

## Immunogenomics Analysis Reveals that *TP53* Mutations Inhibit Tumor Immunity in Gastric Cancer<sup>1</sup>



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### Abstract

Although immunotherapy continues to demonstrate efficacy in a variety of refractory cancers, currently, no any immunotherapeutic strategy is clinically used for gastric cancer (GC) except its microsatellite instable subtype. Thus, it is important to identify molecular biomarkers for predicting the responders to GC immunotherapy. *TP53* mutations frequently occur in GC and are associated with unfavorable clinical outcomes in GC. We performed a comprehensive characterization of the associations between *TP53* mutations and immune activities in GC based on two large-scale GC cancer genomics data. We compared expression and enrichment levels of 787 immune-related genes and 23 immune gene-sets among *TP53*-mutated GCs, *TP53*-wildtype GCs, and normal tissue, and explored the correlations between p53-mediated pathways and immune activities in GC. Strikingly, almost all analyzed immune gene-sets were significantly downregulated in enrichment levels in *TP53*-mutated GCs compared to *TP53*-wildtype GCs. These less active immune pathways and cell types in *TP53*-mutated GCs included 15 immune cell types and function, tumor-infiltrating lymphocytes, regulatory T cells, immune checkpoint, cytokine and cytokine receptor, human leukocyte antigen, pro-inflammatory, and parainflammation. Moreover, we identified a number of p53-mediated pathways and proteins that were significantly associated with immune activities in GC. Furthermore, we demonstrated that the *TP53* mutation itself could result in the depressed immune activities in GC and other cancer types. We revealed that chromosomal instability was an important mechanism for the depressed tumor immunity in *TP53*-mutated cancers. Finally, we showed that immune cell infiltration and immune activities were likely positively associated with survival prognosis in GC. Our findings suggest that p53 may play an important role in activating tumor immunity in GC and other cancer types and that the *TP53* mutation status could be useful in stratifying cancer patients responsive to a certain immunotherapy.

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### Introduction

*TP53* is the most frequently mutated gene in human cancers and are associated with poor prognosis in various cancers [1]. The associations of p53 with immune regulation have been extensively explored [2] including the roles p53 played in tumor immune regulation [3–6]. For example, p53 activation might enhance antitumor immunity [6]. p53 targeted many tumor immunosuppression-associated genes such as *PD-L1*, *VISTA*, and *FOXP3* [3]. p53 played a role in antitumor immunosurveillance by regulating *VISTA* [3].

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Cancer immunotherapies have recently demonstrated high efficacy in treating various cancers such as immune checkpoint blockade [7] and chimeric antigen receptor (CAR) T cell therapy [8]. Several immune checkpoint inhibitors such as ipilimumab (anti-CTLA4), nivolumab (anti-PD1), pembrolizumab (anti-PD1), atezolizumab (anti-PD-L1), and avelumab (anti-PD-L1) are being clinically used to treat various advanced malignancies including the cancers with deficient mismatch repair (dMMR). Unfortunately, these therapies are only beneficial to partial patients. Thus, discovering the biomarkers that are effective in predicting the cancer immunotherapeutic response is a pressing need. Several studies have shown that high tumor mutation burden was associated with a positive clinical response to CTLA4 or PD1 blockade [9–12]. The dMMR, neoantigens, and PD-L1 expressions have been associated with response to cancer immune checkpoint blockade treatment [12–14]. However, few studies have correlated cancer immunotherapy response with the *TP53* mutation status, although a recent phase II clinical trial demonstrated that p53-mutated metastatic breast cancer patients had an overall survival (OS) benefit when treated by the immuno-oncology viral agent REOLYSIN and paclitaxel combination [15].

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer death in the world [16]. GC is particularly prevalent in Asian countries, such as in China [17]. Based on genomic profiles, The Cancer Genome Atlas (TCGA) classified GC into four subtypes: Epstein–Barr virus (EBV) associated, microsatellite instable (MSI), genomically stable (GS), and chromosomal instability (CIN) [18]. The Asian Cancer Research Group (ACRG) classified GC into four subtypes: microsatellite stable (MSS)/epithelial-mesenchymal transition (EMT), MSI, MSS/p53+, and MSS/p53– [19]. The high heterogeneity of GC makes GC treatment a big challenge [20]. Some targeted therapies for GC have been investigated such as targeting HER2, EGFR, and VEGFR. Besides, immune checkpoint blockade targeting CTLA4, PD1, or PD-L1 is being evaluated in the immunotherapy of GC. Recently, FDA has approved the PD1 inhibitor pembrolizumab in treating dMMR (or MSI) cancers including the MSI subtype of GC.

*TP53* is the most frequently mutated gene in GC (approximately 50%) [19]. To explore the effect of *TP53* mutations on GC immunity, we performed comprehensive comparisons of expression or enrichment levels of 787 immune-related genes and 23 gene-sets between *TP53*-mutated and *TP53*-wildtype GCs based on TCGA [18] and ACRG [19] GC genomics datasets. We aimed at addressing several questions, including: Is the immunity of *TP53*-mutated GCs different from *TP53*-wildtype GCs? What factors may explain the differences in the immunity between *TP53*-mutated and *TP53*-wildtype GCs? Are there any associations of *TP53* mutations, immune cell infiltration, and clinical outcomes in GC?

## Materials and Methods

### Materials

We downloaded TCGA RNA-Seq gene expression profiles (Level 3), gene somatic mutations (Level 2), somatic copy number alteration (Level 3), protein expression profiles (Level 3), and clinical data from the genomic data commons data portal (<https://portal.gdc.cancer.gov/>). The ACRG gene expression profiles data were downloaded from NCBI Gene Expression Omnibus (GSE62254), and somatic mutations and clinical data were obtained from the publication [19].

### Comparisons of Gene Expression Levels, Gene-Set Enrichment Levels, and Protein Expression Levels between Two Classes of Samples

We normalized the TCGA GC and colon cancer gene expression values by base-2 log transformation and used the downloaded ACRG gene expression data since they have been normalized. We quantified the enrichment level or activity of an immune cell type or function in a sample using the single-sample gene-set enrichment analysis (ssGSEA) score [21,22]. We compared expression levels of a single gene between two classes of samples using Student's *t* test and compared enrichment levels (ssGSEA scores) of a gene-set between two classes of samples using Mann-Whitney *U* test. Based on the normalized GC protein expression profiles data in TCGA, we compared protein expression levels between *TP53*-mutated and *TP53*-wildtype GCs using Student's *t* test. We used the Benjamini-Hochberg method [23] to calculate the false discovery rate (FDR) in adjusting for multiple tests. The threshold of  $FDR < 0.05$  was used to identify the differentially expressed genes (proteins) and differentially enriched gene-sets. We performed the comparisons of gene expression data between *TP53*-mutated or *TP53*-wildtype GCs and normal tissue only in TCGA since ACRG had no gene expression data in normal tissue available.

### Gene-Set Enrichment Analysis

We performed a pathway analysis of the set of genes that were differentially expressed between *TP53*-mutated and *TP53*-wildtype GCs using the GSEA software [24]. The gene-set enrichment analysis was implemented for the set of genes that were upregulated in *TP53*-mutated GCs and the set of genes that were downregulated in *TP53*-mutated GCs compared to *TP53*-wildtype GCs concurrently in both datasets, respectively (Student's *t* test,  $FDR < 0.05$ ). The threshold of adjusted *P* value  $FDR < 0.05$  was used to identify the KEGG pathways with differential activities between *TP53*-mutated and *TP53*-wildtype GCs.

### Comparison of the Immune Cell Infiltration Degree between *TP53*-Mutated and *TP53*-Wildtype GCs

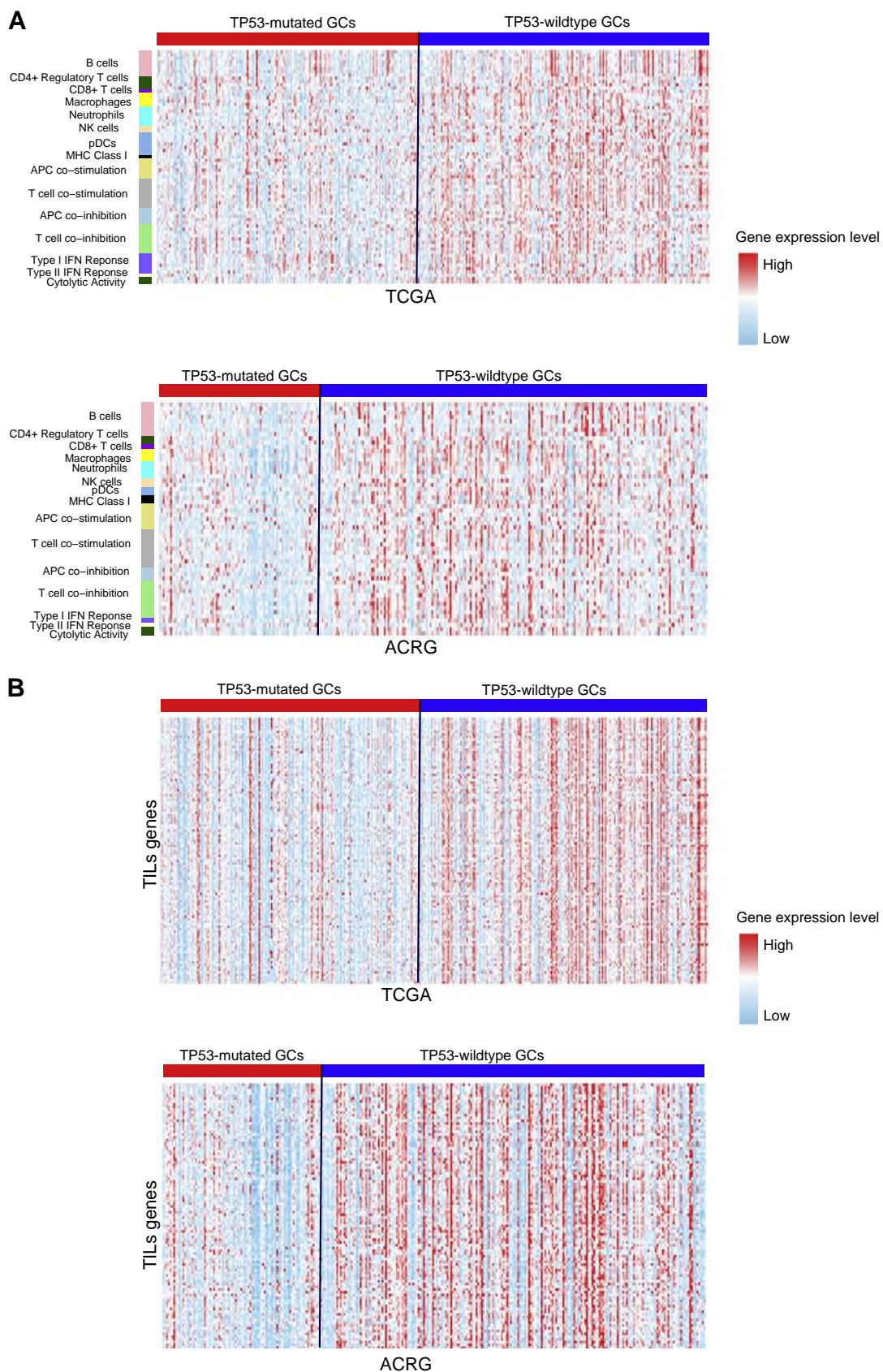
We used ESTIMATE [25] to evaluate the degree of immune cell infiltration in GC. For each GC sample, we obtained an immune score to quantify the degree of immune cell infiltration in the GC tissue. We compared the immune scores between *TP53*-mutated and *TP53*-wildtype GCs using Mann-Whitney *U* test.

### Comparison of Mutation Counts between *TP53*-Mutated and *TP53*-Wildtype GCs

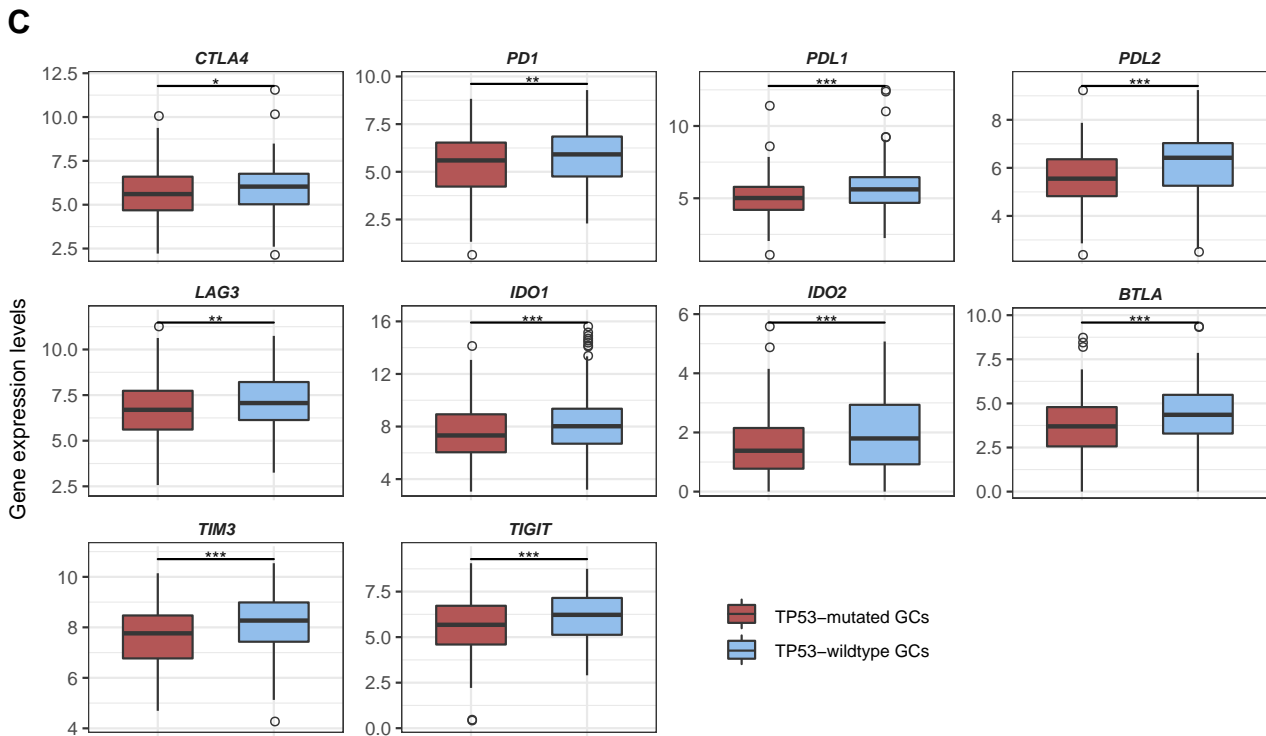
We compared the total mutation counts between *TP53*-mutated and *TP53*-wildtype GCs using Mann-Whitney *U* test. The comparison was performed only in TCGA since the somatic mutation data in TCGA were generated by whole exome sequencing, while data were generated by targeted exome sequencing in ACRG.

### Correlations of Pathway or Protein Activities with Immune Activities in GC

We explored the correlations of the p53-mediated pathway activities with enrichment levels of the 23 immune gene-sets. We obtained the gene-set collections for the p53-mediated pathways from KEGG [26] and quantified the activity of a pathway and the enrichment level of an immune gene-set in a GC sample by the ssGSEA score [21,22]. To correct for the strong correlation between the p53 pathway and the other p53-mediated pathways,



**Figure 1.** TP53-mutated GCs likely have the depressed expression of immune cell types and functional marker, TILs, and immune checkpoint genes compared to TP53-wildtype GCs. (A) Heat map for expression levels of immune cell types and functional genes in TP53-mutated and TP53-wildtype GCs. (B) Heat map for expression levels of TILs genes in TP53-mutated and TP53-wildtype GCs. (C) A number of important immune checkpoint genes show significantly lower expression levels in TP53-mutated GCs than in TP53-wildtype GCs in TCGA. \*:  $P < .05$ , \*\*:  $P < .01$ , \*\*\*:  $P < .001$ , \*\*\*\*:  $P < .0001$ , and it applies to all the following box charts.



**Figure 1.** (continued).

we used the first-order partial correlation to evaluate the correlations between the pathways and the immune gene-sets with the R package “ppcor” [27]. The correlation between a pathway and an immune gene-set was defined as significant if  $FDR < 0.05$ . We quantified the correlation between a protein and an immune gene-set by calculating the Spearman correlation coefficient (“rho”) of expression levels of the protein and ssGSEA scores of the immune gene-set.

### Survival Analyses

We compared OS and disease-free survival (DFS) time between two groups of GC patients classified based on gene expression levels, gene-set enrichment levels, and immune scores, respectively. Kaplan-Meier survival curves were used to show the survival differences between both classes of patients (gene higher-expression-level patients vs. gene lower-expression-level patients, gene-set higher-enrichment-level patients vs. gene-set lower-enrichment-level patients, and higher-immune-score patients vs. lower-immune-score patients). We used the median values of gene expression levels, gene-set enrichment levels (ssGSEA scores), or immune scores to classify patients into two different groups. The log-rank test was used to calculate the significance of survival time differences between two classes of patients with a threshold of  $P < .05$ .

## Results

### TP53 Mutations Are Associated with Depressed Expression of Immune Cell Types and Functional Marker Genes in GC

We analyzed the activities of 15 immune cell types and functional gene-sets in GC. These gene-sets represent B cells, CD4+ regulatory T cells, CD8+ T cells, macrophages, neutrophils,

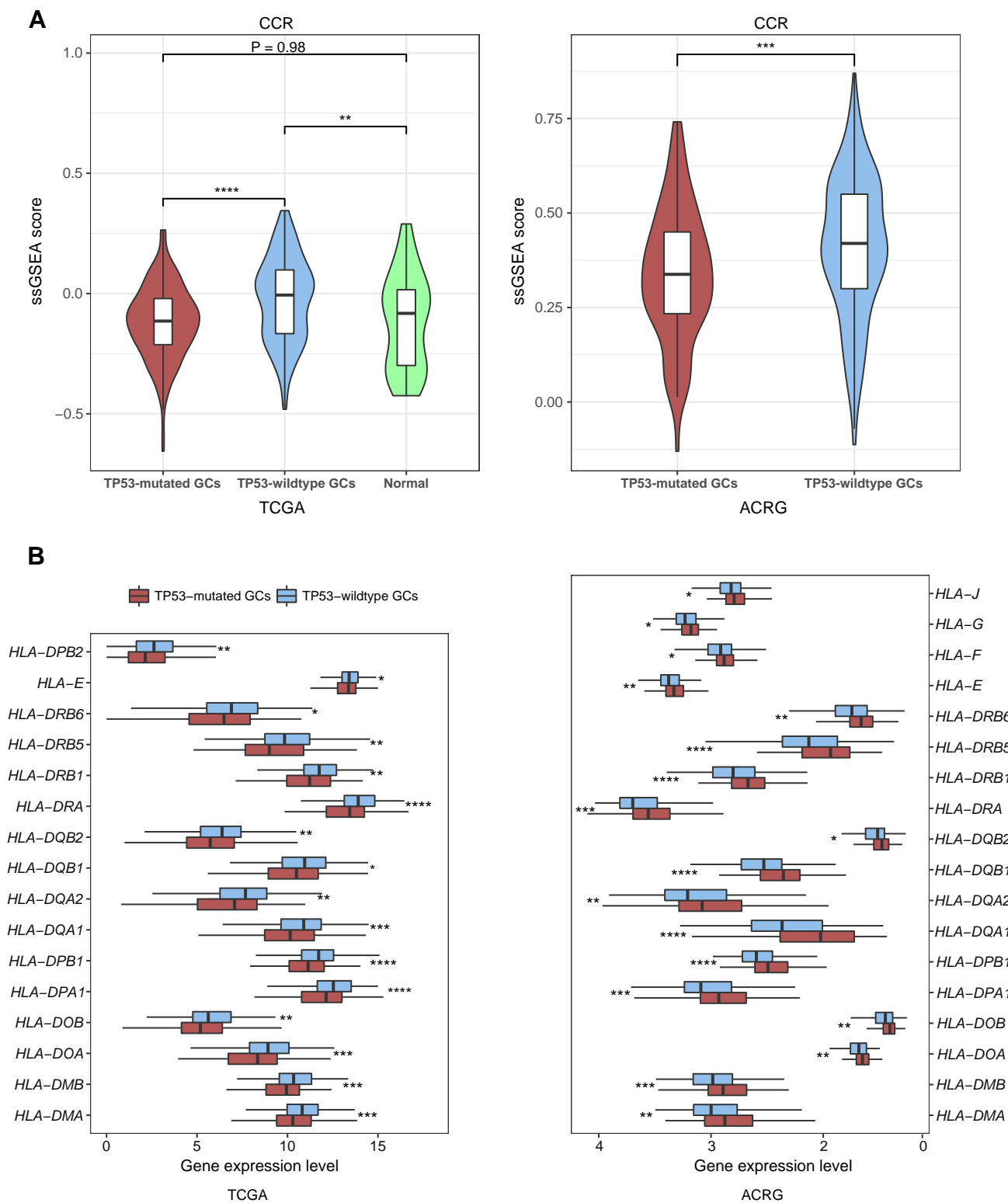
natural killer (NK) cells, plasmacytoid dendritic cells (pDCs), major histocompatibility complex (MHC) class I, APC co-stimulation, T cell co-stimulation, APC co-inhibition, T cell co-inhibition, TYPE I IFN response, type II IFN response, and cytolytic activity, respectively [28]. We found that a substantial number of genes in the 15 gene-sets had significantly lower expression levels in TP53-mutated GCs than in TP53-wildtype GCs (Figure 1A; Supplementary Table S1). For example, 7 of the 10 B cell marker genes, the CD8+ T cell marker gene (*CD8A*), both NK cell marker genes (*KLRC1* and *KLRF1*), 8 of the 10 T cell co-inhibition marker genes, and both cytolytic activity genes (*GZMA* and *PRF1*) were more lowly expressed in TP53-mutated GCs than in TP53-wildtype GCs in both datasets.

Strikingly, we found that all 15 immune cell types and functional gene-sets showed significantly lower ssGSEA scores in TP53-mutated GCs than in TP53-wildtype GCs in at least one dataset and 12 in both datasets (Mann-Whitney *U* test,  $P < .05$ ) (Supplementary Table S1, Figure S1A). These results suggest that TP53-mutated GCs likely have depressed immune activities compared to TP53-wildtype GCs. Compared to normal tissue, the ssGSEA scores for B cells, CD8+ T cells, neutrophils, NK cells, and cytolytic activity were significantly lower in TP53-mutated GCs, while showing no significant differences in TP53-wildtype GCs. These data indicated that TP53 mutations might comprise the activity of immune attack on GC cells by the host immune system. Interestingly, the CD4+ regulatory T cell and its marker genes *FOXP3*, *CTLA4*, and *IL32* had significantly higher enrichment or expression levels in both TP53-mutated and TP53-wildtype GCs than in normal tissue. The higher activity of the CD4+ regulatory T cells in GC may imply the GC immunosuppression mechanism [29].

**TP53 Mutations Are Associated with Decreased Immune Cell Infiltration and Lower Immunosuppressive Activity in GC**

The density of tumor-infiltrating lymphocytes (TILs) is associated with cancer prognosis [30,31]. We compared expression levels of 122

TILs gene signatures [32] between TP53-mutated and TP53-wildtype GCs. Strikingly, we found that 107 (88%) TILs genes were more lowly expressed in TP53-mutated GCs in at least one dataset (100 in both datasets) (Figure 1B; Supplementary Table S2).



**Figure 2.** TP53-mutated GCs likely have the depressed expression of CCR and HLA genes compared to TP53-wildtype GCs. (A) Comparison of enrichment levels of the CCR gene-set between TP53-mutated and TP53-wildtype GCs. (B) A number of HLA genes show significantly lower expression levels in TP53-mutated GCs than in TP53-wildtype GCs.

The ssGSEA scores for the TILs gene-set were significantly lower in *TP53*-mutated GCs than in *TP53*-wildtype GCs (Mann-Whitney *U* test,  $P = 1.29 \times 10^{-7}$ ,  $3.80 \times 10^{-5}$  for TCGA and ACRG, respectively) (Supplementary Figure S1B). It indicates that *TP53* mutations are associated with decreased TILs infiltration in GC.

Regulatory T (Treg) cells and the immune checkpoint pathway play important roles in tumor immunosuppression [7,33]. We compared the ssGSEA scores of the Treg and immune checkpoint gene-sets which, respectively, included 70 and 47 gene signatures [34] between *TP53*-mutated and *TP53*-wildtype GCs, respectively. We found that both gene-sets showed significantly lower enrichment levels in *TP53*-mutated GCs than in *TP53*-wildtype GCs (Mann-Whitney *U* test,  $P < .05$ ). Moreover, many notable immune checkpoint genes were downregulated in *TP53*-mutated GCs such as *CTLA4*, *PD1*, *PD-L1*, *PD-L2*, *IDO1/2*, *BTLA*, *LAG3*, *TIM3*, and *TIGIT* (Figure 1C; Supplementary Table S3, Figure S1, C and D). These results indicated that *TP53* mutations were associated with lower immunosuppressive activity in GC, suggesting that p53 may transactivate tumor immunosuppressive pathways. This finding is in line with prior studies [3].

### *TP53 Mutations Are Associated with Lower Cytokine Activity in GC*

Cytokines are important components of the tumor microenvironment and play important roles in tumor immunity [35]. Of 261 cytokine and cytokine receptor (CCR) genes [36], 91 were downregulated in *TP53*-mutated GCs in at least one dataset versus 16 upregulated in *TP53*-mutated GCs (Fisher's exact test,  $P < 2.2 \times 10^{-16}$ , OR = 8.17) compared to *TP53*-wildtype GCs (Supplementary Table S4, Figure S2A). The CCR gene-set enrichment levels were significantly lower in *TP53*-mutated GCs than in *TP53*-wildtype GCs (Mann-Whitney *U* test,  $P = 2.93 \times 10^{-7}$ ,  $4.22 \times 10^{-4}$  for TCGA and ACRG, respectively) (Figure 2A). It suggests that *TP53* mutations may suppress the activity of cytokines in GC.

### *TP53 Mutations Are Associated with the Upregulation of Cancer-Testis Antigens in GC*

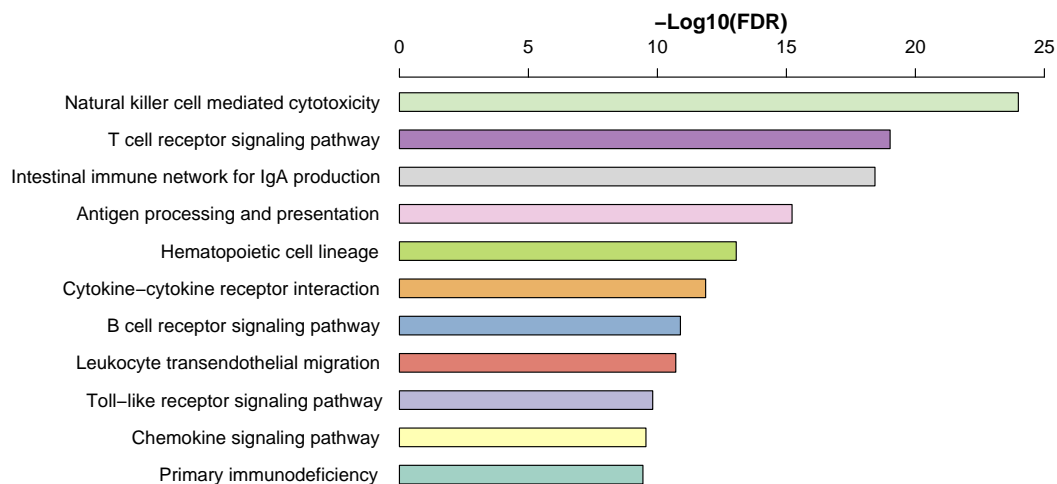
Cancer-testis antigens (CTAs) are a group of immunogenic proteins that are aberrantly activated and expressed in various cancers

[37]. We found that 125 of the 223 CTA genes [38] were more highly expressed in *TP53*-mutated GCs in at least one dataset versus 8 more highly expressed in *TP53*-wildtype GCs (Fisher's exact test,  $P < 2.2 \times 10^{-16}$ , OR = 34) (Supplementary Table S5). Notably, the CTA genes upregulated in *TP53*-mutated GCs included *MAGEAs*, *NY-ESO-1*, and *PRAME* that are promising targets for developing cancer vaccines [37]. Enrichment levels of the CTA gene-set were significantly higher in *TP53*-mutated GCs than in *TP53*-wildtype GCs (Mann-Whitney *U* test,  $P = 1.98 \times 10^{-12}$  and  $3.32 \times 10^{-8}$  for TCGA and ACRG, respectively). These data indicated that p53 could suppress the expression of many CTA genes, a finding consistent with a previous study showing that p53 regulated CTA genes [39]. As expected, enrichment levels of the CTA gene-set were significantly higher in both *TP53*-mutated GCs and *TP53*-wildtype GCs than in normal tissue (Mann-Whitney *U* test,  $P = 2.18 \times 10^{-17}$  and  $2.01 \times 10^{-11}$  for *TP53*-mutated GCs and *TP53*-wildtype GCs, respectively).

### *TP53-Mutated GCs Shows Significant Differences in Human Leukocyte Antigen (HLA) Gene Expression and Somatic Mutations Compared to TP53-wildtype GCs*

HLA genes encode MHC proteins which are involved in the regulation of the immune system in humans [40]. Of 24 HLA genes analyzed, we found that 19 (80%) were downregulated in *TP53*-mutated GCs relative to *TP53*-wildtype GCs in at least one dataset (15 in both datasets) (Figure 2B; Supplementary Table S6). Enrichment levels of the HLA gene-set were significantly lower in *TP53*-mutated GCs than in *TP53*-wildtype GCs (Mann-Whitney *U* test,  $P = 5.0 \times 10^{-4}$  and  $2.25 \times 10^{-6}$  for TCGA and ACRG, respectively). Compared to normal tissue, enrichment levels of the HLA gene-set were significantly higher in *TP53*-wildtype GCs (Mann-Whitney *U* test,  $P = .036$ ), while showed no significant differences in *TP53*-mutated GCs (Mann-Whitney *U* test,  $P = .94$ ). These results indicated that *TP53* mutations might inhibit HLA expression in GC. This finding conforms to a previous study showing that p53 increased the expression of MHC proteins in cancer [41].

Gene mutations may yield neoepitopes that can be recognized by immune cells [42]. Rooney et al. [28] predicted that mutations



**Figure 3.** Immune-related pathways that are downregulated in *TP53*-mutated GCs compared to *TP53*-wildtype GCs by the gene-set enrichment analysis.

introduced novel peptides loading in imputed HLA alleles in TCGA samples. Interestingly, we found that although TP53-mutated GCs had significantly higher total mutation counts than TP53-wildtype GCs (Mann-Whitney U test,  $P = .018$ ), TP53-mutated GCs had fewer mutations that may yield predicted HLA-binding peptides than TP53-wildtype GCs (Mann-Whitney U test,  $P = .015$ ). It suggests that TP53 mutations may suppress tumor immunity in GC.

**TP53 Mutations Are Associated with Depressed Inflammatory Activity in GC**

Inflammatory responses are significantly associated with tumor development [43]. We compared expression levels of 16 pro-inflammatory genes [44] between TP53-mutated and TP53-wildtype GCs. Strikingly, we found that 15 of the 16 genes had significantly

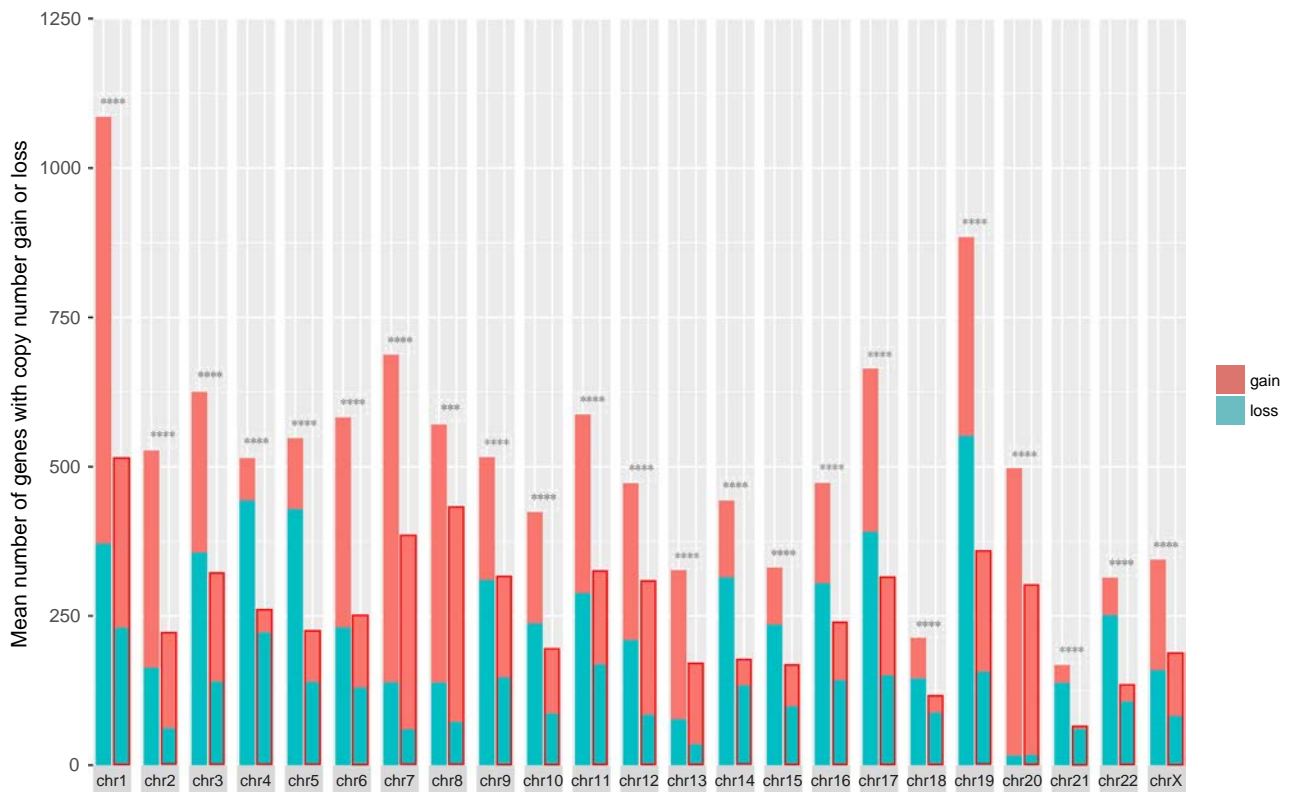
lower expression levels in TP53-mutated GCs in at least one dataset (10 in both datasets) (Supplementary Figure S2B, Table S7). Notably, GZMB (granzyme B) was downregulated in TP53-mutated GCs. The product of this gene and the aforementioned cytolytic activity markers GZMA and PRF1 are mainly secreted by NK cells and cytotoxic T lymphocytes [28]. Accordingly, the decreased expression of GZMA, GZMB, and PRF1 implied the depressed immune cytolytic activity in TP53-mutated GCs. The pro-inflammatory gene-set showed significantly lower enrichment levels in TP53-mutated GCs than in TP53-wildtype GCs (Mann-Whitney U test,  $P = 7.62 \times 10^{-5}$  and  $1.21 \times 10^{-4}$  for TCGA and ACRG, respectively). These data indicated that TP53 mutations might inhibit inflammatory and immune cytolytic activities in GC.

Parainflammation (PI) is a low-grade inflammatory reaction and is associated with carcinogenesis [45]. We compared expression levels of

**A**

|              | # TP53-mutated GCs | # TP53-wildtype GCs | Total number | Fisher's Exact Test  |
|--------------|--------------------|---------------------|--------------|--|
| # CIN        | 97                 | 39                  | 136          | Two-sided $P=9.87 \times 10^{-12}$<br>One-sided $P=8.83 \times 10^{-12}$<br>Odds ratio = 12.86 |
| # GS         | 8                  | 42                  | 50           |  |
| Total number | 105                | 81                  | 186          |  |

**B**



**Figure 4.** TP53-mutated GCs are likely to be chromosomally unstable, and chromosomal instability is associated with depressed immune activities in cancer. (A) TP53-mutated GCs harbor a significantly higher proportion of CIN samples than TP53-wildtype GCs. (B) TP53-mutated GCs had much more genes with copy number alteration than TP53-wildtype GCs. In each chromosome, the left bar is for the TP53-mutated GC, and the right bar is for the TP53-wildtype GC that is surrounded by a pane. (C) CCR genes that show higher frequencies of copy number alteration in TP53-mutated GCs than in TP53-wildtype GCs. The CCR genes with "\*" ahead have higher frequencies of copy number gain, and the others have higher frequencies of copy number loss in TP53-mutated GCs than in TP53-wildtype GCs. (D) Most of the immune gene-sets show significantly lower enrichment levels in CIN cancers than in GS cancers. STAD, stomach adenocarcinoma; COAD, colon adenocarcinoma; NS, not significant. \*:  $P < .05$ , \*\*:  $P < .01$ , \*\*\*:  $P < .001$ , \*\*\*\*:  $P < .0001$ .

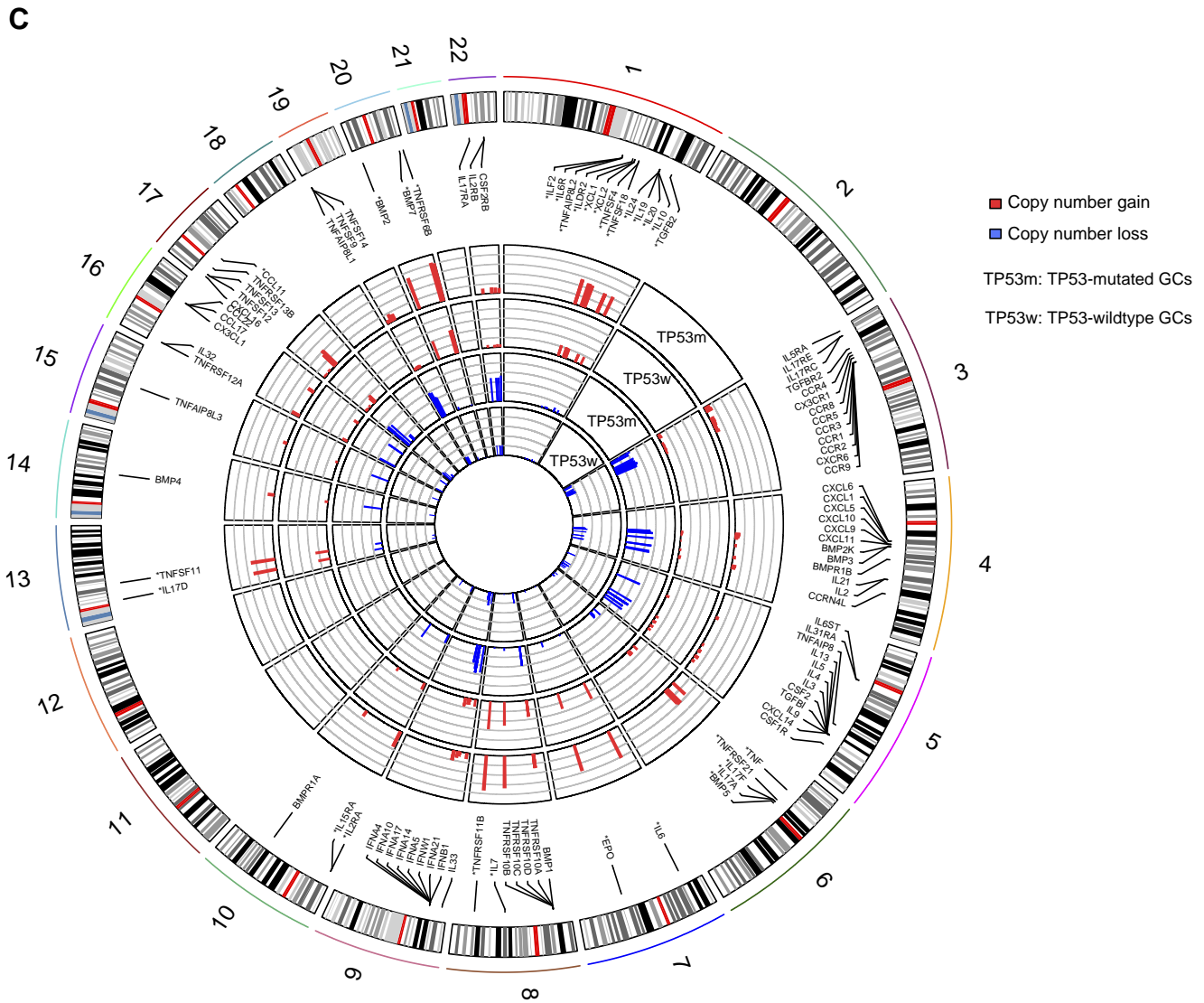


Figure 4. (continued).

40 PI gene signatures [45] between *TP53*-mutated and *TP53*-wildtype GCs. We found that the PI activity was significantly lower in *TP53*-mutated GCs than in *TP53*-wildtype GCs (Mann-Whitney *U* test,  $P = .006$ , 0.01 for TCGA and ACRG, respectively; Supplementary Table S8), suggesting that *TP53* mutations were negatively correlated with the PI activity in GC.

***TP53 Mutations Are Associated with Lower Activities of Immune-Related Pathways in GC***

The GSEA showed that plentiful immune-related pathways significantly correlated with the set of genes that were downregulated in *TP53*-mutated GCs. These pathways included NK cell-mediated cytotoxicity, T cell receptor signaling, intestinal immune network for IgA production, antigen processing and presentation, hematopoietic cell lineage, cytokine-cytokine receptor interaction, B cell receptor signaling, leukocyte transendothelial migration, toll-like receptor signaling, chemokine signaling, and primary immunodeficiency (Figure 3). In contrast, no immune-related pathways significantly correlated with the set of genes that were upregulated in *TP53*-mutated GCs. These results again demonstrate that *TP53*-mutated

GCs have lower immune activities compared to *TP53*-wildtype GCs.

***TP53 Mutations Suppress Tumor Immunity via Regulation of Chromosomal Stability***

We found that *TP53*-mutated GCs harbored a significantly higher proportion of CIN GCs and a significantly lower proportion of GS GCs as compared to *TP53*-wildtype GCs (Fisher's exact test,  $P < .05$ ; Figure 4A). Moreover, we found that *TP53*-mutated GCs had much more genes with copy number alteration (gain and loss) than *TP53*-wildtype GCs in each of the 23 chromosomes (Figure 4B). These results demonstrate that p53 plays a key role in restricting CIN [46]. Interestingly, most of CCR genes showed higher frequencies of copy number alteration in *TP53*-mutated GCs than in *TP53*-wildtype GCs (Fisher's exact test,  $P < .05$ ; Figure 4C). Furthermore, we found that almost all 23 immune gene-sets had significantly lower enrichment levels in CIN GCs than in GS GCs except the CTA gene-set with higher enrichment levels in CIN GCs (Figure 4D). In addition, we used the ESTIMATE algorithm [25] to evaluate the levels of immune cell infiltration (immune score)



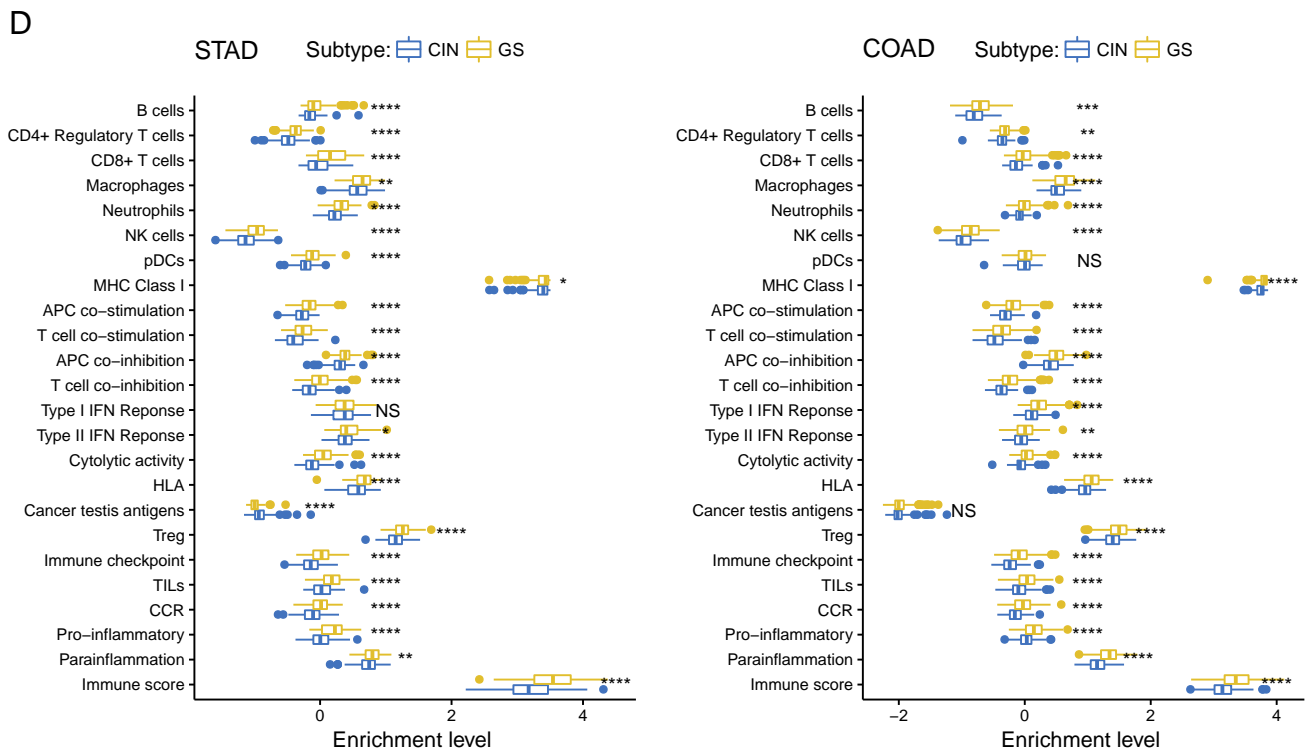


Figure 4. (continued).

in GC and found that the CIN GCs had significantly lower immune scores than the GS GCs (Figure 4D). We further analyzed another TCGA dataset for colon cancer and obtained similar results that the CIN colon cancers had lower enrichment levels of almost all the immune gene-sets and had lower immune scores than GS colon cancers (Figure 4D). These results suggest that the suppressive tumor immunity in TP53-mutated cancers may be attributed to the decreased chromosomal stability by TP53 mutations. It is in line with a recent study showing that CIN could drive tumor metastasis via regulation of immune pathways [47].

**Immune Activities in GC Are Significantly Associated with Activities of p53-Regulated Pathways**

p53 plays an important role in regulating the cell cycle, and cell cycle arrest is one of the prominent functions of p53 in tumor suppression [48]. Thus, the activity of the p53 pathway would have a negative correlation with the activity of the cell cycle pathway in the tumor. Accordingly, the p53 mutation may lead to hypoactivation of the p53 pathway and hyperactivation of the cell cycle pathway in the tumor [49]. Strikingly, we found that all immune gene-sets analyzed significantly correlated with the p53 pathway in a positive direction except the CTA gene-set in a negative direction in GC (Figure 5A). In contrast, all the immune gene-sets significantly inversely correlated with the cell cycle pathway except the CTA gene-set with a positive correlation (Figure 5A). This is consistent with a recent study showing that the inhibition of cell cycle progression could increase tumor immunogenicity [50]. Besides the p53 and cell cycle pathways, p53 is importantly involved in regulation of a number of other cancer-associated pathways, including DNA damage repair, apoptosis, autophagy, metabolism, EMT, inflammation, angiogen-

esis, and metastasis [46]. Indeed, we found that many related pathways were downregulated in TP53-mutated GCs relative to TP53-wildtype GCs by the GSEA [24], including Jak-STAT, apoptosis, MAPK, focal adhesion, NOD-like receptor, VEGF, calcium, and autophagy pathways. Strikingly, we found that most of the immune gene-sets analyzed showed significant positive correlations with each of these pathways except the CTA gene-set with a negative correlation in GC (Figure 5A). These results suggest that TP53 mutations cause disturbances of the pathways targeted by p53, thereby contributing to the depressed immune activities in TP53-mutated GCs.

**Identification of Proteins Differentially Expressed between TP53-Mutated and TP53-wildtype GCs That Are Significantly Correlated with Tumor Immunity in GC**

Based on the TCGA protein expression profiles data, we identified two proteins (CCNE1 and ACACA) and one protein (PIK3CG) with significantly higher and lower expression levels in TP53-mutated GCs than in TP53-wildtype GCs, respectively (Student's t test, FDR < 0.05). Interestingly, both CCNE1 and ACACA had negative expression correlations with immune infiltration levels in GC, while PIK3CG had a positive expression correlation with immune infiltration levels in GC (Figure 5B). CCNE1 (cyclin E1) belongs to the cyclin family that functions as regulators of cyclin-dependent kinases. This protein is a downstream target of p53 and plays an important role in promoting cell cycle progression and CIN [51]. Thus, the elevated expression of CCNE1 may inhibit immune activities by enhancing the cell-cycle and CIN activities in TP53-mutated GCs. Another upregulated protein in TP53-mutated GCs ACACA is related to the metabolism pathway. It has been shown that

p53 induces the AMP-activated protein kinase, which phosphorylates ACACA and ACACB to block fatty acid biosynthesis [52]. Our data indicate that targeting the metabolism pathway may be an approach for p53 to regulate tumor immunity. PIK3CG is involved in MAPK, VEGF, and immune-related pathways [53]. Our results have shown that these pathways are positively associated with immune activities in GC. Altogether, these data indicate that the differential protein activities may contribute to the differential immune activities between *TP53*-mutated and *TP53*-wildtype GCs.

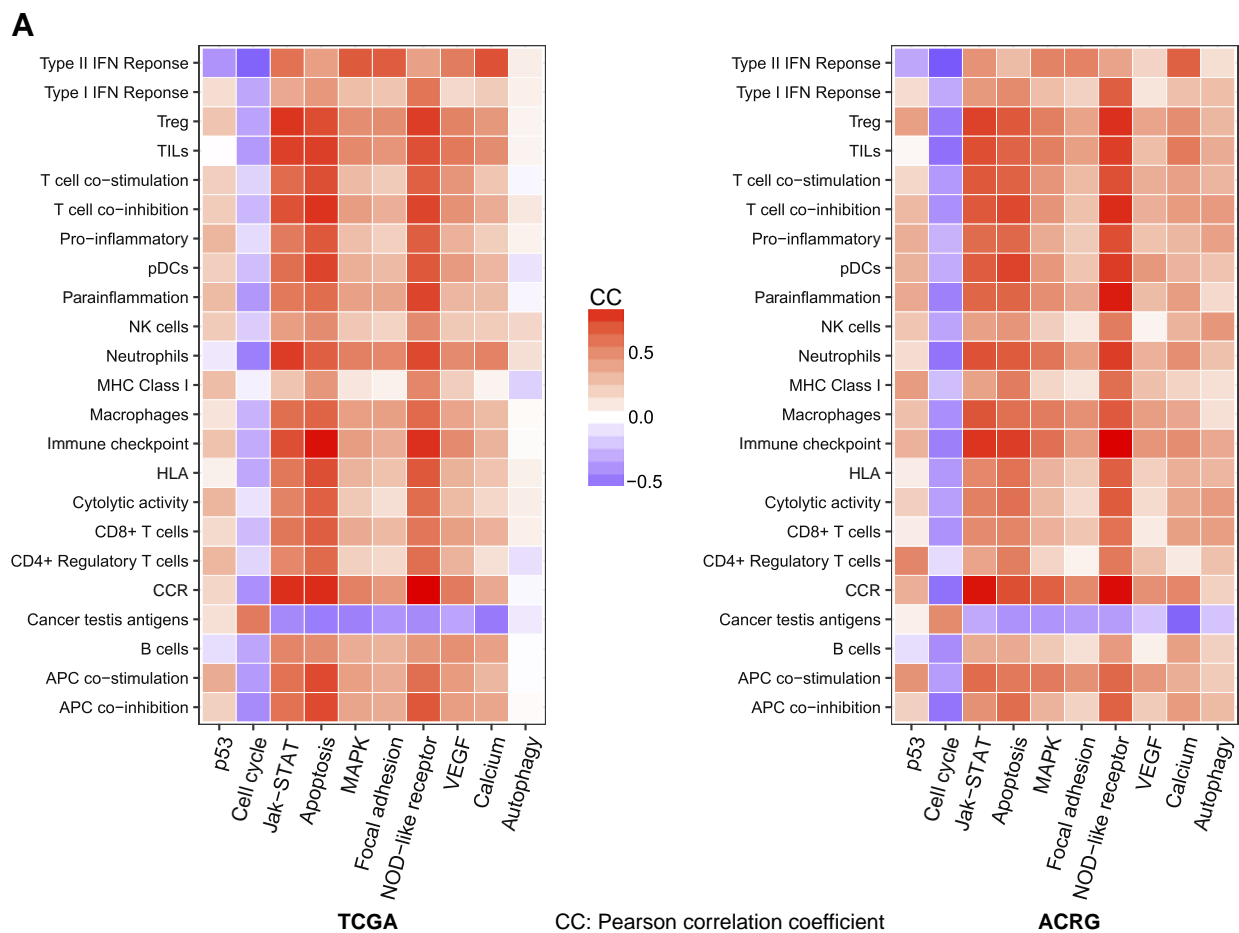
### Immune Activities Are Prone to be Positively Associated with Survival Prognosis in GC

Among the 23 immune gene-sets analyzed, 10 and 3 gene-sets showed significant correlations of enrichment levels with survival prognosis in *TP53*-mutated and *TP53*-wildtype GCs, respectively. Strikingly, elevated enrichment levels of all the gene-sets were consistently associated with better OS and/or DFS prognosis in *TP53*-mutated or *TP53*-wildtype GCs (Figure 6A). Moreover, we found a number of immune genes whose elevated expression was positively associated with OS and/or DFS prognosis in *TP53*-mutated and/or *TP53*-wildtype GCs (Figure 6B; Supplementary

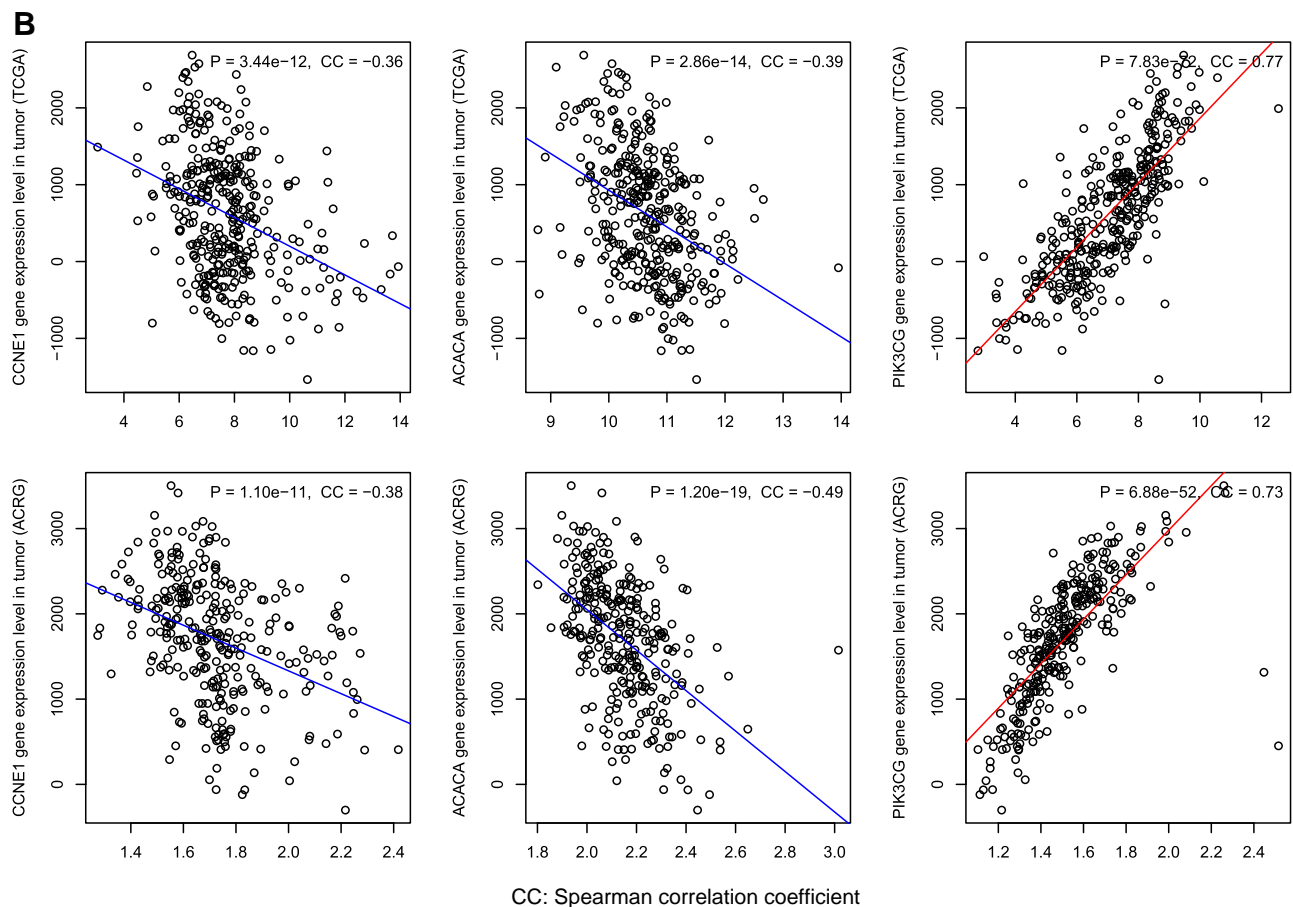
Table S9). For example, elevated expression of immune checkpoint genes *CTLA4* and *PD1* was associated with better survival prognosis in both *TP53*-mutated and *TP53*-wildtype GCs; elevated expression of other three immune checkpoint genes, *PD-L2*, *FOXP3*, and *TIGIT*, was associated with better survival prognosis in *TP53*-mutated GCs; elevated expression of *GZMA*, *GZMB*, *IDO1*, *STAT1*, and *LAG3* was associated with better survival prognosis in *TP53*-wildtype GCs. Furthermore, we found that higher immune scores were associated with better survival prognosis in *TP53*-mutated GCs (Figure 6C; Supplementary Table S9). Altogether, these data show that higher immune activities are likely associated with more favorable clinical outcomes in GC.

### Discussion

In the present study, we performed a comprehensive analysis of the association between *TP53* mutations and tumor immunity in GC based on two large-scale GC genomics data. Strikingly, we found that almost all the immune-related gene-sets analyzed showed significantly lower enrichment levels (ssGSEA scores) in *TP53*-mutated GCs than in *TP53*-wildtype GCs in both datasets except that the CTA gene-set had significantly higher enrichment levels in *TP53*-mutated GCs



**Figure 5.** The p53-mediated pathways and proteins show significant correlations with immune activities in GC. (A) All the immune gene-sets (except cancer testis antigen) show negative correlations with the cell cycle pathway, and positive correlations with the other p53-mediated pathways. B. The differentially expressed proteins between *TP53*-mutated and *TP53*-wildtype GCs show significant expression correlations with immune infiltration scores in GC.



**Figure 5.** (continued).

(Figure 7A; Supplementary Table S10). Moreover, the ESTIMATE [25] evaluation showed that *TP53*-mutated GCs had significantly lower levels of immune cell infiltration than *TP53*-wildtype GCs in both datasets (Mann-Whitney *U* test,  $P = 4.24 \times 10^{-8}$  and  $2.63 \times 10^{-5}$  for TCGA and ACRG, respectively) (Figure 7B), again demonstrating that *TP53* mutations were associated with lower immune activities in GC. It should be noted that the association of *TP53* mutations with tumor immunity is not restricted to GC but also other cancer types. Indeed, we found that the *TP53*-mutated subtype had the significantly lower degree of immune infiltration than the *TP53*-wildtype subtype in various cancer types (Figure 7B).

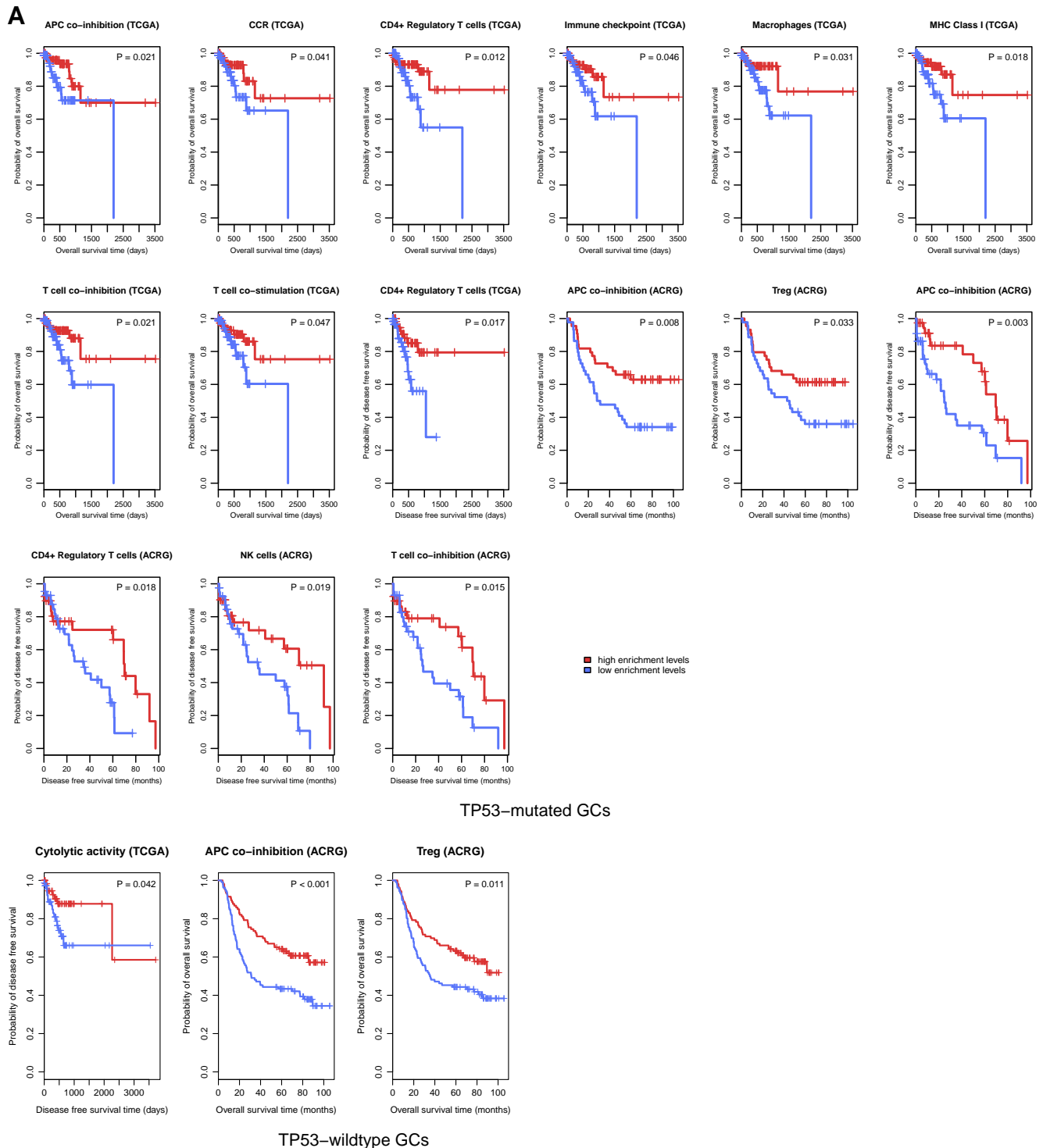
The MSI genomic feature has been associated with high immunogenic activity in cancer [14]. We found that *TP53*-mutated GCs involved a lower proportion of MSI samples compared to *TP53*-wildtyped GCs (Fisher's exact test,  $P = .004$ , OR = 0.45 for TCGA,  $P = .006$ , OR = 0.38 for ACRG). This result may partially explain the lower immunogenic activity in *TP53*-mutated GCs than in *TP53*-wildtyped GCs. However, in comparisons of the enrichment levels of the 23 immune-related gene-sets between *TP53*-mutated and *TP53*-wildtyped GCs within the MSS subtype of GC, we found that almost all 23 gene-sets had significantly lower enrichment levels in *TP53*-mutated MSS GCs than in *TP53*-wildtype MSS GCs in both datasets (Supplementary Table S11). It indicates that MSI is not the essential factor explaining the differential immunogenic activity between *TP53*-mutated and *TP53*-wildtype

GCs. On the other hand, GCs are often associated with infectious agents, including the bacterium *Helicobacter pylori* and EBV. It has been observed that EBV+ GCs have increased immune activities versus EBV- GCs [28]. We found that EBV+ GCs had a much lower *TP53* mutation rate compared to EBV- GCs (Fisher's exact test,  $P = 2.66 \times 10^{-6}$ , OR = 0.04 for TCGA,  $P = .002$ , OR = 0 for ACRG). Thus, the lower immunogenic activity in *TP53*-mutated GCs may be attributed to the rare EBV infection in the GC subtype. However, we found that most of the immune gene-sets had significantly lower enrichment levels in *TP53*-mutated EBV- GCs than in *TP53*-wildtype EBV- GCs in both datasets (Supplementary Table S12). It indicates that the differential immunogenic activity between *TP53*-mutated and *TP53*-wildtype GCs cannot be attributed to the EBV infection status. In fact, when we compared enrichment levels of the immune-related gene-sets between *TP53*- (nonfunctional *TP53*) and *TP53*+ (functional *TP53*) GCs in ACRG [19], we found that almost all 23 gene-sets had significantly lower enrichment levels in *TP53*- GCs than in *TP53*+ GCs (the CTA gene-set had elevated expression levels in *TP53*- GCs) (Supplementary Tables S1-8). Moreover, a substantial number of immune-related genes showed significantly lower expression levels in *TP53*- GCs than in *TP53*+ GCs (Supplementary Tables S1-8). These results confirm that *TP53* dysfunction itself represses immunogenic activity in GC.

We have identified a number of p53-mediated pathways whose activities were significantly associated with immune activities in GC

(Figure 5A). These pathways included p53, cell cycle, Jak-STAT, apoptosis, MAPK, focal adhesion, NOD-like receptor, VEGF, calcium, and autophagy pathways. We have also demonstrated that

CIN was associated with depressed tumor immunity (Figure 4B). Since p53 plays a crucial role in maintaining chromosomal stability [46], mutant p53 may result in CIN that inhibits antitumor



**Figure 6.** Immune activities are positively associated with survival prognosis in *TP53*-mutated and *TP53*-wildtype GCs. A. Kaplan–Meier survival curves show that elevated enrichment of immune gene-sets is associated with better survival prognosis in *TP53*-mutated and *TP53*-wildtype GCs (log-rank test, unadjusted  $P$ -value  $<0.05$ ). B. Kaplan–Meier survival curves show that elevated expression of immune genes is associated with better survival prognosis in *TP53*-mutated and *TP53*-wildtype GCs (log-rank test, unadjusted  $P$ -value  $<0.05$ ). C. Kaplan–Meier survival curves show that a higher degree of immune cell infiltration is associated with better survival prognosis in *TP53*-mutated GCs (log-rank test, unadjusted  $P$ -value  $<0.05$ ).

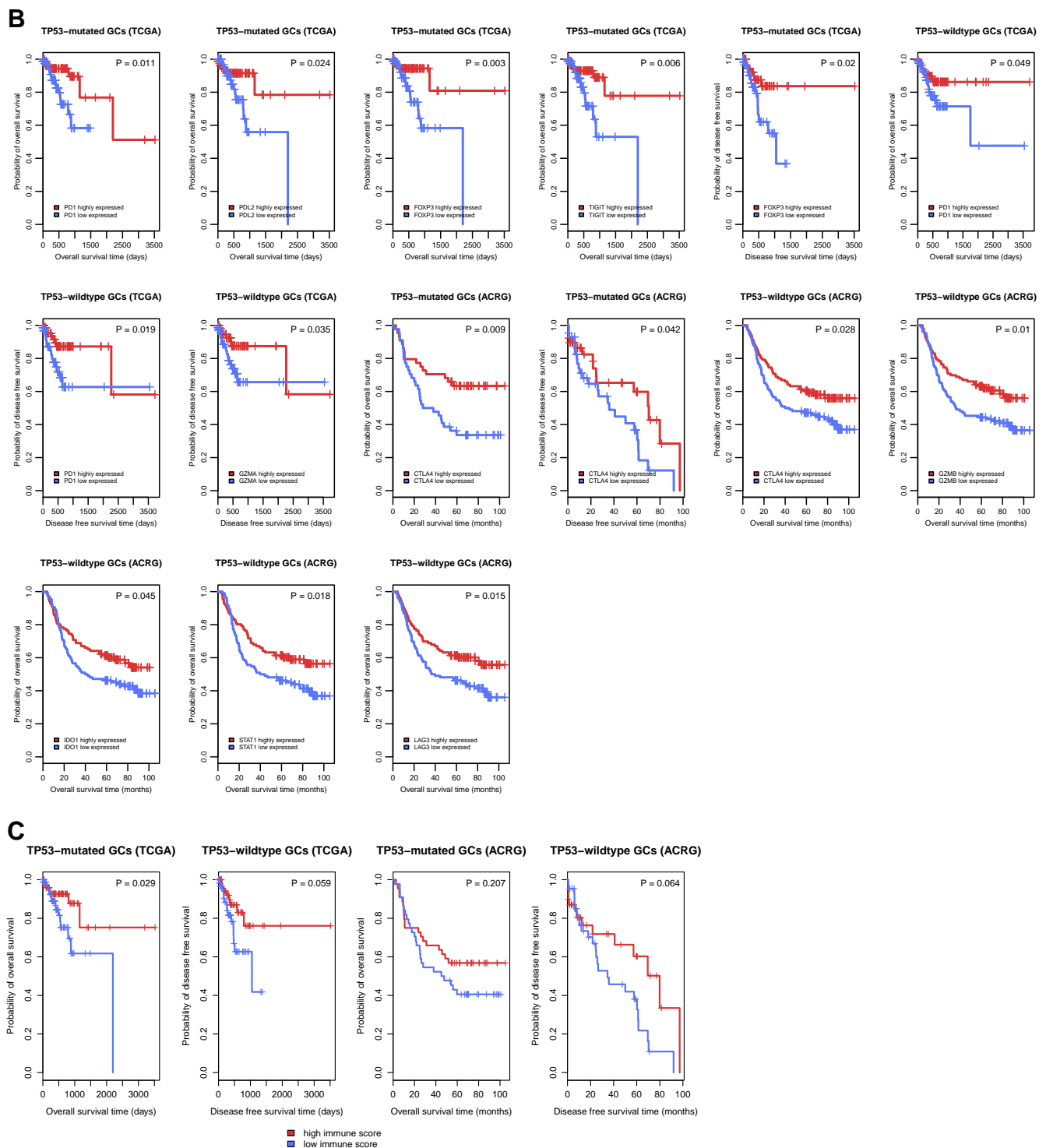


Figure 6. (continued).

immunity. Thus, we argue that CIN is a key clue that explains the depressed tumor immunity in *TP53*-mutated cancers (Figure 7C).

In [54], Smyth et al. listed 26 immunotherapeutic targets currently used in the clinic or in clinical trials, 18 of which were downregulated in *TP53*-mutated GCs versus *TP53*-wildtype GCs in both datasets (Supplementary Table S13). In addition, 9 of 12 targets for immunotherapy agents in preclinical development [54] were downregulated in *TP53*-mutated GCs (Supplementary Table S13). These data

indicate that most of the promising immunotherapy agents may be less effective against *TP53*-mutated GCs than *TP53*-wildtype GCs.

### Conclusions

Our findings suggest that p53 may play an important role in activating tumor immunity in GC and other cancer types and that the *TP53* mutation status could be used for predicting cancer patients responsive to a certain immunotherapy.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tranon.2018.07.012>.

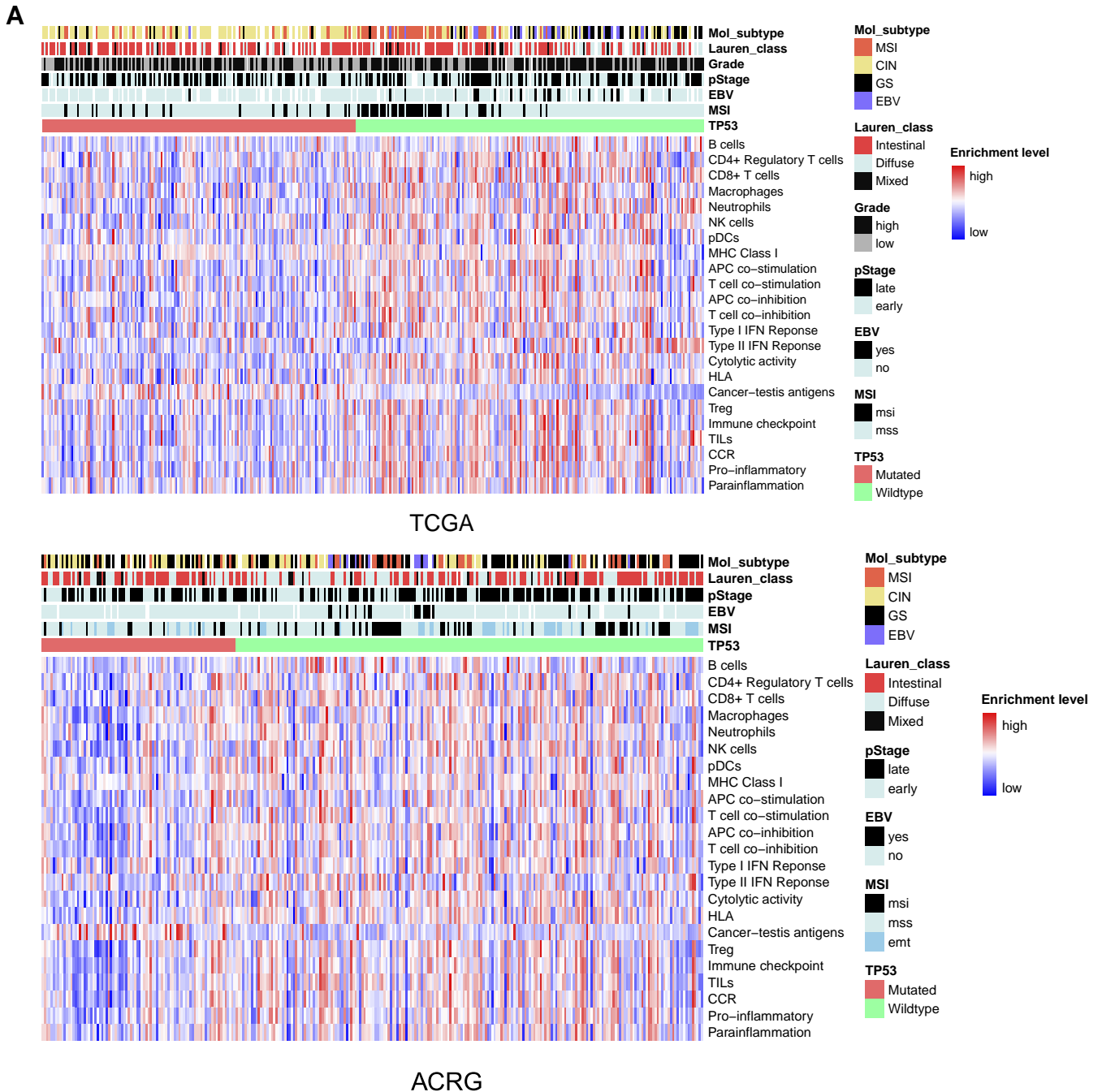
**Authors' Contributions**

ZJ performed the major data analyses, and helped prepare for the manuscript. ZL performed data analyses. ML performed data

analyses. CC performed data analyses. XW conceived the research, designed the analyses strategies, and wrote the manuscript. All the authors read and approved the final manuscript.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.



**Figure 7.** TP53-mutated cancers likely have decreased immune activities compared to TP53-wildtype cancers. (A) All the immune gene sets (except of CTA) likely have lower enrichment levels in TP53-mutated GCs than in TP53-wildtype GCs. Lauren\_class, the Lauren classification of GC; Mol\_subtype, the molecular classification of GC by TCGA. (B) TP53-mutated cancers have the significantly lower degree of immune infiltration than TP53-wildtype cancers in various cancer types. (C) TP53 mutations may disrupt the functions of wildtype p53 that maintain genome stability, and result in decreased tumor immunity in cancer. HNSC, head and neck squamous cell carcinoma; UCEC, uterine corpus endometrial carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; LUSC, lung squamous cell carcinoma; GBM, glioblastoma multiforme; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma.

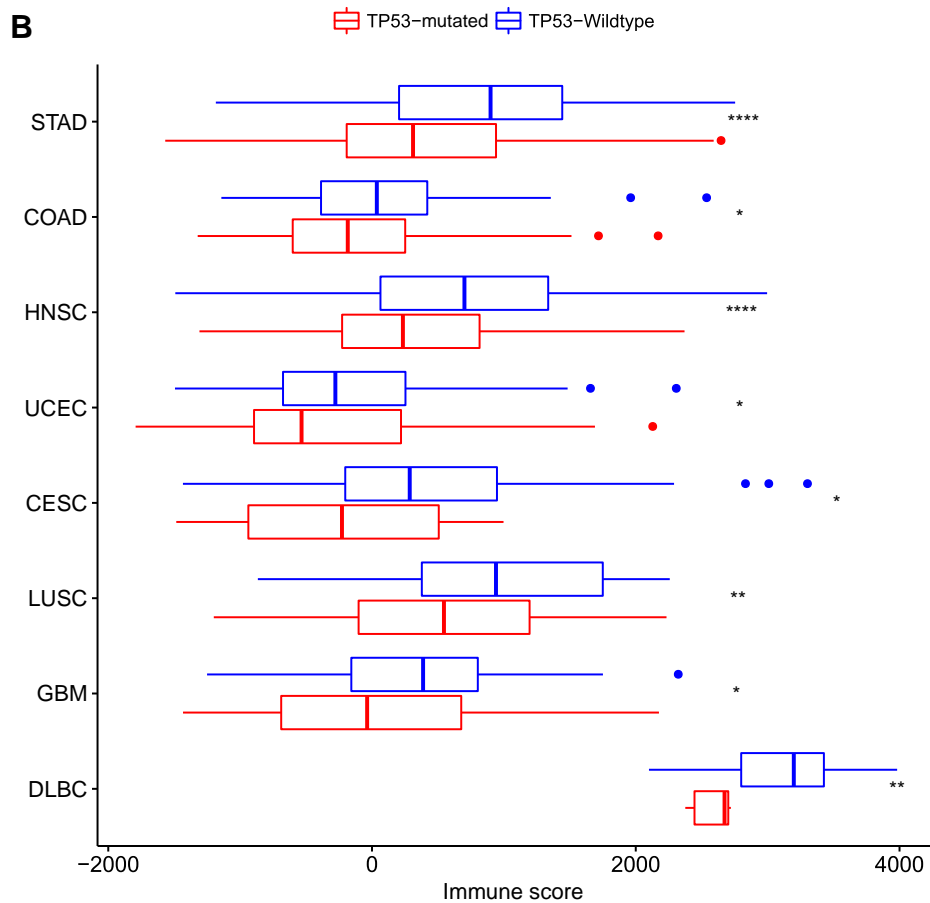


Figure 7. (continued).

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Not applicable.

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