Original Article

Non-disruptive mutation in *TP53* DNA-binding domain is a beneficial factor of esophageal squamous cell carcinoma

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Background: *TP53* is frequently altered in esophageal squamous cell carcinoma (ESCC). However, the landscape of *TP53* mutation and its effects on patients remain controversial.

Methods: Somatic mutations of *TP53* in 161 patients with resectable ESCC were identified by next-generation sequencing (NGS) and verified by immunohistochemistry (IHC). Patients were stratified into seven *TP53* mutations, and depending on the extent of the effect on the encoded protein, it was divided into "disruptive" and "non-disruptive" types. The association of *TP53* mutation with clinicopathological properties and disease outcome was investigated.

Results: *TP53* mutations were discovered in 85.7% patients, of which 68.9% carried mutations in the DNA-binding domain (DBD). A total of 47.8% and 37.9% patients had disruptive and non-disruptive *TP53* mutations, respectively. Most patients carried only one *TP53* mutation, but 15.5% had double mutations. *TP53* mutations were dominant in exons 5 to 8. Missense mutation was the most frequent (97/163, 59.5%), and the top five frequently occurring variations included R273X, Y220X, H193, H179X, and R175H. Multivariable analysis revealed non-disruptive mutation in *TP53* DBD as the independent prognostic predictor for progression-free survival (PFS) and overall survival (OS). The expression of p53 positively correlated with non-disruptive mutation in DBD. Patients with high p53 protein expression showed better outcomes.

Conclusions: Non-disruptive mutation in *TP53* DBD serves as an independent beneficial prognostic factor of prolonged survival in resectable ESCC.

Keywords: Esophageal squamous cell carcinoma (ESCC); *TP53* mutation; next-generation sequencing (NGS); prognosis

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Introduction

Esophageal cancer is one of the deadliest diseases worldwide, and 90% of esophageal cancer cases belong to esophageal squamous cell carcinoma (ESCC) in China (1,2). The tumor suppressor gene *TP53* is the most frequently mutated gene in ESCC. This gene comprises 11 exons and 10 introns. The p53 protein encoded by *TP53*, is a 393 amino acid residue protein with seven functional domains, including an acidic N-terminus transcription activation domain (TAD) from residue 1 to 42 and 55 to 75, an activation domain 2 (AD2) from residue 43 to 63, a DNA-binding domain (DBD) from residue 102 to 292, a nuclear localization signaling (NLS) domain from residue 316 to 325, a C-terminal oligomerization domain (OD) from residue 307 to 355, and a tetramerization domain (TET) from residue 356 to 393 (3,4). The coding sequence of *TP53* gene comprises five regions, namely, 13–19, 117–142, 171–192, 236–258, and 270–286, that show a high degree of conservation among vertebrates, primarily in exons 2, 4, 5, 7, and 8, respectively. Aside from the coding region 13–19, the other four conserved areas are located in the DBD (4-6). The p53 DBD provides a scaffold for a flexible DNA-binding surface, which is formed by two large loops (loop L2, residues 163–195; L3, residues 236–251) that bind to a zinc atom (7). The transcriptional activity mediated by the DBD is the primary mechanism underlying the tumor suppressor activity of p53 (8).

p53 plays a crucial role in many cellular processes, including autophagy (9), metabolism (10), differentiation (11), and DNA repair. It is one of the most commonly mutated genes in human cancers, and over 50% human tumors carry *TP53* mutations (12,13). Mutant p53 has been reported to overturn crucial cellular pathways and promote cancer cell proliferation and survival, invasion, migration, metastasis, and chemoresistance (12-15). However, mutant p53 protein not only loses its tumor suppressive functions but also gains new oncogenic properties (16). The function and prognostic values of mutant p53 are yet incompletely understood (4,17).

Several criteria have been used to classify *TP53* mutations, including mutation status, mutation number, allele frequency, mutation region, degree of disturbance in p53 protein structure or function, and p53 protein expression. Classification into "disruptive" and "non-disruptive" forms based on functional effects on p53 protein has been proposed (18). Disruptive mutations are defined as (I) any mutations that introduce a stop codon (nonsense, frameshift, and intronic) or (II) an in-frame deletion within the L2 or L3 loop or missense mutations in the L2 or L3 loop replacing one residue by another with different polarity or charge. Non-disruptive variations include (I) missense mutations and in-frame deletions outside the L2–L3 loop or (II) missense mutations within the L2–L3 loop without any change in polarity or charge (8,18). Disruptive mutations are likely to cause loss of activity of p53 protein, while non-disruptive variants may retain the functional properties of wild-type p53. Skinner and colleagues proved that disruptive *TP53* mutations lead to locoregional recurrence in head and neck cancers (19). Non-disruptive mutation serves as an independent prognostic factor of shorter survival in advanced non-small lung cancer (8). Considerable efforts have been directed to clarify the impact of *TP53* mutations on the prognosis of patients with ESCC, but the results remain controversial. The number of patients enrolled, differences in follow-up methods and time, and various classifiers of *TP53* mutations have led to contradictory outcomes, particularly the scattered mutation spectrum of *TP53* (20). ESCC is one of the lethal cancers, highlighting the need for the discovery of novel biomarkers to assist disease management (21).

Here, we examined the whole exons of *TP53* gene in 161 patients with resectable ESCC by next-generation sequencing (NGS), and analyzed the expression level of p53 protein by immunohistochemistry (IHC). We stratified patients by multiple *TP53* mutation classifiers and analyzed the correlation of *TP53* mutations with clinical parameters. We identified the most relevant classification of *TP53* mutations with respect to patient outcome.

Methods

Patients and samples

Formalin-fixed paraffin-embedded (FFPE) specimens with matched blood samples as reasonable controls were available from 161 patients with ESCC. These patients underwent surgery from May 2008 to June 2014, and their tissue samples were collected and stored in the Tissue Bank of Zhejiang Cancer Hospital. All subjects had provided written informed consent, and this study was conducted following the Declaration of Helsinki Principles and approved by the Institutional Review Committee of Zhejiang Cancer Hospital. Patient data were available for age, gender, body weight, height, smoking and alcohol consumption status, and tumor size, localization, differentiation, TNM stage, surgery, and treatment. The 8th edition of AJCC/UICC staging system was used for TNM staging. Information on tumor differentiation and histopathologic classification

was collected from pathology reports and independently examined by two senior pathologists.

NGS and data analysis

FFPE samples containing at least 20% tumor cells [as determined from the examination of hematoxylin and eosin (H&E)-stained sections] were deparaffinized and genomic DNA (gDNA) was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) in accordance with manufacturer's instructions, followed by quantification using PicoGreen fluorescence assay (Invitrogen). The gDNA from white blood cell (WBC) samples was extracted using QIAamp DNA Blood Mini Kit (Qiagen) as described by the manufacturer.

All sequencing processes were accomplished in 3DMed Medical Laboratory Co., Ltd (Shanghai) (22). The details of NGS method are described in manuscript communicated for publication (Paper #NCOMMS-18-38299C). Illumina NextSeq 500 was used to sequence samples with the IDT xGen hybridization buffer. To evaluate the quality of the sequencing data, we used FastQC software (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). BWA-MEM was used to map the sequence data to the human genome (hg19) reference. The results were sorted, and duplicate reads were removed with Picard (http://broadinstitute.github.io/picard/) (23,24). In general, the mean sequencing depth of FFPE samples was 394× and that of matched blood samples was 431×.

Classification of TP53 mutations

Mutations were classified as "disruptive" and "non-disruptive", as per a reported article (18). Supplementary *Table S1* shows the other six summarized criteria, including *TP53* mutation status, mutation numbers, mutation frequency, degree of disturbance of p53 protein structure or function, functional domain, and domain and function.

Assessment of IHC

Mouse anti-p53 protein monoclonal antibody (ZM-0408, ZSGB-BIO, Beijing, China) was used to detect the expression of p53 in FFPE specimens. Complete IHC protocols are described in our previous study (25). p53-stained slides were digitally imaged with a Digital slice scanner (KF-PRO-005-EX) and graded by two independent pathologists. Intensity was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining) (26,27). p53 expression level in each sample was assessed as per IHC score, which was calculated using the following formula: staining intensity × percentage of positive cells (28-30). The resulting score ranged from 0 to 300. Receiver operating characteristic (ROC) curve analysis was performed to obtain the best cutoff values by the Youden index (sensitivity + specificity − 1) (31) to divide patients into two cohorts as follows: low expression and high expression.

Statistical analyses

Descriptive statistics were used to summarize the characteristics of patients; the results were expressed as frequencies and percentages for categorical variables. All factors were considered as categorical variables. Spearman's rank correlation analysis was used to assess the correlation between *TP53* mutation status and clinicopathologic features. Differences in the distribution of *TP53* mutation types under various clinicopathologic variables were evaluated using the chi-square test.

Progression-free survival (PFS) was calculated for the patients in our ESCC cohort from time of surgery to cancer recurrence or last follow-up. Overall survival (OS) was defined as the time from surgery to death or last follow-up. The data for patients who were alive without recurrence at the time of analysis were censored at the last follow-up. Median PFS and OS and 95% confidence interval (CI) were evaluated using the Kaplan-Meier method, and survival curves were compared by the log-rank test. The Cox proportional hazard model was used to explore possible survival differences and identify factors affecting survival. Cox regression univariate and multivariate analyses were used to generate survival hazard ratio (HR) and 95% CI. Levels of statistical significance were bilaterally set at $P<0.05$. All calculations were performed with Statistical Package for Social Science (SPSS) for Windows (version 19.0; IBM Corp., Armonk, NY), and figures were created using GraphPad Prism (version 7.0; GraphPad Software, San Diego, CA).

Results

Patient characteristics and TP53 status

In total, 161 patients with ESCC were grouped according to *TP53* mutation status as detected by NGS, and their clinicopathologic features are shown in *Table 1*. The median age of the cohort was 61 years and 50.9% patients

Table 1 Baseline characteristics of the patients

Clinical pathological variables	Cases (%)	Patients with *TP53* mutation (%)	Patients with wide-type *TP53* (%)	P value
Overall	161 (100.0)	138 (85.7)	23 (14.3)	
Age				1
≤60	79 (49.1)	69 (87.3)	10 (12.7)	
>60	82 (50.9)	69 (84.1)	13 (15.9)	
Gender				0.181
Male	140 (87.0)	122 (87.1)	18 (12.9)	
Female	21 (13.0)	16 (76.2)	5 (23.8)	
BMI				0.544
<18.5	31 (19.3)	24 (77.4)	7 (22.6)	
18.5–25	116 (72.0)	101 (87.1)	15 (12.9)	
>25	14 (8.1)	12 (85.7)	2 (14.3)	
Alcohol				0.677
Yes	117 (72.7)	101 (86.3)	16 (13.7)	
No	43 (26.7)	36 (83.7)	7 (16.3)	
NA	1 (0.6)	1 (100.0)	0 (0.00)	
Smoking				0.925
Yes	124 (77.0)	106 (85.5)	18 (14.5)	
No	36 (22.4)	31 (86.1)	5 (13.9)	
NA	1 (0.6)	1 (100.0)	0 (0.00)	
Family history				0.350
Yes	48 (29.8)	43 (89.6)	5 (10.4)	
No	112 (69.6)	94 (83.9)	18 (16.1)	
NA	1 (0.6)	1 (100.0)	0 (0.00)	
Tumor differentiation				0.587
Well	2 (1.2)	2 (100.0)	0 (0.00)	
Moderate	118 (73.3)	103 (87.3)	15 (12.7)	
Poor	37 (23.0)	31 (83.8)	6 (16.2)	
NA	4 (2.5)	2 (50.0)	2 (50.0)	
TNM stage				0.540
I	1 (0.6)	1 (100.0)	0 (0.00)	
II	4 (2.5)	3 (75.0)	1 (25.0)	
III	156 (96.9)	134 (85.9)	22 (14.1)	
Tumor primary site location				0.604
Upper thoracic	13 (8.1)	12 (92.3)	1 (7.7)	
Middle thoracic	91 (56.5)	76 (83.5)	15 (16.5)	
Lower thoracic	57 (35.4)	50 (87.7)	7 (12.3)	

NA, not available.

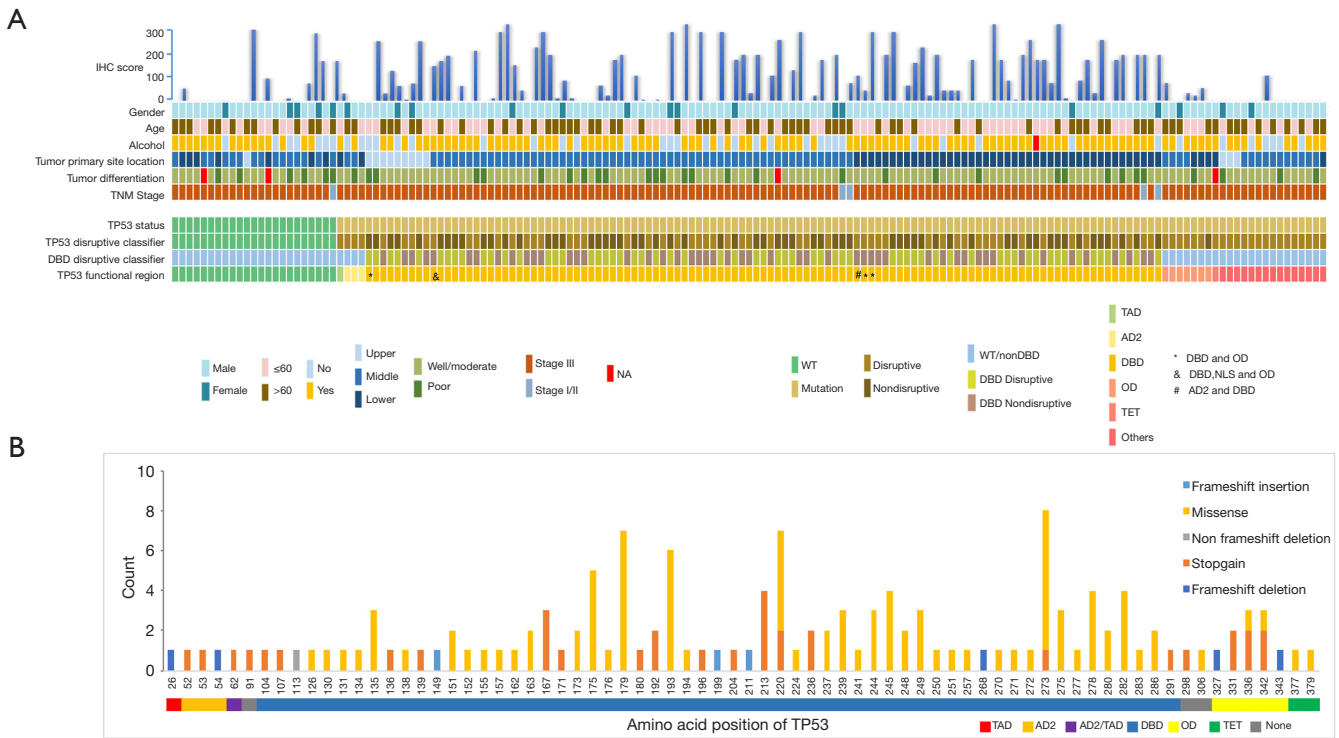

Figure 1 Mutational landscape of *TP53* in 161 resectable ESCC patients (A) mutation spectrum of *TP53* by different classifiers and IHC score of each patients by IHC (B) the location of *TP53* mutations.

were older than 60 years. In total, 87.0% were males. The majority of patients had smoking (77.0%) and drinking (72.7%) habits, and 8.1% patients were considered obese with a body mass index (BMI) >25. Based on pathological characteristics, 96.9% tumors were TNM stage III, 73.3% were moderately differentiated tumor, and 56.5% were located in middle thoracic. For treatment, 3.72% patients received neoadjuvant treatment, 49.07% received adjuvant treatment, and 47.2% [76] patients received neither neoadjuvant nor adjuvant treatment. *TP53* mutations were detected in tumors from 138 patients (85.7%), and the mutation status was not significantly associated with gender, histology, BMI, smoking, alcohol consumption, family history, tumor stage, differentiation, or location in either *TP53* wild-type (*TP53*-wt) or *TP53* mutant (*TP53*-mut) group (*Table 1*).

Mutational landscape of TP53

All coding exons of *TP53* gene were examined by NGS, and 163 mutations were discovered in 138 patients. In general, 85.7% (138/161) patients had *TP53* mutations. The different types of *TP53* mutations detected in our study and their distribution are shown in *Figure 1*. Most patients (113/138, 81.9%) carried only one *TP53* mutation, while 15.5% had double mutations. *TP53* mutations were detected in exons 3 to 11, and were dominant among exons 5 to 8 (109/161, 67.7%) (*Figure 1B*). These mutations were mainly detected in DBD (111/161, 68.9%) (*Figure 1A*). Missense mutation was the most frequently detected mutation (97/163, 59.5%), followed by stop-gain (34/163, 20.9%), splicing (18/163, 11.0%), and frameshift deletion/insertion (8/163, 4.9%) (*Figure 1B*). The most frequently occurring variation was R273X (H/L/C) that accounted for 4.9% (appeared in 8 cases) cases, followed by Y220X (C/*) discovered in 7 patients, H193 (Y/L/R) and H179X (Y/L/R) in 6 patients, and R175H in 5 cases (*Figure 1B*). In addition, 55.8% (77/138) patients with *TP53* mutations showed disruptive mutations, of which 64.9% (50/77) were observed in DBD (*Figure 1A*).

TP53 mutation classification and survival

The follow-up period ranged from 0.1 to 120 months, with

Table 2 Univariate Cox regression analysis of predictors for PFS and OS of ESCC patients

Characteristic	Case (%)	Progression-free survival			Overall survival		
		HR	95% CI	P value	HR	95% CI	P value
Age (years)							
≤60	79 (49.1)	1			1		
>60	82 (50.9)	0.83	0.53–1.31	0.426	0.77	0.49–1.22	0.264
Gender							
Male	140 (87.0)	1			1		
Female	21 (13.0)	0.78	0.39–1.57	0.488	0.70	0.32–1.52	0.368
Family history							
No	112 (69.6)	1			1		
Yes	48 (29.8)	1.13	0.69–1.82	0.632	1.39	0.86–2.24	0.175
Smoking							
No	124 (77.0)	1			1		
Yes	36 (22.4)	1.62	0.91–2.89	0.104	1.52	0.84–2.76	0.171
Alcohol							
No	117 (72.7)	1			1		
Yes	43 (26.7)	1.31	0.78–2.20	0.306	1.56	0.87–2.79	0.134
Tumor primary site location							
Upper thoracic	13 (8.1)	1			1		
Middle thoracic	91 (56.5)	0.96	0.43–2.14	0.918	0.86	0.40–1.83	0.692
Lower thoracic	57 (35.4)	1.15	0.51–2.62	0.738	0.93	0.42–2.10	0.863
Tumor differentiation							
Well + moderate	120 (76.4)	1			1		
Poor	37 (23.6)	1.25	0.74–2.14	0.406	1.40	0.81–2.46	0.225
TNM stage							
I+II	5 (3.1)	1			1		
III	156 (96.9)	0.93	0.13–6.68	0.939	0.94	0.13–6.80	0.954

Statistical analysis does not include cases of "NA" in *Table 1*.

a median of 39.47 months for patients whose data were censored. During follow-up, 88 cases of recurrence and 87 deaths due to tumor progression were reported. Univariate Cox analysis showed that clinical pathological variables were not predictors of PFS and OS (*Table 2*). *TP53*-mut patients had a median OS of 25.57 months versus 38.35 months for *TP53*-wt patients, but the difference was not statistically significant (HR: 0.708; 95% CI, 0.37–1.34; P=0.29, *Table 3*). Different types of mutations in *TP53* gene have different effects on the functionality of the protein. Hence, we stratified patients into multiple *TP53* mutation classifiers based on different mutant features (*Table S1*). Some *TP53* mutation classifiers, including hotspot mutations, mutation numbers, and allele frequency (data not shown), failed to predict the prognosis of patients (*Table 3*).

Mutations in DBD showed benefit in PFS (HR: 0.48, 95% CI: 0.26–0.92, P=0.026, *Table 3*, *Figure 2A*) but no significance with OS (HR: 0.65, 95% CI: 0.34–1.25, P=0.198, *Table 3*, *Figure 2B*). According to the degree of disturbance to the structure and function of p53 protein,

Table 3 Univariate Cox regression analysis of predictors for PFS and OS of ESCC patients by different *TP53* classifier

Characteristic	Case (%)	Progression-free survival			Overall survival		
		HR	95% CI	P value	HR	95% CI	P value
Status							
Wild type	23 (14.3)	1			1		
Mutation	138 (85.7)	0.59	0.32–1.10	0.094	1.63	0.94–2.86	0.084
Mutation frequency							
Wild type	23 (14.3)	1			1		
Hotspot	17 (10.6)	0.49	0.19–1.24	0.129	0.56	0.22–1.46	0.236
Non-hotspot	121 (75.2)	0.61	0.33–1.13	0.116	0.73	0.38–1.40	0.341
Mutation number							
Wild type	23 (14.3)	1			1		
Single mutation	113 (70.2)	0.59	0.31–1.10	0.094	0.66	0.34–1.27	0.211
Double mutations	25 (15.5)	0.62	0.27–1.41	0.253	0.96	0.44–2.11	0.918
Functional domain							
Wild type	23 (14.3)	1			1		
DBD	111 (68.9)	0.48	0.26–0.92	0.026	0.65	0.34–1.25	0.198
Non-DBD	27 (16.8)	1.21	0.59–2.50	0.601	1.06	0.48–2.37	0.886
Disturbance of structure or function							
Wild type	23 (14.3)	1			1		
Disruptive	77 (47.8)	0.78	0.41–1.49	0.451	0.96	0.49–1.82	0.904
Non-disruptive	61 (37.9)	0.41	0.21–0.83	0.013	0.49	0.24–1.00	0.05
Domain and function							
Wild type or non-DBD	50 (31.0)	1			1		
DBD disruptive	50 (31.0)	0.54	0.31–0.94	0.029	0.88	0.51–1.53	0.651
DBD non-disruptive	61 (38.0)	0.36	0.21–0.62	0	0.49	0.28–0.85	0.012

we divided the mutations into two categories, namely the "disruptive" and "non-disruptive" type, and found that patients with non-disruptive mutation had better PFS (HR: 0.41, 95% CI: 0.21–0.83, P=0.013, *Table 3*, *Figure 2C*) and extended OS (HR: 0.49, 95% CI: 0.24–1.00, P=0.050, *Table 3*, *Figure 2D*). Together the results of DBD and disruptive analyses led to the creation of a new classifier, "DBD disruptive" and "DBD non-disruptive". Univariate Cox regression analysis showed that the patients with non-disruptive p53 mutation in DBD had better PFS (P<0.001, *Table 3*, *Figure 2E*) and OS (P=0.005, *Table 3*, *Figure 2F*) than those with *TP53*-WT or *TP53*-mut not located in DBD.

In the multivariate Cox proportional hazard model (*Table 4*), the presence of a DBD non-disruptive *TP53* mutation was significantly associated with increased PFS (HR: 0.34; 95% CI: 0.19–0.61; P=0.000) and OS (HR: 0.42; 95% CI, 0.23–0.77; P=0.005). The presence of non-disruptive *TP53* mutation in DBD was an independent prognostic factor for resectable ESCC.

IHC

The IHC result was shown in *Figure 3*. The best cutoff value of 170 was used to distinguish patients into low and high p53 expression groups. Of these, 77.1% (118/153) patients were categorized into the low expression group and

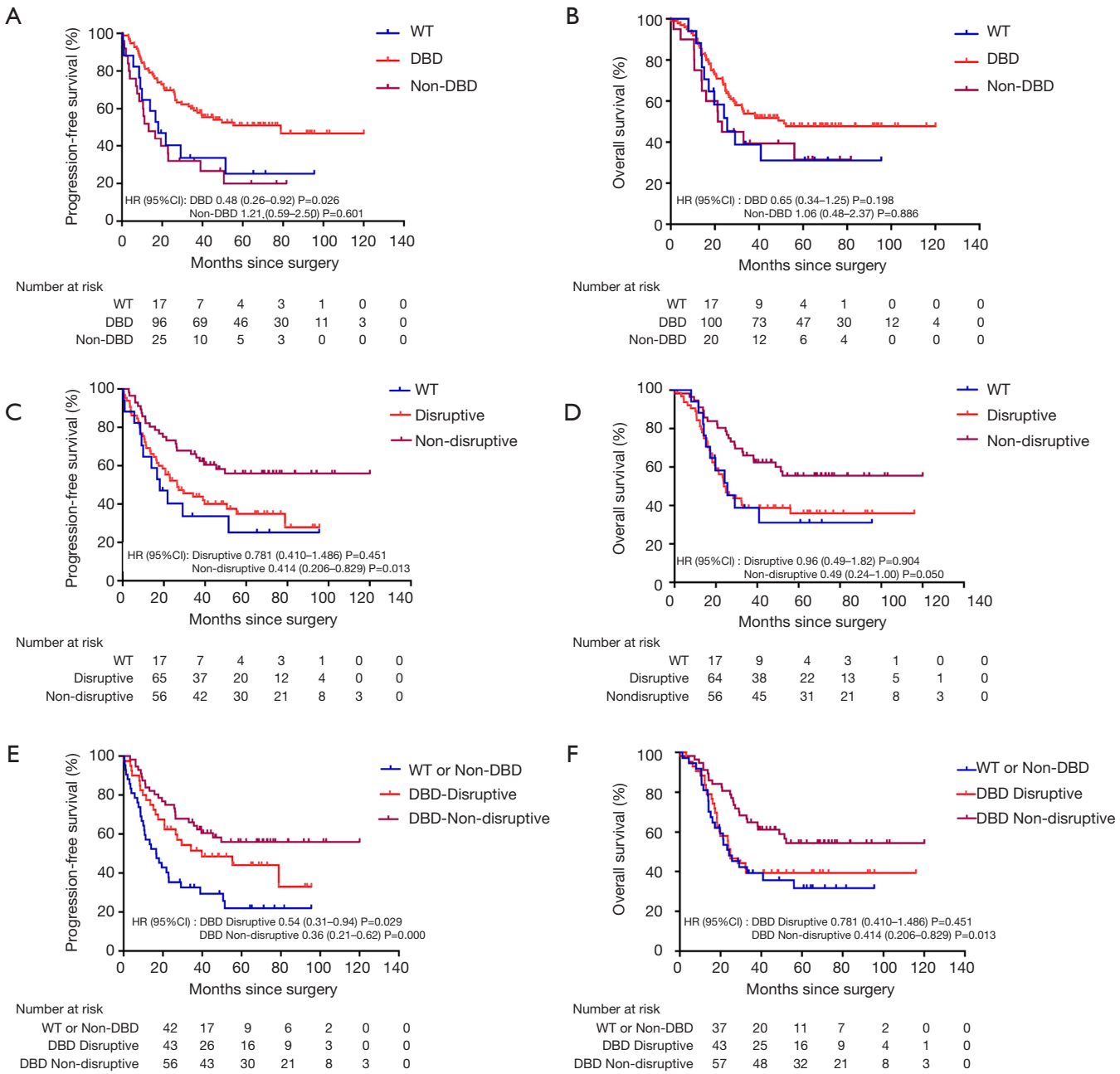

Figure 2 Survival analysis of different classifier of *TP53*. (A) The PFS of mutations in DBD or non-DBD; (B) the OS of mutations in DBD or non-DBD; (C) the PFS of disruptive mutations or non-disruptive mutations; (D) the OS of disruptive mutations or non-disruptive mutations; (E) the PFS of disruptive mutations or non-disruptive mutations in DBD; (F) the OS of disruptive mutations or non-disruptive mutations in DBD.

Table 4 Cox regression multivariate analysis

Characteristic	Progression-free survival			Overall survival		
	HR	95% CI	P value	HR	95% CI	P value
Functional domain						
Wild type	1		0.004	1		0.163
DBD	0.63	0.25–1.06	0.044	0.57	0.28–1.15	0.114
Non-DBD	1.69	0.62–4.58	0.731	0.88	0.38–2.06	0.768
Disturbance of structure or function						
Wild type	1		0.01	1		0.004
Disruptive	0.8	0.29–1.64	0.542	0.87	0.43–1.76	0.699
Non-disruptive	0.39	0.18–0.84	0.017	0.38	0.17–0.81	0.013
Domain and function						
Wild type or Non-DBD	1		0.001	1		0.006
DBD disruptive	0.6	0.34–1.05	0.074	0.94	0.53–1.66	0.838
DBD non-disruptive	0.34	0.19–0.61	0	0.42	0.23–0.77	0.005
Protein expression						
Low TP53 mutation protein	1			1		
High TP53 mutation protein	0.33	0.16–0.70	0.004	0.46	0.24–0.87	0.016

The Cox regression multivariate analysis contains seven patients' clinical pathological variables, including gender, age, family history, TNM, tumor differentiation, smoking and alcohol history.

35 into the high expression group. The median IHC score was 161.8, 106.1, and 89.2 in exon 7, 8, and 5, respectively. Exons 5–8 were the top 4 locations for mutations and mutated protein expression (*Figure 1A*). The results of chi-square test showed that the expression of p53 was associated with missense mutations (P<0.001), mutations in DBD (P=0.001), hotspot mutations (P=0.020), disruptive mutation (P=0.010), and non-disruptive mutation in DBD (P=0.001) (*Table S2*). The expression level of TP53 protein was independent of the mutational status (P=0.117) and mutation numbers (P=0.270). Furthermore, Cox regression univariate analysis showed that the patients from the high p53 expression group showed better outcomes (PFS: HR: 0.33, P=0.004; OS: HR: 0.46, P=0.016) (*Table 3*).

Conclusions

We analyzed *TP53* mutations in 161 patients with resectable ESCC and described a new standard method to classify *TP53* mutations. *TP53* non-disruptive mutation located in DBD characterizes a distinct prognostic group of patients with ESCC with significantly extended survival. We found that patients with high p53 protein expression (IHC score >170) showed better outcomes. *TP53* non-disruptive mutation in DBD and IHC results highlight the clinical usefulness of this prognostic marker in resectable ESCC.

We detected *TP53* mutations in 85.71% patients with ESCC, consistent with the frequency described in The Cancer Genome Atlas (TCGA) database. However, different studies have shown variations in *TP53* mutation frequency in ESCC, as determined by sequence coverage and other methods. Examination of exons 5 to 8 with traditional methods such as Sanger sequencing showed that almost 40% patients carried *TP53* mutations (32-34). *TP53* mutation frequency may reach up to 93% with NGS in ESCC (35). This phenomenon shows that the genomic region is essential for *TP53* genotyping. The most frequently detected *TP53* mutation type in ESCC was C>T transition (up to 85%) that was located in exons 5 to 8 (35). We found similar results. Nonsynonymous SNV was the most dominant mutation. In general, the *TP53* mutational landscape observed in the present study is consistent with that previously reported.

Hotspot mutations are important for driver genes

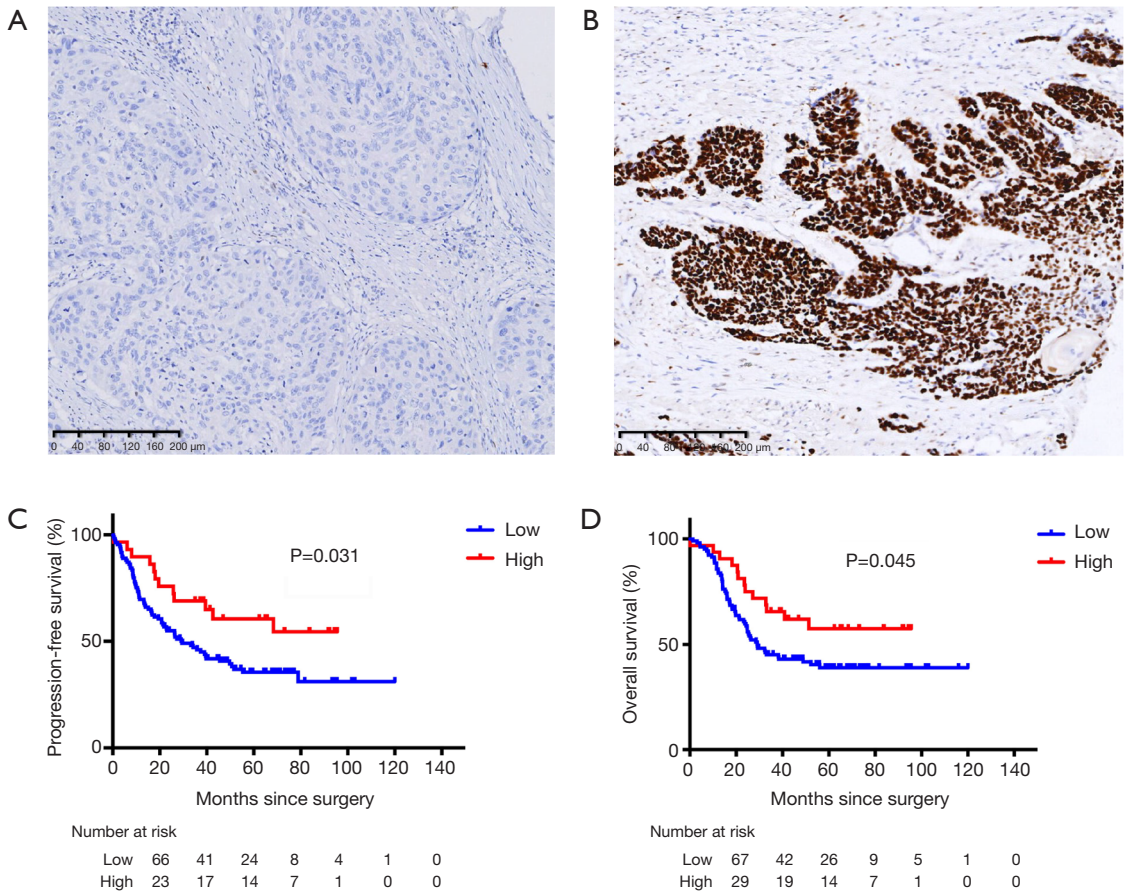

Figure 3 Immunohistochemistry result. (A) TP53 immunohistochemistry result of No.86 patient: (–) score: 0; (B) TP53 Immunohistochemistry result of No.102 patient: (+++, 100%) score: 300; (C) the survival analysis of PFS about immunohistochemistry; (D) the survival analysis of OS about immunohistochemistry.

such as EGFR primarily located in exons 18–21. In such situations, target NGS panel, droplet digital polymerase chain reaction (PCR), or quantitative PCR instead of whole exome sequencing, may reduce the cost and turnaround time. However, *TP53* mutations are dispersed in human cancers, and aside from the "hotspot mutations", several other mutations are known to affect p53 protein functions. Hotspot mutations of *TP53* are inconsistent in different studies. Maeng (36) found *TP53* hotspot mutations in R306, R175H, and R273C, but others have defined hotspot mutations in R175, G245, R248, R249, R273, and R282 in ESCC (37,38). In our study, we found some variants, including R273X, Y220X, H179X, H193, and R175, that showed frequent mutations. However, these "hotspots" were not so frequent, as the most common mutation R273X appeared only in eight cases. Hence, it is much more suitable to detect *TP53* gene by NGS instead of identifying hotspot mutations.

TP53 mutation, one of the most frequently observed mutations in human cancers, has been studied in various carcinomas (39). Studies with *TP53* have mainly focused on mutation status and analyzed the effect of prognosis or clinical features, including smoking, drinking, and family history of cancer (40). However, recent reports have shown the shortcomings associated with these classifications. Efforts have been directed to define *TP53* mutations to understand the exact nature of *TP53*. As per the effects on p53 protein function, Poeta and his colleagues (18) first proposed a standard method in head and neck squamous cell carcinoma (HNSCC) by dividing mutations into "disruptive" and "non-disruptive" forms. Matteo Canale (41) and colleagues tried to use a different exon mutation to classify *TP53* mutations in non-small cell lung cancer (NSCLC). A meta-analysis showed that the OS of ESCC

patients with different *TP53* mutation number, frequency of allele was no differential in survival outcomes (21). Several studies have proved that the expression of p53 is more critical than *TP53* mutations (21,42). Different mutation could result in different proteins, activate or suppress signaling pathways, and produce a range of significant biological effects (43,44). Hence, we considered the impact of risk factors for ESCC on prognosis, including BMI, gender, smoking, and alcohol consumption. However, we failed to observe any direct evidence that these risk factors would reduce PFS or OS.

Several strategies have been used to group *TP53* mutations. After many attempts, we classified *TP53* mutations into "disruptive" or "non-disruptive" types. This classification has been used with HNSCC (18), NSCLC (8), breast cancer (16), and ovarian cancer (45). However, no research report has described this classification in ESCC. In comparison with patients from disruptive mutation group, those from *TP53* non-disruptive mutation group had better treatment response for head and neck cancer (19). However, in NSCLC, *TP53* disruptive cluster showed prolonged OS (8). In our study, we clearly found that non-disruptive *TP53* mutation was associated with good prognosis. In ovarian cancer, disruptive *TP53* mutations showed survival benefits (45). The association between *TP53* non-disruptive mutation and prognosis was significantly different in various cancers and may be related to the following factors: pathological types of tumors (adenocarcinoma versus squamous cell carcinoma) (42), treatment regime (new targeted therapy versus traditional radiotherapy/chemotherapy), and other molecular features. To test and verify our results, we used the whole exome sequencing data by Gao *et al.* (35) available at the European Genome-phenome Archive (EGA) under the accession number EGAS00001000932. It included results of 113 Chinese patients with ESCC. Even with a P value >0.05, a trend of non-disruptive mutation showing longer OS than the other two types was observed (*Figure S1*).

The result of IHC proves our view. p53 expression level and related mutations were associated with the prognosis of patients. IHC of p53 was related to some mutations, which affected protein expression.

In spite of the specificity and sensitivity of IHC and the overexpression of WT p53 (46,47), five samples considered as WT by NGS showed false-positive results. As p53 is a regular routine index in pathological IHC reports, the conversion of staining results into IHC scores is convenient. Hence, the use of this value to estimate prognosis in clinic may be valuable for patients that cannot afford sequencing and may help clinicians to access patient prognosis.

Some limitations of this study include the limited case numbers with *TP53* WT and stage I and II cases and incomplete data (such as smoking and alcohol history did not distinguish between former consumers and non-consumers).

In conclusion, we demonstrate that the non-disruptive mutation in *TP53* DBD and p53 expression level both have significant clinical importance in patients with resectable ESCC. These parameters may help clinicians to assess the prognosis of patients.

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Footnote

Conflicts of Interest: The authors declare no conflicts of interest.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All subjects had provided written informed consent, and this study was conducted following the Declaration of Helsinki Principles and approved by the Institutional Review Committee of Zhejiang Cancer Hospital.

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Supplementary

Table S1 Different criteria of *TP53* mutations classification

Mutation classifiers	Mutation type	Criteria of classification
TP53 status	Wild type	Patients with no mutation detected
	Mutation	Patients with any mutation detected
TP53 mutation numbers	Single	Patients with only one mutation discovered in TP53
	Double	Patients with two mutations found in TP53
TP53 mutation frequency	Hotspot	Mutations at TP53 codons (175, 245, 273 and 248)
	Non-hotspot	Mutations at TP53 codons besides 175, 245, 273 and 248 Stopgain; frameshift deletion; splicing
Degree of disturbance of the p53 protein structure or function	Disruptive	In-frame deletions within L2–L3 (163-195, 236-251)
		Missense within L2–L3 & replacing a residue with another polarity or charge
	Non-disruptive	In-frame deletions outside L2–L3 if within L2-L3, replacing a residue with another of the same polarity or charge
Degree of disturbance of the p53 protein structure or function	Truncate	Stopgain; frameshift deletion; splicing
	Missense	Nonsynonymous SNV
TP53 functional domain	DBD	Mutations at TP53 codons 98–292
	Non-DBD	Mutations at TP53 codons 1–97, 293–393
TP53 domain and function	DBD disruptive	Stopgain; frameshift deletion at TP53 codons 98–292
		Missense within L2–L3 & replacing a residue with another polarity or charge
	DBD non-disruptive	Mutations at TP53 codons 98–162,196–235, 252–292
		If within L2–L3, replacing a residue with another of the same polarity or charge

Table S2 The correlation coefficient between IHC score and mutation type

Mutation type	IHC of p53 mutation protein	
	Correlation coefficient	P
Wild type (disruptive *vs.* non-disruptive)	0.23	0.001
Wild type (DBD *vs.* non-DBD)	–0.08	0.143
Wild type or non-DBD (DBD disruptive *vs.* DBD non-disruptive)	0.28	<0.001

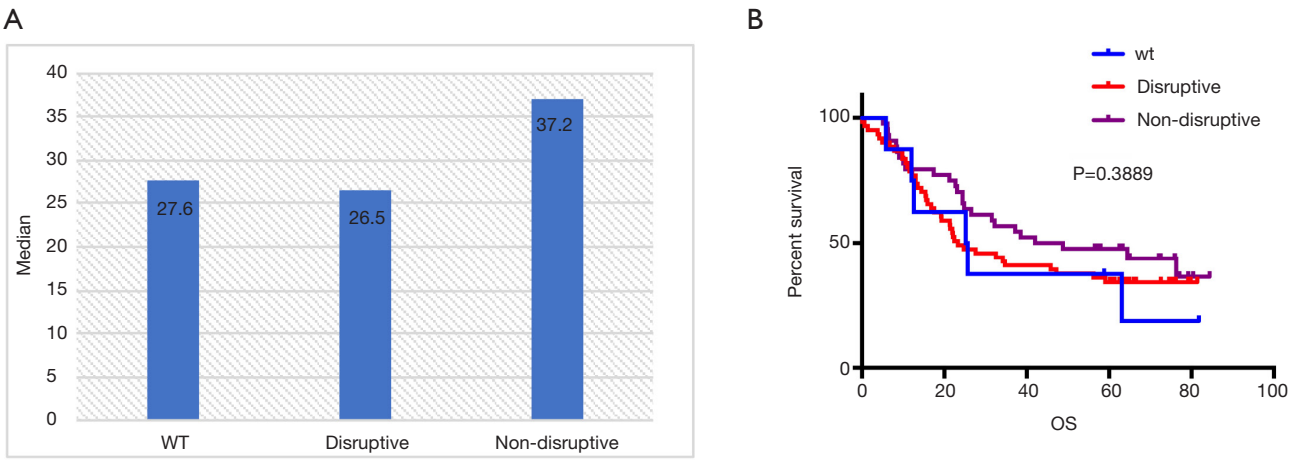

Figure S1 Reanalysis the exome sequencing data files of Nat Genet. 2014 Oct;46(10):1097-102, including 113 Chinese ESCC patients. (A) The median of mutations; (B) survival analysis of different classified type.