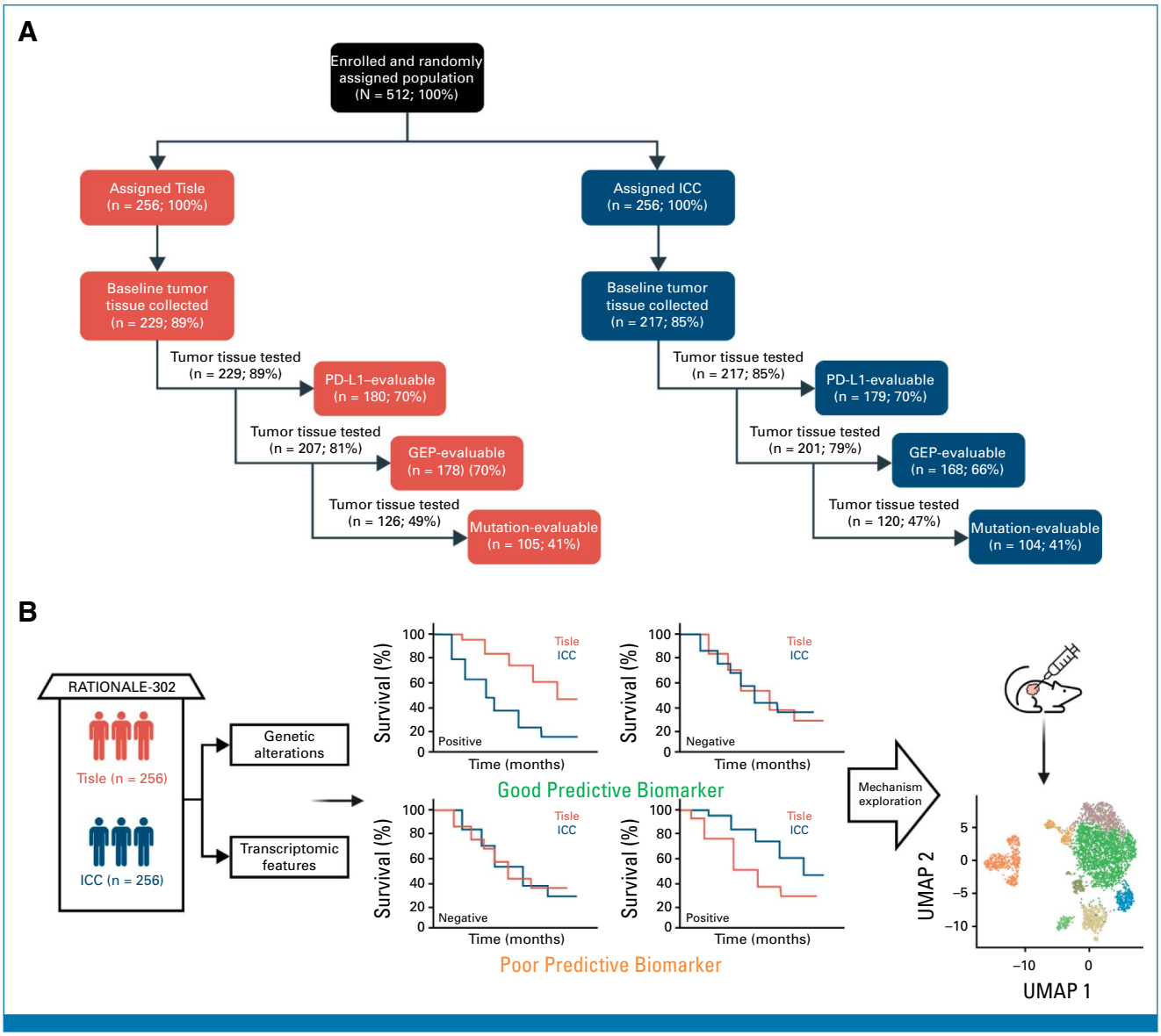

FIG 1. Study design. (A) Flowchart showing patient enrollment and sample collection workflow of the RATIONALE-302 study. Tumor tissue samples obtained from patients in the RATIONALE-302 study were tested sequentially for PD-L1 IHC, GEP, and mutation profiling. Consequently, many samples were exhausted after the first two assays, with only 48% of patients having sufficient tissue samples remaining for mutation profiling. (B) Schematic overview of the analysis workflow. GEP, gene expression profiling; ICC, investigator-chosen chemotherapy; IHC, immunohistochemistry; Tisle, tislelizumab; UMAP, uniform manifold approximation and projection.

alterations in *NOTCH1* and *KMT2D* were observed to be associated with prominent OS improvements for tislelizumab over ICC. However, a trend toward reduced OS benefit with tislelizumab versus chemotherapy was observed for *EGFR* alterations.

NOTCH1 Mutation Was Identified as a Predictive Biomarker for Improved Outcome With Tislelizumab Monotherapy Over ICC

NOTCH1 mutations (extended data given in Fig 1A) showed the strongest association with tislelizumab OS benefit (Fig 2B). Specifically, OS of patients with *NOTCH1* mutations was prolonged when treated with tislelizumab compared with ICC (median OS [mOS], 18.4 months *v* 5.3 months). This OS improvement was superior in patients with *NOTCH1* mutations compared with those with wild-type (WT) *NOTCH1*, with a hazard ratio (HR) of 0.35 (95% CI, 0.17 to 0.71) versus 0.81 (0.57 to 1.14; two-sided interaction $P = .0372$; Fig 2C). The objective response rate (ORR) of patients with WT *NOTCH1* (17%; 28 of 163) was similar for the tislelizumab (ORR, 18%; 15 of 84) and ICC (ORR, 16%; 13 of 79) groups. However, ORR was numerically higher after tislelizumab treatment (ORR, 33%; 7 of 21) compared with ICC (ORR, 8%; 2 of 25) in patients with *NOTCH1* mutations (extended data given in Fig 1B). Furthermore, progression-

TABLE 1. Overview of Baseline Characteristics and Clinical Efficacy in Different Study Populations

Baseline Characteristic and Clinical Efficacy	ITT (N = 512)		PD-L1 BEP (n = 359)		GEP BEP (n = 346)		Mutation BEP (n = 209)	
	Tislelizumab (n = 256)[a]	ICC (n = 256)[a]	Tislelizumab (n = 180)	ICC (n = 179)	Tislelizumab (n = 178)	ICC (n = 168)	Tislelizumab (n = 105)	ICC (n = 104)
Age, years, mean (SD)	61.45 (8.43)	61.56 (8.01)	61.16 (8.46)	62.13 (7.97)	61.19 (8.50)	62.08 (7.68)	60.94 (8.07)	62.34 (8.22)
Sex, male, No. (%)	217 (84.8)	215 (84.0)	149 (82.8)	150 (83.8)	144 (80.9)	139 (82.7)	88 (83.8)	89 (85.6)
ECOG PS 1, No. (%)	190 (74.2)	196 (76.6)	134 (74.4)	139 (77.7)	133 (74.7)	133 (79.2)	75 (71.4)	83 (79.8)
Region, No. (%)								
Asia excluding Japan	176 (68.8)	178 (69.5)	119 (66.1)	126 (70.4)	118 (66.3)	115 (68.5)	67 (63.8)	67 (64.4)
Japan	25 (9.8)	25 (9.8)	14 (7.8)	12 (6.7)	13 (7.3)	15 (8.9)	8 (7.6)	12 (11.5)
EU/United States	55 (21.5)	53 (20.7)	47 (26.1)	41 (22.9)	47 (26.4)	38 (22.6)	30 (28.6)	25 (24.0)
PD-L1 status, No. (% in total/% in PD-L1–evaluable)								
PD-L1 ≥10%	80 (31.2/44.4)	62 (24.2/33.7)	80 (44.4)	60 (33.5)	69 (38.8/44.8)	50 (29.8/33.3)	45 (42.9/48.9)	35 (33.7/37.6)
PD-L1 <10%	100 (39.1/55.6)	122 (47.7/66.3)	100 (55.6)	119 (66.5)	85 (47.8/55.2)	100 (59.5/66.7)	47 (44.8/51.1)	58 (55.8/62.4)
Missing	76 (29.7)	72 (28.1)	0	0	24 (13.5)	18 (10.7)	13 (12.4)	11 (10.6)
Previous therapies, No. (%)								
Surgery	94 (36.7)	99 (38.7)	71 (39.4)	77 (43.0)	67 (37.6)	70 (41.7)	55 (52.4)	51 (49.0)
Radiotherapy	169 (66.0)	163 (63.7)	116 (64.4)	113 (63.1)	118 (66.3)	103 (61.3)	67 (63.8)	66 (63.5)
Platinum therapy	249 (97.3)	252 (98.4)	177 (98.3)	177 (98.9)	175 (98.3)	166 (98.8)	104 (99.0)	103 (99.0)
Former or current smoker	188 (73.4)	192 (75.0)	136 (75.6)	132 (73.7)	131 (73.6)	125 (74.4)	81 (77.1)	75 (72.1)
ORR, % (95% CI)[b]	20.3 (15.6 to 25.8)	9.8 (6.4 to 14.1)	20.6 (14.9 to 27.2)	10.1 (6.1 to 15.4)	19.1 (13.6 to 25.7)	11.9 (7.4 to 17.8)	21.0 (13.6 to 30.0)	14.4 (8.3 to 22.7)
mPFS, months (95% CI)[c]	1.64 (1.45 to 2.66)	2.07 (1.51 to 2.66)	1.84 (1.45 to 2.69)	2.04 (1.48 to 2.69)	1.64 (1.45 to 2.66)	1.54 (1.45 to 2.60)	1.54 (1.41 to 2.73)	2.60 (1.48 to 2.79)
PFS, HR (95% CI)[d]	0.83 (0.68 to 1.01)		0.81 (0.64 to 1.03)		0.79 (0.62 to 1.01)		0.89 (0.65 to 1.21)	
mOS, months (95% CI)[c]	8.61 (7.49 to 10.41)	6.28 (5.29 to 7.03)	8.67 (6.97 to 10.41)	5.82 (4.86 to 6.93)	8.57 (6.90 to 10.28)	5.59 (4.86 to 6.93)	7.95 (5.16 to 11.56)	6.78 (4.60 to 7.62)
OS, HR (95% CI)[d]	0.69 (0.57 to 0.84)		0.70 (0.56 to 0.89)		0.68 (0.54 to 0.86)		0.69 (0.51 to 0.93)	

Abbreviations: BEP, biomarker-evaluable population; ECOG PS, Eastern Cooperative Oncology Group performance status; GEP, gene expression profiling; HR, hazard ratio; ICC, investigator-chosen chemotherapy; ITT, intent-to-treat; mOS, median OS; mPFS, median PFS; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; SD, standard deviation.

[a]Planned treatment.

[b]The 95% CI was estimated using the Clopper-Pearson method.

[c]Medians were estimated using the Kaplan-Meier method with 95% CIs estimated using the Brookmeyer and Crowley method with log-log transformation.

[d]HR and 95% CIs were estimated using a Cox regression model.

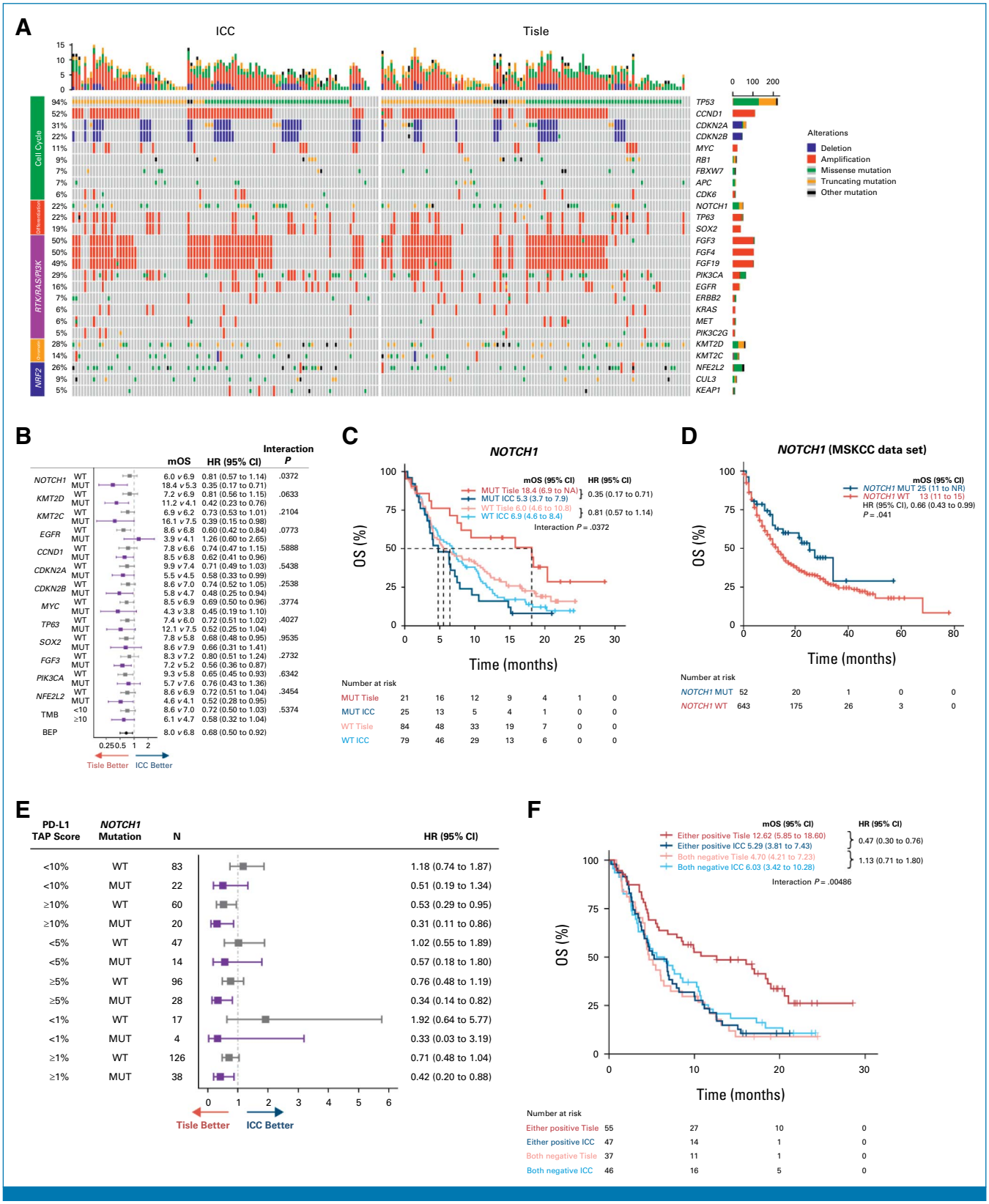

FIG 2. Genomic alterations associated with patient outcomes for Tisle. (A) Oncoplot depicting genomic alterations in 209 patients with ESCC in the RATIONALE-302 cohort. (B) Forest plot depicting OS improvements for Tisle versus ICC in patients with and without mutations altered in >10% of patients and patients with TMB ≥10 mut/Mb or TMB <10 mut/Mb. (C) Kaplan-Meier curve depicting OS in patients with ESCC treated with Tisle or ICC stratified by the status of *NOTCH1* (mutated *v* WT). (D) Kaplan-Meier curve depicting OS in patients with mixed solid tumors treated with anti–PD-(L)1 monotherapy stratified by the status of *NOTCH1* mutation from the MSKCC data set. Patients with annotated GOF *NOTCH1* mutations and indications where *NOTCH1* was reported mostly as the oncogenic (continued on following page)

FIG 2. (Continued). activator or GOF mutations were excluded from the analysis. (E) Forest plot depicting OS in patients with ESCC treated with Tisle or ICC stratified by the combination of PD-L1 TAP score (cutoffs 1%, 5%, and 10%) and *NOTCH1* mutation status. (F) Kaplan-Meier curves depicting OS in patients with ESCC treated with Tisle or ICC stratified by the combination of PD-L1 status and *NOTCH1* mutation. Either positive: *NOTCH1* mutant or PD-L1 TAP ≥10%; both negative: *NOTCH1* WT and PD-L1 TAP<10%. ESCC, esophageal squamous cell carcinoma; GOF, gain-of-function; HR, hazard ratio; ICC, investigator-chosen chemotherapy; mOS, median OS; MSKCC, Memorial Sloan Kettering Cancer Center; MUT, mutation; NA, not applicable; NR, not reached; OS, overall survival; TAP, tumor area positivity; Tisle, tislelizumab; TMB, tumor mutational burden; WT, wild-type.

free survival showed a trend of improvement for tislelizumab versus ICC in patients with *NOTCH1* mutations (HR, 0.71 [95% CI, 0.37 to 1.37]), but not WT *NOTCH1* (HR, 0.94 [95% CI, 0.66 to 1.34]; extended data given in Fig 1C). These results suggest that mutations in the *NOTCH1* gene may be a novel predictive biomarker of tislelizumab efficacy.

On the basis of available OncoKB annotations for 36% of samples and previous reports in ESCC, mutations in the *NOTCH1* gene were highly indicative of loss-of-function mutations.[19-22] We observed that the presence of both annotated and unannotated *NOTCH1* mutations correlated with reduced expression of *NOTCH1* and its downstream targets *HEY2* and *HEYL* (extended data given in Figs 1D and 1E). This indicated that these mutations were associated with functional deficiency of NOTCH1. In addition, OS was improved in *NOTCH1*-mutant patients with tislelizumab versus ICC for both annotated and unannotated mutations (HR, 0.21 [95% CI, 0.05 to 1.00] *v* 0.31 [95% CI, 0.12 to 0.78]; extended data given in Figs 1F and 1G).

To determine whether *NOTCH1* mutations could serve as a tumor-agnostic predictive biomarker, association of *NOTCH1* mutations with OS was analyzed in an independent cohort from Memorial Sloan Kettering Cancer Center database. Patients in this cohort received anti–PD-1/PD-L1 treatment. Patients with annotated gain-of-function (GOF) *NOTCH1* mutations and indications where *NOTCH1* was reported mostly as the oncogenic activator or GOF mutations were excluded from analysis.[23,24] Consistently, patients with *NOTCH1* mutations exhibited better OS than those without (mOS, 25 months [95% CI, 11 to not reached] *v* 13 months [95% CI, 11 to 15]; HR, 0.66 [95% CI, 0.43 to 0.99]; two-sided P = .041; Fig 2D). However, in a cohort from The Cancer Genome Atlas (TCGA) with the same indications but without ICI treatment, *NOTCH1* mutations were not identified as prognostic for OS (mOS, *NOTCH1* mutations: 54 months [95% CI, 44 to 75] *v* WT: 63 months [95% CI, 57 to 67]; extended data given in Fig 1H).

The Predictive Value of *NOTCH1* Mutation Was Not Dependent on TMB or PD-L1

Using the generally accepted cutoff of 10 mutations per megabase (mut/Mb),[25] a numerically greater OS benefit with tislelizumab over ICC was observed in the high TMB subgroup versus the low subgroup, but not statistically significant (two-sided interaction P = .5374; Fig 2B). We observed that patients with *NOTCH1* mutations displayed higher TMB (P = .0005; extended data given in Fig 2A). This relationship led us to investigate whether the predictive role of *NOTCH1* mutations depends on TMB status. Interestingly, patients with *NOTCH1* mutations had improved OS when treated with tislelizumab versus ICC regardless of TMB (*NOTCH1* mutation, TMB-high: HR, 0.34 [95% CI, 0.12 to 0.98]; *NOTCH1* mutation, TMB-low: HR, 0.38 [95% CI, 0.14 to 1.02]; extended data given in Fig 2B).

In patients with both PD-L1 tumor area positivity (TAP) score and mutation results (n = 185) from the RATIONALE-302 study, those with high PD-L1 expression benefited more from tislelizumab than ICC compared with patients with low PD-L1 expression, especially at the 10% cutoff (two-sided interaction P = .0298; extended data given in Fig 2C). We compared PD-L1 expression between *NOTCH1* mutation and WT groups and found no difference (extended data given in Fig 2D). According to PD-L1 stratification analysis, we observed that in the PD-L1-high subgroup (TAP score ≥10%), patients with *NOTCH1* mutations showed increased OS benefit for tislelizumab versus ICC (HR, 0.31 [95% CI, 0.11 to 0.86]; Fig 2E). Even in patients with low PD-L1 (TAP score <10%), those with *NOTCH1* mutations showed a trend toward longer OS from tislelizumab versus ICC (HR, 0.51 [95% CI, 0.19 to 1.34]; Fig 2E). These results suggest that *NOTCH1* mutation may be an independent predictive marker of efficacy for tislelizumab.

We further investigated whether combining PD-L1 expression and *NOTCH1* mutation status could serve as a better predictive biomarker. Patients with either *NOTCH1* mutation or PD-L1 TAP ≥10% experienced greater survival benefit from tislelizumab over ICC compared with patients who were negative in both biomarkers (either positive HR, 0.47 [95% CI, 0.30 to 0.76]; both negative HR, 1.13 [95% CI, 0.71 to 1.80]; two-sided interaction P = .00486; Fig 2F). These findings suggest that combining *NOTCH1* mutation status and PD-L1 may better identify the population that is likely to benefit from tislelizumab, potentially expanding the clinical benefit to patients with low or negative PD-L1 expression.

Transcriptomic Markers Associated With Clinical Outcomes

Features of tumor microenvironment (TME) have been associated with efficacy of ICIs and are usually defined by published GEP scores, such as IFN-γ and T effector

signatures.[26,27] To assess whether a given TME feature was associated with the therapeutic effects of tislelizumab, tumor-immune relevant functional gene signatures were analyzed and the results are shown in Figure 3A. Our analysis showed potential predictive value for two novel signatures: the type I IFN (IFN-I) signature and the toll-like receptor (TLR) signature. Higher levels of the two signatures showed strong associations with longer OS from tislelizumab versus ICC (IFN-I: HR, 0.55 [95% CI, 0.39 to 0.78]; TLR: HR, 0.53 [95% CI, 0.38 to 0.76]; Fig 3B; extended data given in Fig 3A). This OS improvement was more pronounced in patients with higher versus lower levels of the TLR or IFN-I signatures. The genes *IFI27* and *IFI6* appeared to be the main drivers of the predictive associations in these two signatures (extended data given in Fig 3B). Interestingly, several immune cell signatures, such as those for B cells, neutrophils, Tregs, and immature dendritic cells (two-sided interaction P = .0002, .0853, .0656, and .0292, respectively), were negatively associated with OS in the tislelizumab arm and positively or not associated with OS in the ICC arm (Figs 3C and 3D; extended data given in Figs 3C and 3D), giving them poor predictive potential. This was further confirmed by the respective marker genes (extended data given in Fig 3E).

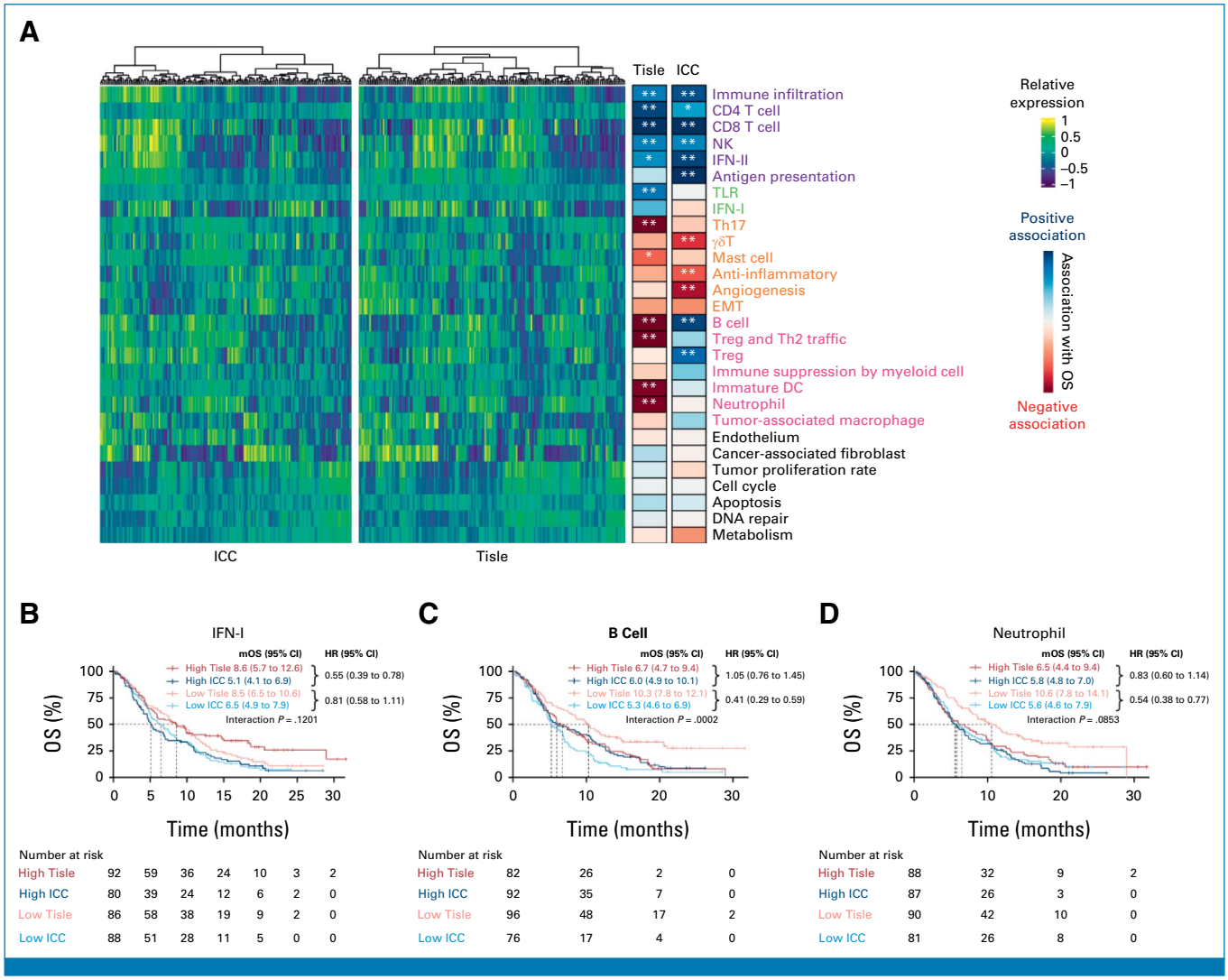

FIG 3. Relationship between key transcriptomic markers and clinical outcomes for Tisle. (A) Heatmaps showing the expression level of representative signatures in 346 patients with ESCC in the RATIONALE-302 cohort and the association of indicated signatures with OS in each treatment arm. Color in the OS association heatmap indicates the hazard risk between high versus low within each arm, which was calculated as −log10 (P value) × (HR − 1)/|HR − 1|. Red indicates negative association; blue indicates positive association. The color of the signature name indicates the prognostic/predictive role: purple, good prognostic; green, good predictive; orange, poor prognostic; magenta, poor predictive; black, no association. (B-D) Kaplan-Meier curves depicting OS in patients with ESCC treated with Tisle or ICC stratified by the median value of indicated signatures: IFN-I, B cell, and neutrophil. *P < .1; **P < .05. DC, dendritic cell; EMT, epithelial to mesenchymal transition; ESCC, esophageal squamous cell carcinoma; HR, hazard ratio; ICC, investigator-chosen chemotherapy; IFN-I, type I IFN; mOS, median OS; NK, natural killer; OS, overall survival; Tisle, tislelizumab; TLR, toll-like receptor.

NOTCH1 Mutations Correlated With an IFN-I Enriched, Less Immunosuppressive TME in ESCC

To better understand how the presence of *NOTCH1* mutations may associate with better outcomes from tislelizumab versus ICC, we compared differentially expressed genes between tumor tissues expressing WT *NOTCH1* and mutated *NOTCH1*. Expression of genes associated with B cells, neutrophils, M2 macrophages, and endothelial cells was upregulated in WT tumors, whereas IFN-I-related genes were upregulated in tumors with *NOTCH1* mutations (Fig 4A). Top signatures enriched in tissues from the *NOTCH1*-mutant subgroup included the IFN-I signature and interferon-stimulated gene signature, whereas M2 macrophage, B-cell, and neutrophil signatures were among the top signatures enriched in the *NOTCH1* WT subgroup (Fig 4B). Downregulation of B cells and neutrophils in tumors with *NOTCH1* mutations compared with WT was also confirmed by multiplex IHC, showing decreased infiltration of these cell types in an independent ESCC cohort (Fig 4C).

To further validate the *NOTCH1* mutation–mediated mechanisms in ESCC and indications with similar histologic characteristics, differentiated gene signatures in squamous cancer types from TCGA database were analyzed. Similarly, gene set enrichment analysis revealed that IFN-I signature was enriched in tumors with *NOTCH1* mutations, whereas B-cell signature was enriched in tumors without *NOTCH1* mutations (Fig 4D). These distinctive gene expression profiles observed in tumors with *NOTCH1* mutations align with the identified predictive signatures (Fig 3A), suggesting that the favorable clinical outcomes observed in patients with *NOTCH1* mutations receiving tislelizumab may be attributed to a modified TME.

Notch1 Knockdown Sensitized Murine ESCC Tumors to Anti–PD-1 Treatment by Altering the Immune Infiltration

The observation that *NOTCH1* mutations correlated with reduced *NOTCH1* expression in the RATIONALE-302 cohort (extended data given in Fig 1D) suggests that *NOTCH1* mutations in ESCC may lead to defects in NOTCH1 signaling.[28] We used short hairpin RNAs to generate the *Notch1* knockdown (sh-*Notch1*) murine ESCC cell lines (extended data given in Fig 4A) and examined whether *NOTCH1* inhibition could affect tumor growth and sensitize anti–PD-1 treatment. As expected, tumor cells with *Notch1* knockdown showed increased sensitivity to anti–PD-1 therapies ($P < .0001$; Fig 5A; $P = .0002$; extended data given in Fig 4B), whereas no differences in tumor growth were observed between anti–PD-1 therapies or vehicle in tumors expressing sh-Ctrl.

We next performed scRNA-seq to assess TME alterations and associated molecular mechanisms after *Notch1* knockdown. Unbiased clustering and visualization using the uniform manifold approximation and projection algorithm revealed 16 cell clusters on the basis of expression of known lineage markers (extended data given in Figs 4C and 4D). scRNA-seq analysis revealed a higher proportion of cytotoxic ($CD8^+$) T cells and a lower proportion of neutrophils in *Notch1* knockdown tumors (sh-*Notch1*) compared with control (sh-Ctrl) tumors (Fig 5B). Notably, gene set enrichment analysis revealed that $CD8^+$ T cells from the sh-*Notch1* group enriched the MHC class I protein binding, antigen binding, and interleukin 2–mediated signaling pathway (Fig 5C). Among myeloid cells, subclusters $Ly6i^+$ Mac/Mono and $Plac8^+$ Mac/Mono, which expressed the M1 signature, were upregulated after *Notch1* knockdown. Conversely, the subcluster $Arg1^+$ Mac, which expressed the M2 macrophage signature, was downregulated (Fig 5B; extended data given in Fig 4E). Moreover, tumor-associated macrophages from sh-*Notch1* mice displayed upregulated pathways associated with Ifn-γ response, Tnfα signaling via Nfκb, Ifn-α response, and antigen processing and presentation (Fig 5D). Comparison of differentially expressed genes revealed that monocyte/macrophages from the sh-*Notch1* group expressed higher levels of genes associated with antigen presentation (*H2-Ab1, H2-Aa, H2-Eb1*) and T-cell activation (*Cxcl9/10, Cd86, Cd40, Irf1*), but lower levels of genes associated with immunosuppressive function (*Spp1, Ctsa, Fn1*; Fig 5E). These shifts in immune cells in our experimental models are consistent with observations derived from the aforementioned analysis in clinical samples.

DISCUSSION

In this study, we performed an integrative analysis of genomic and transcriptional data from patients with ESCC from the RATIONALE-302 study. Through comprehensive evaluation, *NOTCH1* mutation was identified as a novel biomarker for predicting ICI outcomes in ESCC. Our data suggest that ESCCs with inactivating *NOTCH1* mutations harbor immune-activated microenvironments with higher IFN-I gene signatures and lower immune-suppressive cell infiltration. Our findings provide conceptual evidence that *NOTCH1* mutation or knockdown reshaped the TME, suggesting that targeting the NOTCH pathway may be a relevant immunotherapeutic strategy for ESCC.

To the best of our knowledge, this is the first analysis of genome- and transcriptome-wide biomarkers for ICIs in ESCC from samples derived from a large, randomized Phase III study. Among genomic biomarkers, *NOTCH1* mutations ranked as having the highest predictive value, displaying the most significant interaction *P* value for all mutations altered in ≥10% of patients. At present, most often-used biomarkers to select for ICI treatment in patients with ESCC are PD-L1 expression and TMB. However, challenges in tissue sample acquisition, preservation, and preparation may limit predictive values, as shown in our study and other ESCC studies. Inability of TMB to predict ICI efficacy in ESCC suggests that it fails to consider the contribution of immunogenic mutations and does not reflect the impact of certain specific mutations. Notably, the mOS of 18.4 months in the *NOTCH1*-

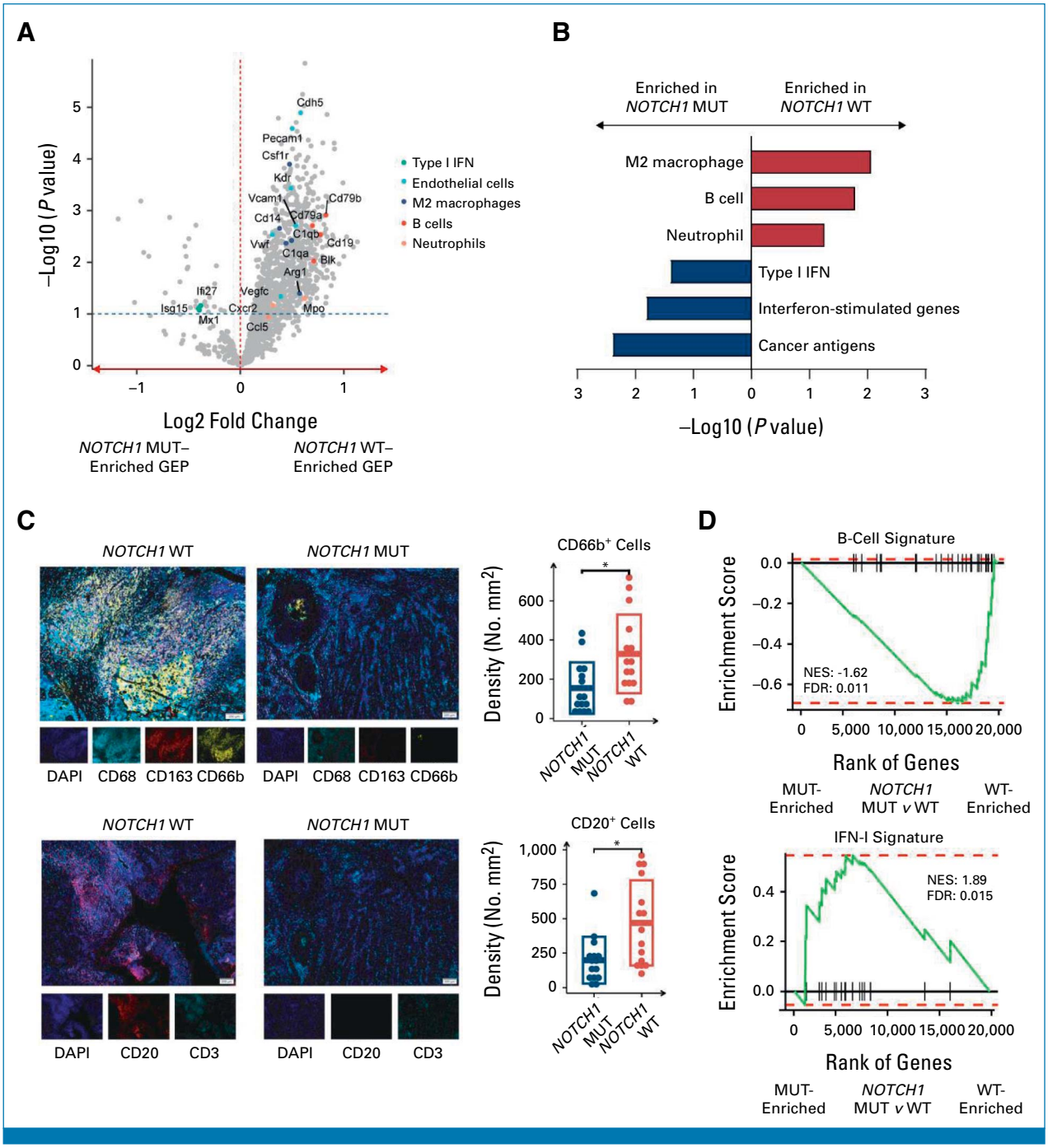

FIG 4. TME features of *NOTCH1* mutation in ESCC. (A) Volcano plot showing differentially expressed genes between *NOTCH1* WT and *NOTCH1*-mutant subgroups. (B) Depiction of top signatures significantly enriched in *NOTCH1* WT versus *NOTCH1*-mutant subgroups. (C) Representative images and quantification of CD66b+ neutrophils and CD20+ B cells in *NOTCH1* WT and *NOTCH1*-mutant tumor tissues from an independent ESCC cohort. (D) Gene set enrichment analysis results of *NOTCH1*-mutant versus WT from the TCGA-SC cohort (ESCC and head and neck squamous cell carcinoma). *$P < .01$ by *t*-test. ESCC, esophageal squamous cell carcinoma; FDR, false discovery rate; GEP, gene expression profiling; MUT, mutation; NES, normalized enrichment score; SC, squamous cancer; TCGA, The Cancer Genome Atlas; TME, tumor microenvironment; WT, wild-type.

mutated subgroup is clinically remarkable for second-line ESCC, more than doubling that of the overall population (8.6 months) and surpassing the mOS in the subgroups with PD-L1 TAP ≥10% (10.3 months)[4] and TMB ≥10 (6.1 months). Furthermore, patients with *NOTCH1* mutations in the PD-L1–negative or TMB-low subgroup still had a trend toward greater benefit from tislelizumab over ICC (Fig 2E; extended data given in Fig 2B). This suggests that the presence of *NOTCH1* mutations may be an additional criterion to optimize treatment selection by expanding the candidate

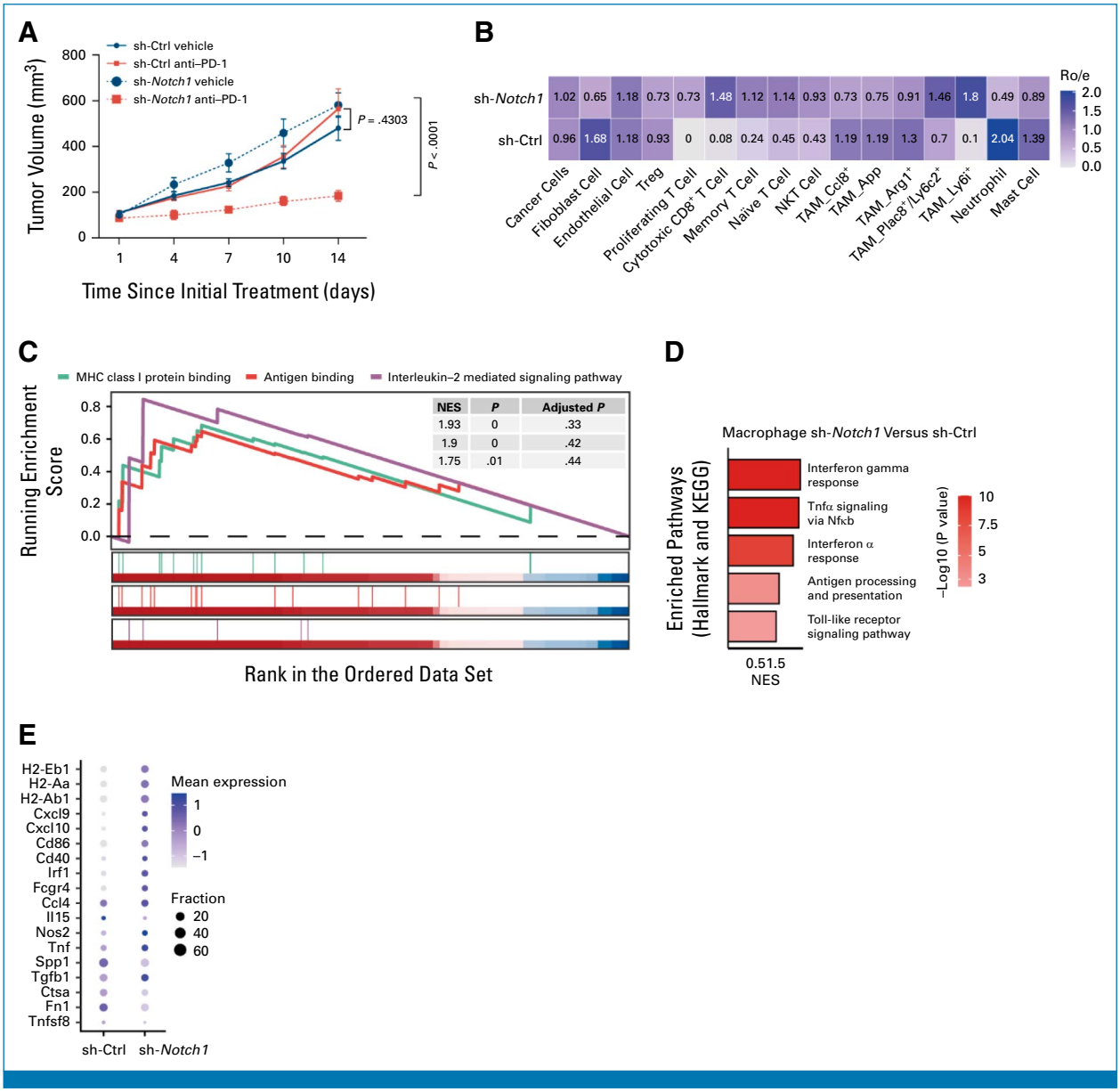

FIG 5. *Notch1* knockdown sensitized murine ESCC tumors to anti–PD-1 by reprogramming the TME. (A) HNM007 tumor volume over time after initial treatment. Treatment of HNM007 tumors was performed with anti–PD-1 therapy (BioXCell, Cat number BE0146) or saline (control). Tumor volume was monitored for ≥10 days after treatment in tumors transfected with vehicle expressing sh-Ctrl and sh-*Notch1* (n = 6 mice/group, data shown as mean ± SEM). One-way analysis of variance with multiple comparisons was used for statistical analysis. (B) Proportion of different clusters in ESCC subcutaneous tumors from WT *Notch1* or *Notch1* knockdown mice. (C) GSEA analysis of enriched pathways in CD8 T cells from tumors with WT *Notch1* or *Notch1* knockdown. (D) GSEA analysis of enriched pathways in macrophages from tumors with WT *Notch1* or *Notch1* knockdown. (E) Dot plot showing differentially expressed genes in macrophages from tumors with WT *Notch1* or *Notch1* knockdown. ESCC, esophageal squamous cell carcinoma; GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; MHC, major histocompatibility complex; NES, normalized enrichment score; Ro/e, ratio of observed to expected cell numbers; SEM, standard error of the mean; WT, wild-type.

patient population relative to PD-L1 or TMB alone. In addition, *NOTCH1* mutation identification is a straightforward process that can be achieved with different technology platforms. Therefore, the detection of *NOTCH1* mutations may be relatively easier to implement in the clinic.

Considering the predictive value of *NOTCH1* mutation, our integrative study further provides mechanistic insights into the microenvironment features of *NOTCH1* mutations. This analysis revealed a significant upregulation of the IFN-I signature in tumors with *NOTCH1* mutations. Potential mechanisms underlying IFN-I activation may be related to *NOTCH1* mutation–induced DNA damage response activity and immunogenicity, as reported in lung cancer.[29-32] This finding points to the importance of NOTCH signaling in regulating the TME and was further validated by

observation of the immune infiltration alterations (decrease in B cells and neutrophils) in tumors with *NOTCH1* mutations. Interestingly, the IFN-I signature was identified as a positive predictive biomarker, whereas B-cell and neutrophil signatures correlated with poor prognosis with ICI treatment. Several studies have revealed the heterogeneity of B cells and neutrophils, and some subclusters may acquire an immunoregulatory function of dampening antitumor T-cell responses and fostering an immunosuppressive microenvironment.[33-36] Moreover, Chen et al found that the cross talk between neutrophils and B cells promoted formation of an immunosuppressive microenvironment,[37] which may provide theoretical explanation for why these signatures correlated with less benefit from ICIs. Collectively, this suggests an important role of *NOTCH1* mutation in generating a unique, less immunosuppressive TME in ESCC, partially contributing to the favorable clinical outcomes with ICIs.

In line with the findings in human tumor samples, the *Notch1* knockdown ESCC model successfully replicated a more activated and less immunosuppressive TME. Furthermore, improved immune activation produced synergistic effects with immunotherapy in *Notch1*-deficient tumors, strengthening the rationale for combining ICIs with agents targeting the NOTCH1 pathway.

This study has several limitations that need to be acknowledged. First, our integrative analysis showed a trend of survival benefit in *NOTCH1*-mutant patients regardless of PD-L1 or TMB status. Because of limited sample size, these findings from retrospective analysis of RATIONALE-302 need validation in additional data sets. Second, because of lack of mutation hotspots, clonality classification, and annotations for the majority of samples, further efforts are required to determine the TME regulatory function and predictive role of individual mutations. Finally, the association between *NOTCH1* mutations and improved clinical outcomes requires prospective validation in a randomized, controlled trial. We plan to conduct a prospective clinical trial to assess whether ICI monotherapy is sufficient for patients with ESCC with *NOTCH1* mutations. We expect ongoing translational research to provide clinically significant insights to support biomarker-guided immuno-oncology treatment of ESCC.

AFFILIATIONS

[1]Department of Gastrointestinal Oncology, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Peking University Cancer Hospital & Institute, Beijing, China
[2]Clinical Biomarker, BeiGene (Shanghai) Co, Ltd, Shanghai, China
[3]Clinical Biomarker, BeiGene (Beijing) Co, Ltd, Beijing, China
[4]The First Affiliated Hospital of Nanjing Medical University (Jiangsu Province Hospital)—Cancer Center, Nanjing, China
[5]Shandong Cancer Hospital—Oncology, Jinan, China
[6]Saitama Cancer Center—Gastroenterology, Kitaadachi-gun, Japan
[7]The First People's Hospital of Changzhou—Oncology, Changzhou, China
[8]National Taiwan University Hospital, Taipei, Republic of China
[9]University Hospitals Gasthuisberg/Leuven & KU Leuven, Leuven, Belgium
[10]Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD
[11]Statistics, BeiGene (Beijing) Co, Ltd, Beijing, China
[12]Clinical Development, BeiGene (Beijing) Co, Ltd, Beijing, China
[13]Department of Gastrointestinal Oncology, State Key Laboratory of Holistic Integrative Management of Gastrointestinal Cancers, Beijing Key Laboratory of Carcinogenesis and Translational Research, Peking University Cancer Hospital & Institute, Beijing, China

CORRESPONDING AUTHOR

Zhihao Lu, MD; e-mail: zhihaolupku@bjmu.edu.cn.

EQUAL CONTRIBUTION

Z.L., W.D., X.J., and Y.W. contributed equally to this work.

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AUTHOR CONTRIBUTIONS

Conception and design: Zhihao Lu, Wenting Du, Xi Jiao, Eric Van Cutsem, Ningning Ding, Lin Shen
Administrative support: Zhirong Shen, Lin Shen
Provision of study materials or patients: Yongqian Shu, Zuoxing Niu, Hiroki Hara, Jun Wu, Chih-Hung Hsu, Eric Van Cutsem, Malcolm V. Brock, Lin Shen
Collection and assembly of data: Zhihao Lu, Wenting Du, Xi Jiao, Yanni Wang, Yongqian Shu, Zuoxing Niu, Hiroki Hara, Jun Wu, Chih-Hung Hsu, Eric Van Cutsem, Malcolm V. Brock, Lin Shen
Data analysis and interpretation: Zhihao Lu, Wenting Du, Xi Jiao, Jingwen Shi, Yang Shi, Chih-Hung Hsu, Eric Van Cutsem, Zhang Zhang, Ningning Ding, Yun Zhang, Zhirong Shen, Lin Shen
Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

***NOTCH1* Mutation and Survival Analysis of Tislelizumab in Advanced or Metastatic Esophageal Squamous Cell Carcinoma: A Biomarker Analysis From the Randomized, Phase III, RATIONALE-302 Trial**

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Wenting Du
Employment: BeiGene
Stock and Other Ownership Interests: BeiGene

Jingwen Shi
Employment: BeiGene
Stock and Other Ownership Interests: BeiGene

Yang Shi
Employment: BeiGene
Stock and Other Ownership Interests: BeiGene

Hiroki Hara
Honoraria: Chugai Pharma, Taiho Pharmaceutical, Merck Serono, Yakult Honsha, Lilly, Ono Pharmaceutical, Takeda, Bristol Myers Squibb, MSD, Daiichi Sankyo/UCB Japan, Miyarisan pharmaceutical, Nippon Kayaku, Astellas Pharma
Consulting or Advisory Role: Boehringer Ingelheim, Chugai Pharma, Daiichi Sankyo/UCB Japan
Research Funding: AstraZeneca (Inst), Merck Serono (Inst), MSD (Inst), Ono Pharmaceutical (Inst), Taiho Pharmaceutical (Inst), Daiichi Sankyo (Inst), Astellas Pharma (Inst), Bayer (Inst), Amgen (Inst), Chugai Pharma (Inst), Janssen Oncology (Inst), ALX Oncology (Inst), Bristol Myers Squibb Japan (Inst), Jazz Pharmaceuticals (Inst), AbbVie (Inst), Henlius (Inst), Innovent Biologics (Inst), Toray Industries (Inst)

Chih-Hung Hsu
Honoraria: Bristol Myers Squibb, Ono Pharmaceutical, Merck Sharp & Dohme, Roche, Eisai, Daiichi Sankyo
Consulting or Advisory Role: Ono Pharmaceutical, Bristol Myers Squibb, Roche/Genentech, AstraZeneca, Daiichi Sankyo, BeiGene
Research Funding: Ono Pharmaceutical (Inst), AstraZeneca (Inst), MSD (Inst), Taiho Pharmaceutical (Inst), Bristol Myers Squibb (Inst), BeiGene (Inst), Johnson & Johnson (Inst), Roche/Genentech (Inst), NGM Biopharmaceuticals (Inst), Eucure Biopharma (Inst), Surface Oncology (Inst), Ipsen (Inst), Gilead Sciences (Inst), Pfizer (Inst)
Travel, Accommodations, Expenses: Daiichi Sankyo, Roche

Eric Van Cutsem
Consulting or Advisory Role: Bayer, Lilly, Servier, Bristol Myers Squibb, Merck Sharp & Dohme, Merck KGaA, Novartis, AstraZeneca, Daiichi Sankyo, Pierre Fabre, Taiho Pharmaceutical, Astellas Pharma, GlaxoSmithKline, Nordic Group, Pfizer, Takeda, ALX Oncology, AbbVie, BeiGene, Boehringer Ingelheim, Mirati Therapeutics, Seagen, Ipsen, Agenus, Amgen, Arcus Biosciences, BioNTech SE, Debiopharm Group, ElmediX, Eisai, Simcere, Bexon Clinical Consulting, Cantargia AB, Fosum, Galapagos NV, ITeos Therapeutics, Microbial Machines, Novocure, Sanofi, Trishula Therapeutics

Malcolm V. Brock
Stock and Other Ownership Interests: Johnson & Johnson/Janssen (I)

Zhang Zhang
Employment: BeiGene
Stock and Other Ownership Interests: BeiGene

Ningning Ding
Employment: BeiGene
Stock and Other Ownership Interests: BeiGene

Yun Zhang
Employment: BeiGene
Stock and Other Ownership Interests: BeiGene
Patents, Royalties, Other Intellectual Property: I have patents when working at BeiGene

Zhirong Shen
Employment: BeiGene
Stock and Other Ownership Interests: BeiGene
Patents, Royalties, Other Intellectual Property: I have patents when working in BeiGene

Lin Shen
Consulting or Advisory Role: MSD, AstraZeneca, Boehringer Ingelheim, Servier, Transcenta Holding Limited
Research Funding: BeiGene (Inst)

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