

Δ 133p53/FLp53 Predicts Poor Clinical Outcome in Esophageal Squamous Cell Carcinoma

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Background: p53 isoform Δ 133p53 is directly transactivated by p53 and antagonizes p53 activities in cancer progression. However, its correlation with prognosis and cancer recurrence in esophageal squamous cell carcinoma (ESCC) is still unclear.

Patients and Methods: Expression of Δ 133p53 and Δ 133p53/full-length p53 (FLp53) in tissues and serums of 180 ESCC patients was evaluated using qRT-PCR. Patients were divided into high- and low-expression groups according to the cutoff value determined by X-tile 3.6.1 software. Survival analysis was performed by the Kaplan–Meier method. Univariate and multivariate Cox survival analyses were applied to assess the hazard ratios (HRs).

Results: Tissue Δ 133p53 expression and Δ 133p53/FLp53 ratio were significantly increased in ESCC tissue compared with adjacent normal tissue. Pre-operative Δ 133p53 expression and Δ 133p53/FLp53 ratio in tissue or serum samples were positively associated with TNM stage and post-operative recurrence. Kaplan–Meier curve and multivariate cox regression analyses revealed that the tissue and serum Δ 133p53/FLp53 ratios (cutoff value: 2.9160) were independent prognostic factors for overall survival (OS) and progression-free survival (PFS) in ESCC patients and showed no statistical difference in receiver-operating characteristic curve (ROC) analysis, while serum Δ 133p53 showed no significant prognostic value. More importantly, the serum Δ 133p53/FLp53 ratio in ESCC patients was significantly decreased within 72 h post tumor resection and patients with a consistently high serum Δ 133p53/FLp53 ratio (≥ 2.9160) had higher recurrence rates than those with consistently low ratio values. In addition, dynamic detection in each follow-up timepoint showed that serum Δ 133p53/FLp53 ratios were higher than 2.9160 upon recurrence, and they even increased prior to radiologic progression.

Conclusion: The serum Δ 133p53/FLp53 ratio can be a novel predictor for survival outcome and may serve as a real-time parameter for monitoring recurrence in ESCC patients after surgery.

Keywords: esophageal squamous cell carcinoma, p53, Δ 133p53, prognosis, recurrence

Introduction

Esophageal cancer is the fourth leading cause of cancer-related death in both men and women in China,¹ and approximately 90% of Chinese patients with esophageal cancer were diagnosed with esophageal squamous cell carcinoma (ESCC).² Recently, multiple approaches have been developed to improve the outcome of ESCC, including esophagectomy combined with radiotherapy or chemotherapy. However, the 5-year overall survival rate is still inferior and ranges from 15 to 25% in all patients.³ This is thought to be due to a combination of late stage of diagnosis, rapid development of ESCC and lack of effective therapies.⁴ Moreover, molecular biomarkers capable of predicting

recurrence and metastasis, which frequently developed in ESCC patients diagnosed with localized disease, have not yet been developed and thus treatment decisions regarding systemic therapy have been delayed.

Events involved in driving cancer evolution affect tumor aggressiveness, response to treatment and patient prognosis.⁵ To identify prognostic biomarkers, several recent large-scale genomic studies have characterized hundreds of somatic mutations, copy number alterations (CNAs) and structural variants (SVs) in ESCC.^{4,6-10} Mutations in *SLC39A6*,⁴ *EP300* (10%),^{9,10} and *FAM135B* (6.8%)⁷ as well as amplification or overexpression of *EGFR*⁵ were associated with a poor prognosis of ESCC. Moreover, many genes involved in cell cycle regulation, DNA repair and apoptosis were mutated or amplified in 99% of cases, including *TP53* (93%), *CCND1* (33%), *CDK4/CDK6* (23.6%), *CDKN2A* (20%), *NFE2L2* (10%) and *RB1* (9%).^{7,9} As the highest frequency of mutated gene, mutation of *TP53* in the DNA binding domain has been regarded as the main cause for p53 inactivation and proposed as a key factor in driving ESCC progression.⁹ Two compounds (ie, APR-246, COTI-2) that can reactivate missense-mutant p53 protein have been tested in clinical Phase I/II trials.¹¹ However, a growing body of evidence has suggested that abundance of p53 isoforms in cancer tissues, due to alternative splicing or usage of alternative promoters, also modulates the biological processes regulated by full-length p53 (FLp53).¹² Importantly, the tumor-promoting functions of p53 mutations also depend on the shorter p53 isoforms from the mutated FLp53 in some specific conditions, like missense mutations.¹³

The human *p53* gene encodes 12 natural p53 isoforms, including $\Delta 133p53$, an N-terminally truncated isoform (lacking the first 132 amino acids).¹⁴ $\Delta 133p53$ is directly transactivated by FLp53 in response to DNA-damaging signals and antagonizes p53 apoptotic activity.¹⁵ Furthermore, $\Delta 133p53$ also stimulates angiogenesis, metastasis and generation of cancer stem cells regardless of p53 mutations, and its mRNA expression is associated with increased risk of recurrence and poor outcome in various cancers.¹⁶⁻²⁰ Recently, several studies further clarified that elevated $\Delta 133p53$ promotes an immunosuppressive and chemoresistant environment by inducing secretion of CCL-2, IL-6 and the recruitment of tumor-associated macrophages.^{21,22} Thus, $\Delta 133p53$ isoform serves as an important oncogene by antagonistically modulating p53 functions.^{23,24} Previously, we have demonstrated that $\Delta 133p53$ promotes DNA double-strand break (DSB) repair and tumorigenesis by coordinating with p73 in a p53-independent manner;^{12,25} however, the expression level of

$\Delta 133p53$ and its prognostic performance in ESCC remains unknown.

In the present study, we first measured the levels of $\Delta 133p53$ and p53 and calculated their ratio ($\Delta 133p53/FLp53$) in cancerous tissue compared to adjacent normal tissues; subsequently, we analyzed their prognostic value in ESCC patients undergoing tumor resection. Circulating p53 mutations were significantly associated with drug resistance and disease progression of patients with metastatic breast cancer.²⁶ Based on promising results in the tissue, we further investigated dynamic changes and prognostic significance of the serum $\Delta 133p53/FLp53$ ratio in ESCC patients.

Patients and Methods

Patients and Samples

FFPE cancerous tissue samples and parallel serum samples of 180 ESCC patients with overall survival information were collected from the Central Hospital of Enshi Autonomous Prefecture (China) between January 2009 to December 2012. All cases were pathologically confirmed as ESCC and did not receive chemotherapy and radiotherapy before tumor resection. Serum samples were obtained before and within 72 h following surgery, and then stored at -80°C for further use. FFPE tissue samples were obtained at the time of surgery and adjacent normal tissue samples were also collected from 42 of 180 ESCC patients. Due to the fact that 31 patients had no regular follow-up checks for recurrence or progression after surgery (confirmed by CT scan), 149 in 180 ESCC patients with recurrence information were included for PFS analysis. Patient clinical information, including age, gender, BMI, smoking, clinicopathological features (tumor location, TNM and differentiation stage) were obtained from the clinical or pathologic records. All cases of ESCC were staged based on the 7th edition of the AJCC/UICC TNM classification system. This study was approved by the Ethics Committee of Central Hospital of Enshi Autonomous Prefecture. All participants were informed about the purpose, procedures, benefits and potential risks of the study, and their written or oral consents (if patients are without culture, or culture is very shallow, the informed oral consent process was approved by the Ethics Committee of the Central Hospital of Enshi Autonomous Prefecture) were obtained.

RNA Isolation and cDNA Synthesis

Five 8- μm -thick FFPE tissue samples were cut from each block and placed in sterile 1.5-mL centrifuge tubes ready

for extraction. Total RNA was extracted using an RNAPrep Pure FFPE Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. Total RNA of 300 μ L serum was isolated by using a Liquid Total RNA Isolation Kit (BioTeke, Beijing, China) as we described previously²⁷ and treated with DNase I prior to reverse transcription. The concentration of RNA was measured by Nanodrop 2000 spectrophotometer (Thermo Fisher) following purification with RNeasy mini kit (QIAGEN). First strand cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen) and conditions were as follows: 65°C for 5 min, and then 37°C for 50 min, 70°C for 15 min.

Quantitative Real-Time PCR (qRT-PCR)

The expressions of Δ 133p53 and full-length p53 (FLp53) were determined on the ABI-7300 (Thermo Fisher) using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. The primers used were as follows: Δ 133p53 (GenBank accession No. NM_001126115.1)¹⁵ (Forward: 5'-TGGGTTGCAGGAG GTGCTTAC-3', Reverse: 5'-CCACTCGGATA AGATGCT GAGG-3'), FLp53 (Forward: 5'-GCTCAAGACTGGCGCT AAA-3', Reverse: 5'-GTTTTC A GGAAGTAGTTCCAT AGG-3'), and GAPDH (Forward: 5'-GAGTCAACGGATTT GGTCTGAT-3', Reverse: 5'-ATGGGTGGAATCATATTGG AAC-3') was used as the endogenous control.²⁸ The reactions were started at 95°C for 5 min, followed by 42 cycles of 95°C for 30 s, 59.3°C for 30 s, and 72°C for 30 s. All experiments were carried out in triplicate for each data point. Relative quantification of Δ 133p53 and FLp53 expression was calculated with the $2^{-\Delta C_t}$ method.

Statistical Analysis

SPSS software (version 17.0; USA) and Graphpad Prism software (version V.5.00, USA) were used to perform the statistical analyses in this study. Differences in the Δ 133p53/FLp53 ratio between ESCC and paired adjacent normal tissues were compared using a paired Student's *t*-test. Patients were divided into relatively high- and low-expression groups according to the cutoff value determined by X-tile 3.6.1 software (Yale University, New Haven, CT, USA)²⁹ in serum and tissue samples for survival analysis. The χ^2 test or Fisher's exact test was used to determine the correlations of Δ 133p53/FLp53 in tissue or serum samples with the clinicopathological factors of ESCC patients. Pearson's correlation coefficient analysis was used to determine the association of the Δ 133p53/FLp53 ratio in the tissue and in the serum.

Survival analysis was carried out by the Kaplan-Meier method, and subjected to the log rank test. Univariate and multivariate Cox survival (Forward Stepwise Likelihood Ratio method) analyses were applied to assess the hazard ratios (HRs). For the Cox proportional hazards regression model, all covariate, including age, gender, BMI, smoking history, tumor location, pathologic M, N and T stage, grade, recurrence, tissue Δ 133p53/p53 ratio and serum Δ 133p53/p53 ratio were treated as binary variable and adjusted for the multivariate Cox proportional hazard regression model on tissue and serum Δ 133p53/p53 ratio. $P < 0.05$ was considered statistically significant. Receiver operating characteristic (ROC) curve analysis was conducted by MedCalc statistical software (Version 18.11.6, USA) to determine the predictive value of the parameters.

Results

Δ 133p53 and Δ 133p53/FLp53 Ratio Significantly Increased in ESCC Tissues

As there is no specific antibody for the Δ 133p53 isoform,³⁰ we first examined the mRNA abundance of Δ 133p53 in 42 pairs of tumor and adjacent normal tissues to determine whether Δ 133p53 was associated with ESCC progression. Δ 133p53 isoform significantly increased in cancerous tissues (0.10 ± 0.09 vs 0.05 ± 0.03 , $P = 0.0019$) (Figure 1A). We simultaneously detected mRNA expression of FLp53 in these paired samples and confirmed that FLp53 is significantly decreased in cancerous tissues of ESCC patients (0.06 ± 0.04 vs 0.17 ± 0.14 , $P < 0.0001$) (Figure 1B). In addition, we also noticed that the expression ratio of Δ 133p53 to FLp53 significantly increased in cancerous tissues (2.40 ± 3.16 vs 0.50 ± 0.64 , $P = 0.0006$) (Figure 1C).

Elevated Tissue Δ 133p53/FLp53 Ratio Predicts Poor Outcome of ESCC Patients

To confirm the association of Δ 133p53 and FLp53 with clinical outcomes of ESCC patients, we then measured Δ 133p53 and FLp53 expression in 180 FFPE ESCC tissue samples. The optimal cut-off values of Δ 133p53, FLp53 and Δ 133p53/FLp53 ratio for predicting the prognosis of ESCC were estimated by X-tile 3.6.1 software (Figure S1). ESCC patients were divided into two groups (Δ 133p53 < 0.0425 and ≥ 0.0425 ; FLp53 < 0.0195 and ≥ 0.0195 ; Δ 133p53/FLp53 ratio < 2.6470 and ≥ 2.6470). The clinicopathological characteristics of the ESCC patients divided by the cut-off values of Δ 133p53, FLp53 and Δ 133p53/FLp53 ratio are shown in Table 1. Our results indicated that the elevated Δ 133p53 was significantly

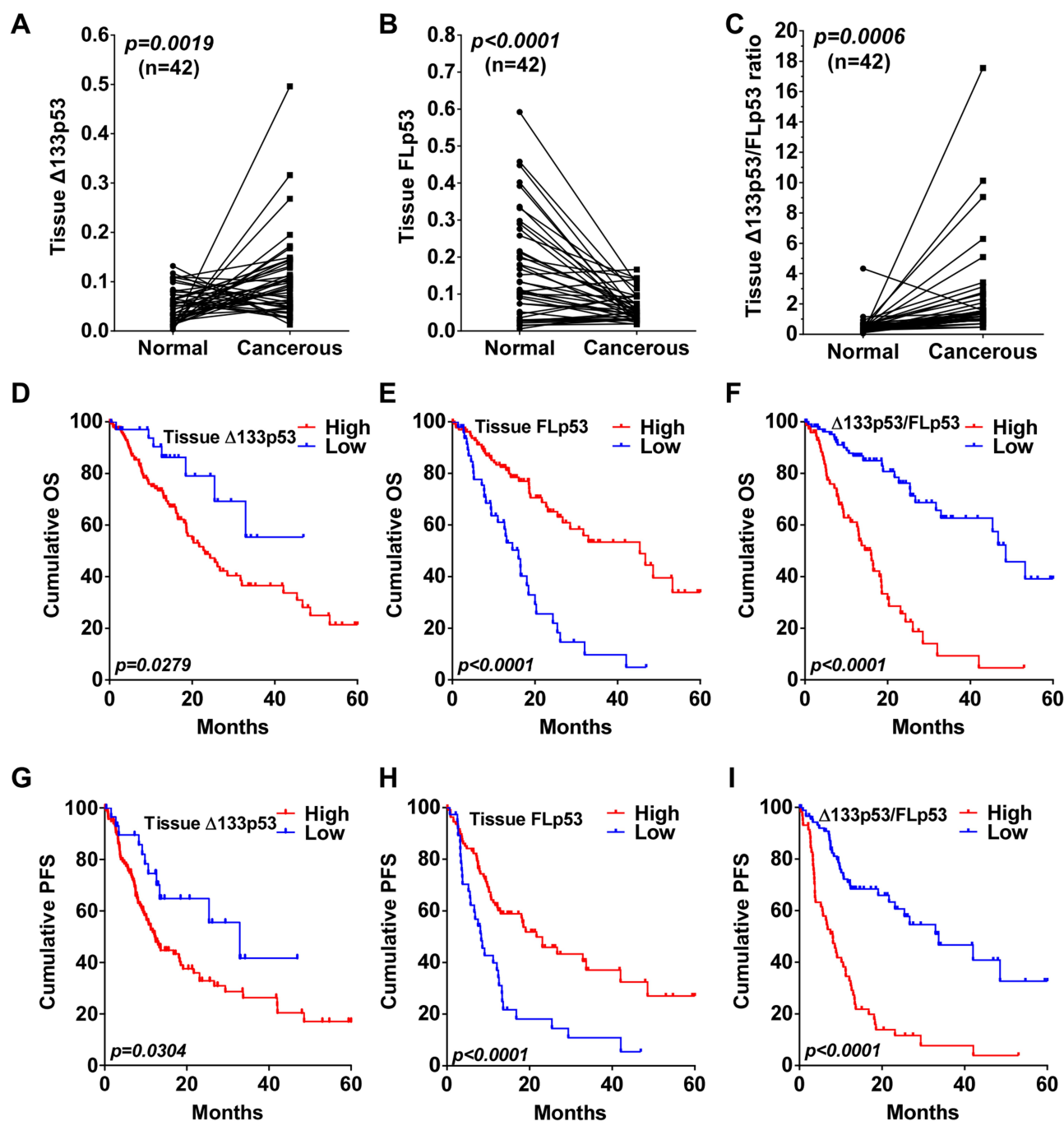


Figure 1 Kaplan-Meier curves of tissue $\Delta 133p53$, FLp53 and $\Delta 133p53$ /FLp53 ratio in ESCC. mRNA expression of $\Delta 133p53$ (A), FLp53 (B) and $\Delta 133p53$ /FLp53 ratio (C) in 42 paired adjacent normal and cancerous tissue of ESCC patients. ESCC, esophageal squamous cell carcinoma; FLp53, full-length p53. OS of 180 ESCC patients divided by tissue $\Delta 133p53$ (D), FLp53 (E) and $\Delta 133p53$ /FLp53 ratio (F). PFS of 149 ESCC patients divided by tissue $\Delta 133p53$ (G), FLp53 (H) and $\Delta 133p53$ /FLp53 ratio (I).

Abbreviations: OS, overall survival; PFS, progression-free survival.

associated with increased TNM stage ($P = 0.0011$), differentiation grade ($P = 0.0030$) and recurrence ($P = 0.0371$), although it had no significant correlations with other clinicopathological features such as age, gender, and BMI index (Table 1). Decreased FLp53 was significantly associated with male gender ($P = 0.0166$), higher BMI ($P = 0.0078$),

lower tumor location ($P = 0.0295$), increased TNM stage ($P < 0.0001$), differentiation grade ($P < 0.0001$) and recurrence ($P < 0.0001$) (Table 1). Increased $\Delta 133p53$ /FLp53 ratio was significantly associated with Lower tumor location ($P = 0.0091$), increased TNM stage ($P < 0.0001$), differentiation grade ($P < 0.0001$) and recurrence ($P < 0.0001$) (Table 1).

Table I Associations of Tissue $\Delta 133p53$, p53 and $\Delta 133p53/p53$ Ratio with Clinicopathological Features in ESCC

	$\Delta 133p53$			p53			$\Delta 133p53/p53$ ratio		
	<0.0425	≥ 0.0425	P value	<0.0195	≥ 0.0195	P value	<2.6470	≥ 2.6470	P value
Age			0.3944			0.6353			0.1389
<60	18	63		23	58		53	28	
≥ 60	17	82		25	74		54	45	
Gender			1.0000			0.0166			0.1375
Male	30	124		46	108		88	66	
Female	5	21		2	24		19	7	
BMI			0.4820			0.0078			0.4763
<24	17	80		18	79		60	37	
≥ 24	18	65		30	53		47	36	
Smoking history			0.8146			1.0000			0.3624
No	6	30		9	27		19	17	
Yes	29	115		39	105		88	56	
Tumor location			0.9731			0.0295			0.0091
Upper/Middle	11	46		9	48		42	15	
Lower	24	99		39	84		65	58	
Pathologic M			0.1304			<0.0001			<0.0001
M0	34	126		32	128		106	54	
M1	1	19		16	4		1	19	
Pathologic N			0.4211			<0.0001			<0.0001
N0–N1	32	122		32	122		106	48	
N2–N3	3	23		16	10		1	25	
Pathologic T			0.0662			0.0009			<0.0001
T1–T2	20	58		11	67		67	11	
T3–T4	15	87		37	65		40	62	
TNM stage			0.0011			<0.0001			<0.0001
I–II	28	71		6	93		98	1	
III–IV	7	74		42	39		9	72	
Grade			0.0030			<0.0001			<0.0001
G1–G2	24	59		9	74		71	12	
G3–G4	11	86		39	58		36	61	
Recurrence ^a			0.0371			<0.0001			<0.0001
No	23	65		10	78		73	15	
Yes	7	54		28	33		18	43	

Note: ^a149 of 180 patients with regular follow-up checks for recurrence after surgery.

The median survival time of all patients was 25.47 months (range 0.3–60 months), 106 patients (58.89%) were censored and 74 patients (41.11%) died during our follow-up period. Kaplan-Meier analysis revealed that increased tissue abundance of $\Delta 133p53$ ($\chi^2 = 4.83$, $P = 0.0279$) (Figure 1D) and decreased p53 expression ($\chi^2 = 27.76$, $P < 0.0001$) (Figure 1E), as well as increased $\Delta 133p53/FLp53$ ratio ($\chi^2 = 42.34$, $P < 0.0001$) (Figure 1F) were significantly related to worse

OS. By the time of analysis, 149 of 180 ESCC patients had regular follow-up checks for recurrence after surgery. Recurrence occurred in 61 of 149 patients, with a median follow-up time of 13.4 months (range 0.8–42 months), and 88 patients had clear evidence of no recurrence. Tissue $\Delta 133p53$ and $\Delta 133p53/FLp53$ ratio were significantly increased in recurrent patients compared with non-recurrent ones ($P = 0.0032$ and $P < 0.0001$, respectively), and FLp53

was decreased in recurrent patients ($P = 0.0023$) (Figure S2). Kaplan-Meier analysis revealed that increased $\Delta 133p53$ ($\chi^2 = 4.69$, $P = 0.0304$) (Figure 1G), decreased p53 expression ($\chi^2 = 15.53$, $P < 0.0001$) (Figure 1H) and increased $\Delta 133p53/FLp53$ ratio ($\chi^2 = 39.18$, $P < 0.0001$) (Figure 1I) were significantly associated with worse PFS. Thus, these results demonstrate that tissue $\Delta 133p53$ and FLp53 expression, tissue $\Delta 133p53/FLp53$ ratio are associated with OS and PFS of ESCC patients.

Serum $\Delta 133p53/FLp53$ Ratio Shows Prognostic Significance in ESCC Patients

p53 could exist as circulating DNA or mRNA in cancers,^{28,31} we next further evaluated $\Delta 133p53$ and FLp53 mRNA expression in serum of 180 ESCC patients. Pearson's correlation coefficient analysis revealed that serum $\Delta 133p53$, FLp53 and $\Delta 133p53/FLp53$ ratio were positively correlated with their tissue expression, and Pearson's correlation coefficients were 0.7019 ($P < 0.0001$) (Figure 2A), 0.4030 ($P < 0.0001$) (Figure 2B) and 0.4137 ($P < 0.0001$) (Figure 2C), respectively. ESCC patients were divided into two groups ($\Delta 133p53 < 0.0870$ and ≥ 0.0870 ; FLp53 < 0.0300 and ≥ 0.0300 ; $\Delta 133p53/FLp53$ ratio < 2.9160 and ≥ 2.9160) by X-tile 3.6.1 software (Figure S3). Correlation analysis revealed that the elevated serum $\Delta 133p53$ was significantly associated with male gender ($P = 0.0105$), increased M stage ($P = 0.0086$), N stage ($P = 0.0184$) and TNM stage ($P = 0.0008$), as well as recurrence ($P = 0.0005$) (Table 2). Increased $\Delta 133p53/FLp53$ ratio was significantly associated with high BMI index ($P = 0.0287$), increased M stage ($P < 0.0001$), N stage ($P = 0.0133$), TNM stage ($P < 0.0001$), differentiation grade ($P = 0.0131$) and recurrence ($P < 0.0001$) (Table 2). Whereas decreased serum FLp53 was only associated with increased TNM stage ($P = 0.0276$) and recurrence ($P = 0.0007$) (Table 2).

Serum $\Delta 133p53$ and $\Delta 133p53/FLp53$ ratio were significantly increased in recurrent patients compared with non-recurrent ones ($P = 0.0002$ and $P < 0.0001$, respectively) (Figure S4A and S4C), FLp53 was decreased in recurrent patients ($P = 0.0001$) (Figure S4B). However, survival analysis indicated that increased serum $\Delta 133p53$ showed no association with OS ($\chi^2 = 2.89$, $P = 0.0890$) (Figure 2D) and PFS ($\chi^2 = 2.93$, $P = 0.0871$) (Figure 2E). Decreased p53 expression correlated with decreased OS ($\chi^2 = 5.80$, $P = 0.0160$) (Figure 2F) and PFS ($\chi^2 = 10.27$, $P = 0.0014$) (Figure 2G); and increased $\Delta 133p53/FLp53$ ratio correlated with poor OS

($\chi^2 = 17.26$, $P < 0.0001$) (Figure 2H) and PFS ($\chi^2 = 56.16$, $P < 0.0001$) (Figure 2I). Taken together, these results suggest that serum FLp53 expression and $\Delta 133p53/FLp53$ ratio are associated with OS and PFS of ESCC patients.

Tissue and Serum $\Delta 133p53/FLp53$ Ratios are Poor Independent Prognostic Factors for ESCC

The univariate survival analysis indicated that tissue and serum $\Delta 133p53/FLp53$ ratio were correlated with OS (Table 3) and PFS (Table 4) of ESCC patients. Furthermore, the multivariate analyses identified that the tissue $\Delta 133p53/FLp53$ ratio was an independent prognostic factor for OS (HR = 3.864; 95% CI = 2.293–6.511, $P < 0.0001$) (Table 3) and PFS (HR = 2.283; 95% CI = 1.387–3.760, $P < 0.0001$) (Table 4) of ESCC patients. The serum $\Delta 133p53/FLp53$ ratio was also an independent prognostic factor for OS (HR = 2.503; 95% CI = 1.465–4.276, $P = 0.0011$) (Table 3) and PFS (HR = 3.230; 95% CI = 1.947–5.359, $P < 0.0001$) (Table 3) of ESCC patients. Besides, M stage and recurrence were also predictive indicators for OS and PFS. However, serum $\Delta 133p53$ or FLp53 and tissue $\Delta 133p53$ were not independent prognostic factors for OS and PFS of ESCC patients, except tissue FLp53 (data not shown).

Tissue and Serum $\Delta 133p53/FLp53$ Ratios Show No Difference in Prognostic Performance for OS and PFS

We also conducted a ROC analysis of tissue and serum $\Delta 133p53/FLp53$ ratio. The AUC values of tissue and serum $\Delta 133p53/FLp53$ ratio in the Cox regression model for OS were 0.695 ($P = 0.0351$) and 0.641 ($P = 0.0360$), respectively (Figure 3A), and 0.692 ($P = 0.0337$) and 0.649 ($P = 0.0340$) for PFS, respectively (Figure 3B). Prognostic performance of tissue and serum $\Delta 133p53/FLp53$ ratio showed no statistical difference for OS and PFS ($P = 0.2066$ and $P = 0.3207$, respectively).

The Dynamic Change of Serum $\Delta 133p53/FLp53$ Ratio After Surgery and Its Association with Recurrence

To evaluate whether serum $\Delta 133p53/FLp53$ ratio dynamically correlates with treatment response, we further measured its postoperative level in 180 ESCC patients within 72 h after resection. Postoperative serum $\Delta 133p53/FLp53$ ratio significantly dropped after surgery (4.31 ± 7.69 vs

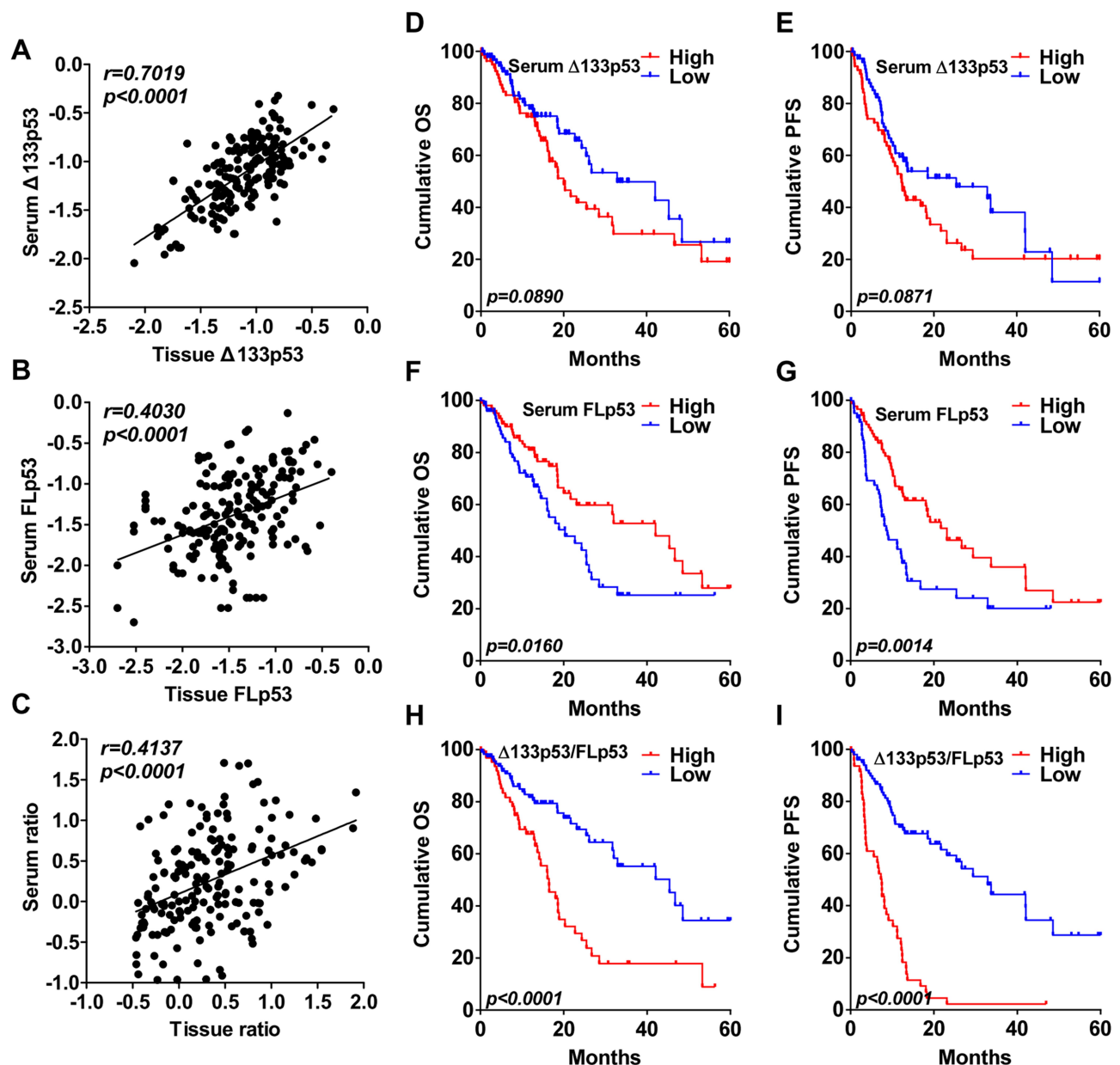


Figure 2 Kaplan-Meier curves of serum $\Delta 133p53$, FLp53 and $\Delta 133p53/FLp53$ ratio in ESCC. Pearson's correlation analysis between tissue $\Delta 133p53$ (A), FLp53 (B), $\Delta 133p53/FLp53$ ratio (C) and their respective level in serum of ESCC patients. OS of 180 ESCC patients divided by preoperative serum $\Delta 133p53$ (D), FLp53 (F) and $\Delta 133p53/FLp53$ ratio (H). PFS of 149 ESCC patients divided by preoperative serum $\Delta 133p53$ (E), FLp53 (G) and $\Delta 133p53/FLp53$ ratio (I).

2.81 ± 4.12 , $P = 0.0098$) (Figure 4A). Based on changes between preoperative and postoperative serum $\Delta 133p53/FLp53$ ratio, patients were divided into four groups: I, persistent high levels at the two time points; II, preoperative high followed by postoperative low; III, preoperative low followed by postoperative high, and IV, preoperative low followed by postoperative low, using the same cutoff value of preoperative serum $\Delta 133p53/FLp53$ ratio (2.9160). Persistent high levels of serum $\Delta 133p53/FLp53$ ratio predicted poor OS (Figure 4B), higher recurrence

rates (Figure 4C) and worse PFS (Figure 4D) in ESCC patients. Patients in group I showed significantly shorter time to relapse (TTR) and higher recurrence rates than group IV (median TTR of 5.57 months vs not reached; recurrence of 27/28 vs 14/88, $P < 0.0001$), as well as group III (median TTR of 5.57 months vs 29.4 months; recurrence of 27/28 vs 4/13, $P < 0.0001$), while there was a propensity of increased recurrence compared with group II (median TTR of 5.57 months vs 8.07 months; recurrence of 27/28 vs 16/20; $P = 0.0643$) (Figure 4C).

Table 2 Associations of Serum $\Delta 133p53$, p53 and $\Delta 133p53/p53$ Ratio with Clinicopathological Features in ESCC

	$\Delta 133p53$			p53			$\Delta 133p53/p53$ Ratio		
	<0.0870	≥ 0.0870	P value	<0.0300	≥ 0.0300	P value	<2.9160	≥ 2.9160	P value
Age									
<60	41	40	0.5088	34	47	0.9394	50	31	0.5852
≥ 60	55	44		41	58		65	34	
Gender									
Male	76	78	0.0105	68	86	0.1322	94	60	0.0759
Female	20	6		7	19		21	5	
BMI									
<24	52	45	0.9363	34	63	0.0516	69	28	0.0287
≥ 24	44	39		41	42		46	37	
Smoking history									
No	16	20	0.2320	14	22	0.7055	24	12	0.6981
Yes	80	64		61	83		91	53	
Tumor location									
Upper/Middle	27	30	0.2748	20	37	0.2229	38	19	0.5974
Lower	69	54		55	68		77	46	
Pathologic M									
M0	91	69	0.0086	64	96	0.2331	112	48	<0.0001
M1	5	15		11	9		3	17	
Pathologic N									
N0–N1	88	66	0.018	60	94	0.0870	104	50	0.0133
N2–N3	8	18		15	11		11	15	
Pathologic T									
T1–T2	47	31	0.1035	29	49	0.2856	53	25	0.3214
T3–T4	49	53		46	56		62	40	
TNM stage									
I–II	64	35	0.0008	34	65	0.0276	78	21	<0.0001
III–IV	32	49		41	40		37	44	
Grade									
G1–G2	50	33	0.0857	32	51	0.4333	61	22	0.0131
G3–G4	46	51		43	54		54	43	
Recurrence ^a									
No	56	32	0.0005	26	62	0.0007	83	5	<0.0001
Yes	21	40		35	26		18	43	

Note: ^a149 of 180 patients with regular follow-up checks for recurrence after surgery.

Compared with group IV, patients in groups I and II had significantly shorter TTR and higher recurrence rates (all $P < 0.0001$, [Figure 4C](#)).

Twelve patients with no history of radio- or chemotherapies after tumor resection were selected for measurement of real-time changes of serum $\Delta 133p53/FLp53$ ratio in each time of follow-up (tumor recurrence was monitored by CT scans every 4 months in the first two years). Six patients were confirmed to have lung, brain or lymph node metastasis,

and the other 6 showed no evidence of recurrence. The serum $\Delta 133p53/FLp53$ ratios were higher than 2.9160 at the time of detected recurrence in 6 patients and even increased prior to radiologic progression in 2/6 patients, while they were lower than 2.9160 in 4/6 patients with no recurrence ([Figure 4E](#)). In addition, healthy donors exhibited significantly lower serum $\Delta 133p53/FLp53$ ratios than did ESCC patients preoperatively, mostly lower than 2.9160 ([Figure S4D](#)). The best cutoff value of the serum $\Delta 133p53/FLp53$ ratio to distinguish

Table 3 Univariate and Multivariate Survival Analyses of OS in Patients with ESCC

	Univariate Analysis		*Multivariate Analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Age (≥ 60 vs < 60)	1.203 (0.758–1.908)	0.4332	2.296 (1.247–4.225)	0.0076
Gender (Male vs Female)	2.012 (0.873–4.641)	0.100		
BMI (≥ 24 vs < 24)	1.188 (0.750–1.882)	0.4624		
Smoking history (Yes vs No)	1.618 (0.923–2.836)	0.0931		
Tumor location (Lower vs Upper + Middle)	1.231 (0.694–2.182)	0.4777		
Pathologic M (M1 vs M0)	3.695 (2.134–6.397)	< 0.0001		
Pathologic N (N2–N3 vs N0–N1)	2.066 (1.182–3.610)	0.0109		
Pathologic T (T3–T4 vs T1–T2)	1.591 (0.992–2.551)	0.054	2.184 (1.298–3.672)	0.0032
Grade (G3–G4 vs G1–G2)	2.107 (1.293–3.433)	0.0028		
Recurrence (Yes vs No) ^a	2.546 (1.549–4.184)	< 0.0001		
Tissue $\Delta 133p53/p53$ ratio (≥ 2.6470 vs < 2.6470)	4.496 (2.742–7.375)	< 0.0001		
Serum $\Delta 133p53/p53$ ratio (≥ 2.9160 vs < 2.9160)	2.559 (1.617–4.050)	< 0.0001	2.503 (1.465–4.276)	0.0011

Notes: ^a149 of 180 patients with regular follow-up checks for recurrence after surgery. Univariate and multivariate Cox survival (Forward Stepwise Likelihood Ratio method) analyses were applied to assess the hazard ratios (HRs) and P value. *Adjusting for age, gender, BMI, smoking history, tumor location, pathologic M, N and T stage, grade, recurrence, tissue $\Delta 133p53/p53$ ratio and serum $\Delta 133p53/p53$ ratio, respectively.

Table 4 Univariate and Multivariate Survival Analyses of PFS in Patients with ESCC

	Univariate Analysis		*Multivariate Analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Age (≥ 60 vs < 60)	1.352 (0.877–2.084)	0.1717	2.171 (1.219–3.866)	0.0085
Gender (Male vs Female)	1.580 (0.836–2.986)	0.1589		
BMI (≥ 24 vs < 24)	1.320 (0.863–2.020)	0.2009		
Smoking history (Yes vs No)	2.030 (1.164–3.542)	0.0127		
Tumor location (Lower vs Upper + Middle)	1.363 (0.833–2.230)	0.2173		
Pathologic M (M1 vs M0)	2.638 (1.522–4.574)	< 0.0001		
Pathologic N (N2–N3 vs N0–N1)	2.066 (1.226–3.482)	0.0065		
Pathologic T (T3–T4 vs T1–T2)	1.438 (0.932–2.218)	0.1007	5.212 (3.062–8.873)	< 0.0001
Grade (G3–G4 vs G1–G2)	1.835 (1.180–2.853)	0.0070		
Recurrence (Yes vs No) ^a	6.760 (4.138–11.041)	< 0.0001		
Tissue $\Delta 133p53/p53$ ratio (≥ 2.6470 vs < 2.6470)	3.677 (2.378–5.684)	< 0.0001		
Serum $\Delta 133p53/p53$ ratio (≥ 2.9160 vs < 2.9160)	4.735 (3.038–7.379)	< 0.0001	3.230 (1.947–5.359)	< 0.0001

Notes: ^a149 of 180 patients with regular follow-up checks for recurrence after surgery. Univariate and multivariate Cox survival (Forward Stepwise Likelihood Ratio method) analyses were applied to assess the hazard ratios (HRs) and P value. *Adjusting for age, gender, BMI, smoking history, tumor location, pathologic M, N and T stage, grade, recurrence, tissue $\Delta 133p53/p53$ ratio and serum $\Delta 133p53/p53$ ratio, respectively.

ESCC patients from healthy donors was 0.6798 (Sensitivity: 76.11%; Specificity: 77.55%, AUC = 0.8376), which was also less than 2.9160 (Figure S4E). These results indicate the potential usefulness of serum $\Delta 133p53/FLp53$ ratio as a prognostic predictor for ESCC and may also reflect recurrence risk in a real-time manner.

Discussion

This investigation provides the novel finding that the pre-operative tissue abundance of $\Delta 133p53$ as well as the $\Delta 133p53/FLp53$ ratio were significantly elevated in recurrent ESCC patients compared with non-recurrent patients. This

finding is consistent with previous reports that $\Delta 133p53$ increases risk of cancer recurrence in breast¹⁸ and ovarian cancer.³² Furthermore, clinicopathological factor distribution analyses also revealed that $\Delta 133p53$ and FLp53 expression, as well as the $\Delta 133p53/FLp53$ ratio correlated significantly with TNM stage or recurrence state. Most importantly, the Kaplan-Meier curve revealed that increased tissue $\Delta 133p53$ and $\Delta 133p53/FLp53$ ratio and decreased FLp53 were correlated to worse OS and PFS in ESCC patients. Thus, $\Delta 133p53$ may also play an oncogenic role in ESCC progression.

$\Delta 133p53$ constituted an independent prognostic marker in patients with advanced serous ovarian cancer.³² In our

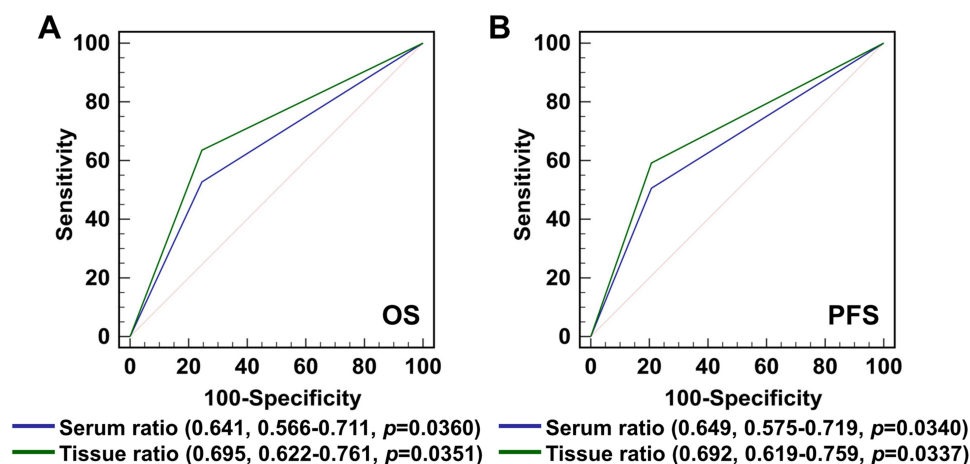


Figure 3 The ROC analysis of tissue and serum $\Delta 133p53/FLp53$ ratio in Cox regression model. Patients were divided into two groups according to the cutoff value of tissue (2.6470) and serum (2.9160) $\Delta 133p53/FLp53$ ratio, separately. **(A)** Receiver operating curve in the OS model. **(B)** Receiver operating curve in the PFS model.

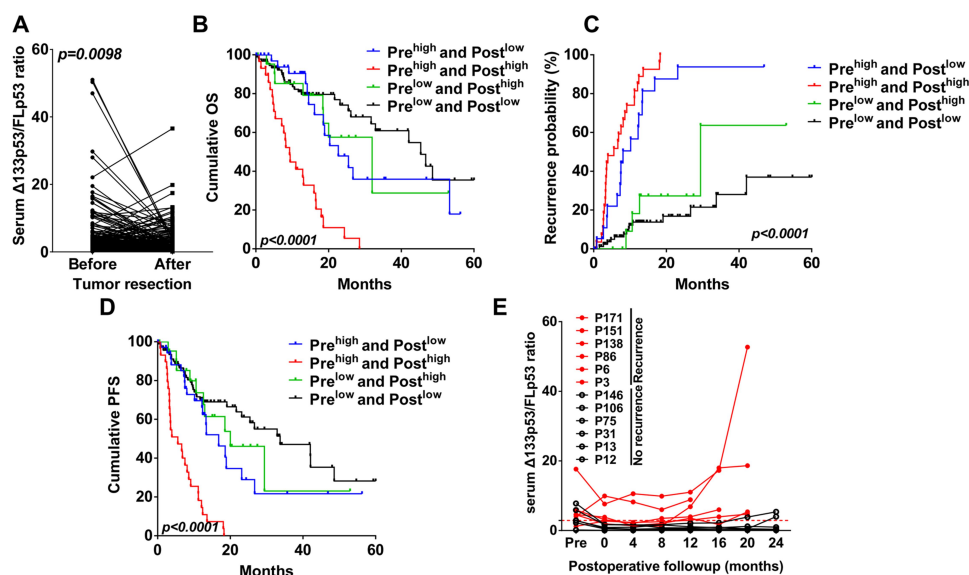


Figure 4 Dynamic changes of serum $\Delta 133p53/FLp53$ ratio reflect treatment response and recurrence risk of ESCC. **(A)** Changes of serum $\Delta 133p53/FLp53$ ratio before versus after (within 72 h following) resection. **(B)** OS of 180 ESCC patients divided by pre- and post-operative serum $\Delta 133p53/FLp53$ ratio, cutoff value was 2.9160. **(C)** Prognostic significance for time to recurrence in patients with persistent high serum $\Delta 133p53/FLp53$ ratio, conversion of serum $\Delta 133p53/FLp53$ ratio from high to low, conversion of serum $\Delta 133p53/FLp53$ ratio from low to high, and persistent low (cutoff value was 2.9160). **(D)** PFS of 149 ESCC patients divided by pre- and post-operative serum $\Delta 133p53/FLp53$ ratio, cutoff value was 2.9160. **(E)** 12 patients were monitored by CT scans every 4 months in a time range of 24 months, serum $\Delta 133p53/FLp53$ ratio were measured at each time until recurrence occurred. Dashed line means $Y = 2.9160$.

study, tissue FLp53 and $\Delta 133p53/FLp53$ ratio were shown to serve as independent prognostic factors for OS and PFS, while $\Delta 133p53$ was not in a further univariate and multivariate analysis. Although we have previously confirmed that $\Delta 133p53$ can act synergistically with p73 to upregulate the transcription of rad51, lig4 and rad52 by joining together to bind to a novel type of $\Delta 133p53$ -responsive element in their promoters,^{12,25} the detailed molecular mechanism of $\Delta 133p53$ function is still unclear, the possibility of this inconsistency may result from the fact that

$\Delta 133p53$ has three isoforms (α , β and γ), which have been shown to have specific functions in a cancer or disease-specific manner and could be regulated by different factors.¹⁸ As in breast cancer, $\Delta 133p53\beta$ promotes cancer cell invasion and associates with cancer progression, while $\Delta 133p53\alpha$ and γ are not.¹⁸ In human glioblastoma, the $\Delta 133p53$ angiogenic effect is due to $\Delta 133p53\alpha$ and γ , but not the β isoform.¹⁶ SRSF1, an essential splicing factor, promotes vascular smooth muscle cell (VSMC) proliferation and injury-induced neointima formation via

inducing $\Delta 133p53\alpha$, but not the γ and β isoforms.³³ It is worth noting that the expression level of SRSF1 differs among 33 cancer types as confirmed by Cancer Genome Atlas (TCGA) Program. The detailed roles of individual $\Delta 133p53$ isoforms (α , β and γ) and their functional molecular mechanisms should be investigated in future studies.

Circulating DNA/RNAs are not affected by tissue sampling timing and conditions, and thus are optimal diagnostic or prognostic biomarkers for various cancers, including ESCC.^{34,35} We previously reported that circulating RNAs could be used as diagnostic biomarkers for non-small cell lung carcinoma.²⁷ In this investigation, we then further measured $\Delta 133p53$ and FLp53 expression in serum samples by using GAPDH²⁸ as an internal control. The Pearson's correlation coefficient of $\Delta 133p53$ was 0.7019 between serum and tissue, significantly higher than that of FLp53 ($r = 0.4030$) and $\Delta 133p53/FLp53$ ratio ($r = 0.4137$), which indicated that cancer cells may be the main source of $\Delta 133p53$. $\Delta 133p53$ is significantly increased during the progression from premalignant adenoma to malignant carcinoma.³⁶ Similar to results obtained from tissue samples, the serum abundance of $\Delta 133p53$ as well as the $\Delta 133p53/FLp53$ ratio were significantly increased in recurrent ESCC patients, and were related to TNM stage and recurrence state. Although serum $\Delta 133p53$ showed no prognostic value in ESCC patients, the $\Delta 133p53/FLp53$ ratio showed a better prognostic performance than FLp53 alone for OS (χ^2 value, 17.26 vs 5.80) and PFS (χ^2 value, 56.16 vs 10.27) prediction. Furthermore, ROC analysis revealed that prognostic performance of the serum $\Delta 133p53/FLp53$ ratio showed no statistical difference from the tissue ratio, both of which were confirmed to be independent prognostic biomarkers for OS and PFS in multivariate analysis.

The clinical utility of monitoring circulating DNA/RNA changes with treatment and recurrence has been reported in various types of cancers.^{37,38} In this study, we reported for the first time that a significant decrease of serum $\Delta 133p53/FLp53$ ratio was observed soon after resection, which may well be attributed to surgical resection of the primary tumor. Patients with consistently high serum $\Delta 133p53/FLp53$ ratio (higher than 2.9160 before and after surgery) showed increased recurrence rate and shorter TTR than those with postoperatively or consistently low levels. Furthermore, serum $\Delta 133p53/FLp53$ ratios were higher than 2.9160 and even increased prior to the time of radiologically detected recurrence. Based on these results, we propose that serum $\Delta 133p53/FLp53$ ratio may be a real-time surrogate indicator

for treatment response and recurrence risk following the ESCC curative resection.

Conclusions

In summary, the results of this study demonstrate that preoperative high serum or tissue $\Delta 133p53/FLp53$ ratio are independent prognostic factors for OS or PFS in ESCC patients undergoing curative resection. Serum $\Delta 133p53/FLp53$ ratio in ESCC patients was significantly decreased within 72 h post tumor resection and patients with a consistently high serum $\Delta 133p53/FLp53$ ratio (≥ 2.9160) (pre- and post-operative) had higher recurrence rates than those with consistently low ratio values. In addition, dynamic detection at each follow-up timepoint showed that serum $\Delta 133p53/FLp53$ ratios were increased upon radiologically detected progression. Thus, the serum $\Delta 133p53/FLp53$ ratio can be a novel predictor for survival outcome and may serve as a real-time parameter for monitoring recurrence in ESCC patients after surgery.

Abbreviations

ESCC, esophageal squamous cell carcinoma; FLp53, full-length p53; OS, overall survival; PFS, progression-free survival; ROC, receiver-operating characteristic curve; CNAs, copy number alterations; TTR, time to relapse; VSMC, vascular smooth muscle cell.

Data Sharing Statement

We are willing to share anonymized data after required ethical approval by the Ethics Committee of Central Hospital of Enshi Autonomous Prefecture.

Ethics Approval and Informed Consent

The study was approved by the Ethics Committee of Central Hospital of Enshi Autonomous Prefecture. All participants were informed about the purpose, procedures, benefits and potential risks of the study, and their written or oral consents were obtained. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions.

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Disclosure

The authors report no conflicts of interest for this work.

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