



53BP1 expression and immunoscore are associated with the efficacy of neoadjuvant chemoradiotherapy for rectal cancer

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Abstract

Purpose Considering the effects of P53 binding protein 1 (53BP1) expression and T lymphocyte infiltration density on tumor radiosensitivity, we investigated the relation of 53BP1 expression and immunoscore based on T lymphocyte infiltration density with the efficacy of neoadjuvant chemoradiotherapy (CRT) for rectal cancer.

Methods Fifty-five patients with rectal cancer receiving neoadjuvant CRT followed by surgery were enrolled. The 53BP1 expression level and the density of CD3⁺, CD8⁺, and CD45RO⁺ T lymphocytes in the tumor tissues were examined by immunohistochemistry, and the relation of these findings to the rates of tumor regression, disease-free survival (DFS), and overall survival (OS) was analyzed.

Results The levels of 53BP1 and the CD3/CD8 immunoscore were closely correlated with the response to CRT ($p < 0.05$), with an area under the receiver operating characteristic curve for CRT efficacy prediction of 0.626 and 0.717, respectively. Further survival analysis revealed that high 53BP1 expression effectively prolonged 2-year DFS compared with low 53BP1 expression (87.5% [95%CI 77.3–97.7] vs. 53.3% [95%CI 28.1–78.6]; $p < 0.05$), while the effect of immunoscore on survival was restricted by the expression status of 53BP1. Cox multivariate analysis confirmed 53BP1 as an independent prognostic factor in DFS.

Conclusion The pretreatment levels of 53BP1 and the immunoscore based on CD3⁺/CD8⁺ T cell infiltration density in tumor tissues are effective predictors for the CRT response, and 53BP1 has a more pronounced impact on prognosis.

Keywords Rectal cancer · Tumor infiltrating T cells · Immune scores · Radiation sensitivity · P53 binding protein 1

Introduction

The authors Ai Huang and Yong Xiao contributed equally to this work.

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Preoperative neoadjuvant chemoradiotherapy (CRT) has become a standard treatment for locally advanced rectal cancer [1]. However, due to certain characteristics of the tumor itself, such as specific gene mutations or abnormalities in the tumor immune microenvironment, the efficacy of CRT varies among individuals [2–4]. Therefore, how to identify the relevant characteristics in the tumor and immune microenvironment is a prerequisite for achieving individualized neoadjuvant CRT [1].

The DNA damage response (DDR) pathway has been found to be closely related to the occurrence, development, and treatment resistance of various tumors [5]. P53 binding protein 1 (53BP1) is a newly discovered tumor suppressor gene, which is also an important member of the DDR pathway family. The protein participates in the repair of DNA damage through the interaction with p53 and ATM protein, to maintain the stability and integrity of the genome and regulate cell cycle and apoptosis [6]. The abnormal expression of 53BP1, including deletions and mutations, is involved in disease progression and poor prognosis. Our previous studies have also verified the close correlation between 53BP1

expression and the radiosensitivity of colorectal cancers, and found that the loss of 53BP1 could inhibit the radiosensitivity of colorectal cancer by inducing cell proliferation and inhibiting apoptosis [7, 8]. Based on existing research results, 53BP1 could be regarded as a predictive marker for neoadjuvant therapy efficacy, but it still requires further support with clinical evidence.

In addition to the tumor's own biological characteristics, the tumor response to treatment depends on the density of tumor-infiltrating lymphocytes (TIL) in the microenvironment [9, 10]. Normally, if tumor can be effectively recognized by the host immune system, a variety of effector cells, such as helper CD3⁺ T cells, memory CD45RO⁺ T cells, and cytotoxic CD8⁺ T cells, can be activated to prevent tumor development. However, tumors themselves can escape the host immune surveillance, leading to a lack of effector T cell infiltration into the tumor microenvironment and inducing therapeutic resistance [11]. Numerous clinical studies have shown that T lymphocyte infiltration density in the central region of the tumor (CT) and the invasion margin (IM) is positively correlated with clinical outcomes [10–13]. Therefore, the current expert consensus has suggested and established an immunoscore approach based on the density of CD3/CD8, CD3/CD45RO, and CD8/CD45RO TILs in the tumor CT and IM area to evaluate treatment response and predict prognosis [9, 14].

Increasing literature contributions have revealed the interaction between tumor molecular characteristics and immune infiltration in the microenvironment. Similarly, 53BP1 has been found to serve a key role in maintaining T cell function and the loss of 53BP1 could result in the deficiency of T cell function [15]. Thus, we speculated that changes in 53BP1 expression and consequent variations of T cell infiltration density in the tumor microenvironment may work together to affect radiosensitivity in colorectal cancer. The purpose of this study was to evaluate the correlation of pretreatment 53BP1 expression and the immunoscore based on TIL density in tumor tissues with a neoadjuvant CRT response, to explore the internal connection between 53BP1 and T lymphocyte infiltration, and determine the role of these two factors on CRT response and prognosis in locally advanced rectal cancer.

Materials and methods

Research design and patient enrollment

This was a retrospective observational study approved by the Ethics Committee of Huazhong University of Science and Technology, China. Patients with locally advanced rectal cancer undergoing neoadjuvant CRT and total mesorectal excision (TME) from January 2015 to February 2018

were enrolled according to the following criteria: (1) the patient's age was in the range of 18 to 75 years; (2) the lower end of the patient's tumor was less than 10 cm from the anal margin and pathologically confirmed as rectal adenocarcinoma; (3) the patient presented with no distant metastasis (stage cT3-4 and/or N+, M0) according to the 7th edition of the American Joint Committee on Cancer (AJCC) staging system; (4) complete pretreatment magnetic resonance imaging (MRI) data and pretreatment tissue specimens from the patient were available; (5) the patient had undergone standard preoperative CRT, TME, and follow-up; and (6) complete postoperative data were collected and postoperative tumor regression grade (TRG) assessments were performed according to the Dworak grading system [16]. Informed consent was obtained from all patients. The patients' clinical characteristics, including age, gender, tumor differentiation status, primary tumor location, stage, and postoperative TRG data, etc., were collected and analyzed.

Treatment regimen and efficacy evaluation

All patients enrolled in the study received a total radiotherapy dose of 45–50 Gy (single doses of 1.8 Gy or 2 Gy), which was administered concurrently with intravenous 5-fluorouracil (5-FU; King York, Tianjing, China) or oral capecitabine (Roche, Shanghai, China). TME was performed six to eight weeks after the completion of radiotherapy. In all cases, baseline and postradiotherapy efficacy assessments were performed according to the Response Evaluation Criteria in Solid Tumors (RECIST 1.1), and postoperative specimens were used to assess the TRG according to the Dworak system [16].

Immunohistochemical analysis

The pretreatment 53BP1 expression and CD3⁺, CD8⁺, and CD45RO⁺ T cell density in tumor tissue were analyzed via immunohistochemistry. According to a previous study [17], paraffin sections were pretreated and incubated with primary anti-CD3 (Abcam, Cambridge, MA, USA, ab16669, 1:150), anti-CD8 (Abcam, ab4055, 1:150), anti-CD45RO (Abcam, ab216024, 1:300), or anti-53BP1 (Abcam, ab175933, 1:300) antibody. Subsequently, the sections were treated with a secondary antibody according to the manufacturer's instructions. The staining results were evaluated according to the staining intensity and percentage of the positively staining cells according to our previous protocol. The expression of 53BP1 localizes to the nucleus of the tumor cells, while CD3, CD8, and CD45RO are membrane proteins of immune cells [6, 7]. Two independent pathologists assessed the data in a single-blind fashion.

Scoring of the immune status of the central and marginal tumor regions

Recently, a tumor immunoscore approach was proposed based on the numeration of any two populations of TILs in the CT and IM of the tumor, such as CD3/CD8, CD3/CD45RO, or CD8/CD45RO [9]. Therefore, in our study, the densities of CD3+, CD8+, and CD45RO+ TILs in pretreatment tissue samples were assessed by immunohistochemistry. A score of 1 was indicative of high density and a score of 0 was indicative of low density. The sum scores of the CD3/CD8, CD3/CD45RO, and CD8/CD45RO in the CT and IM regions were calculated as a total score of 0–4: a score of 0–2 was considered a low immunoscore and a score of 3–4 was considered a high immunoscore.

Statistical analysis

Data were analyzed using SPSS 17.0 statistical software (IBM, Armonk, NY, USA). A chi-squared test was used to assess the correlation of the clinical features, the 53BP1 expression level, and the immunoscore with the treatment response. The area under the receiver operating characteristic (ROC) curve (AUC) and logistic regression analysis were used to evaluate the value of the 53BP1 expression level and immunoscore for predicting the response to CRT, and the corresponding sensitivity, specificity, accuracy, and optimal cutoff values were determined. The Kaplan–Meier method and Cox regression analysis were employed to determine differences in disease-free survival (DFS) and overall survival (OS), and the factors influencing survival between the two groups. $P < 0.05$ (two-sided) was considered statistically significant.

Results

53BP1 expression and CD45RO+, CD3+, and CD8+ TIL density are associated with sensitivity to neoadjuvant CRT in rectal cancer

In total, 55 patients diagnosed with locally advanced rectal cancer from January 2015 to February 2018 undergoing neoadjuvant CRT plus TME were enrolled in this study. According to the postoperative TRG, 34 of the 55 patients (62%) achieved a grade 3–4 pathological response and were included in the response group; the other 21 patients (38%) achieved a grade 0–2 response and were included in the nonresponse group. No significant differences in clinical features, such as age, gender, clinical stage, tumor location, histological grade, tumor markers, or circumferential resection margin (CRM) and extramural venous invasion (EMVI) on conventional MRI were observed between the

Table 1 Patient clinical characteristics and response to neoadjuvant CRT

Clinical characteristic	Response (n = 34; %)	Nonresponse (n = 21; %)	P-value
<i>Gender</i>			0.70
Male	26 (76)	17 (81)	
Female	8 (24)	4 (19)	
<i>Age (years)</i>			0.78
>60	7 (21)	5 (24)	
≤60	27 (79)	16 (76)	
<i>Location (distance to anus; cm)</i>			0.93
>5	15 (44)	9 (43)	
≤5	19 (56)	12 (57)	
<i>Tumor grade (differentiation)</i>			0.61
Well–moderate	17 (50)	12 (57)	
Poor	17 (50)	9 (43)	
<i>Clinical tumor (T) stage</i>			0.21
cT2-3	25 (74)	12 (57)	
cT4	9 (26)	9 (43)	
<i>Clinical nodal (N) stage</i>			0.79
cN0	4 (12)	3 (14)	
cN+	30 (88)	18 (86)	
<i>Pretreatment clinical stage</i>			0.57
IIA–IIIA	5 (15)	3 (14)	
IIIB	19 (56)	9 (43)	
IIIC	10 (29)	9 (43)	
<i>Pretreatment MRI evaluation of CRM</i>			0.31
CRM (+)	10 (29)	9 (43)	
CRM (–)	24 (71)	12 (57)	
<i>Pretreatment MRI evaluation of EMVI</i>			0.46
EMVI (+)	4 (12)	4 (19)	
EMVI (–)	30 (88)	17 (81)	
<i>Pretreatment CEA level (ng/mL)</i>			0.14
>5	14 (41)	13 (62)	
≤5	20 (59)	8 (38)	
<i>53BP1 expression level</i>			0.04
High	28 (82)	12 (57)	
Low	6 (18)	9 (43)	
<i>Immunoscore of CD3/CD8</i>			0.03
>2	16 (47)	4 (19)	
≤2	18 (53)	17 (81)	
<i>Immunoscore of CD3/CD45RO</i>			0.04
>2	21 (62)	7 (33)	
≤2	13 (38)	14 (67)	
<i>Immunoscore of CD8/CD45RO</i>			0.13
>2	15 (44)	5 (24)	
≤2	19 (56)	16 (76)	

CRM circumferential resection margin, CRT chemoradiation therapy, EMVI extramural vascular invasion

two groups ($p > 0.05$). However, the 53BP1 expression level and CD45RO+, CD3+, and CD8+ TIL density were significantly different between the groups. A significantly greater

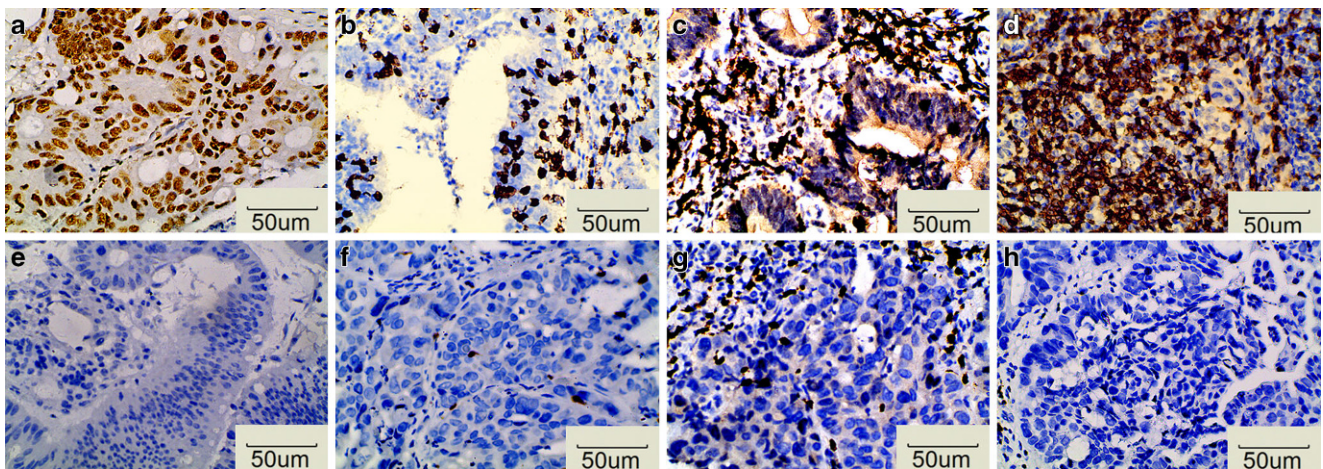


Fig. 1 Representative immunohistochemical staining for 53BP1, CD8, CD3, and CD45RO. **a** High expression of 53BP1. **b** High expression of CD8. **c** High expression of CD3. **d** High expression of CD45RO. **e** Low expression of 53BP1. **f** Low expression of CD8. **g** Low expression of CD3. **h** Low expression of CD45RO

proportion of patients with high 53BP1 expression than with low 53BP1 expression showed clinical downgrading (70% vs. 40%, respectively, $p < 0.05$). Similarly, patients with high levels of CD45RO⁺, CD3⁺, and CD8⁺ TILs and high CD3/CD8 and CD3/CD45RO immunoscore showed significantly better CRT responses than those with low TIL levels and immunoscore ($p < 0.05$). The relevant data and representative images of staining for 53BP1 and T cell markers are shown in Table 1 and Fig. 1.

53BP1 expression is closely related to tumor infiltration by lymphocytes

To understand the internal connection between 53BP1 and degree of T lymphocyte infiltration, the correlations between 53BP1 expression and the three kinds of immunoscore, CD3/CD8, CD3/CD45RO, and CD8/CD45RO, were evaluated (Fig. 2). Lymphocyte infiltration level was positively correlated with 53BP1 expression: low 53BP1 expression was accompanied by decreased CD45RO⁺, CD3⁺, CD8⁺ TIL density and decreased all three kinds of immunoscore. However, with high 53BP1 expression, both the T lymphocyte infiltration levels and the immunoscore were increased. Among the three kinds of immunoscore, the CD3/CD8 immunoscore showed the strongest correlation with the 53BP1 expression level ($p < 0.05$; Supplementary Table 1).

Pretreatment 53BP1 expression level and CD3/CD8 immunoscore enabled effective prediction of response to neoadjuvant therapy

Because the pretreatment 53BP1 expression level and CD3/CD8 immunoscore were closely related to the re-

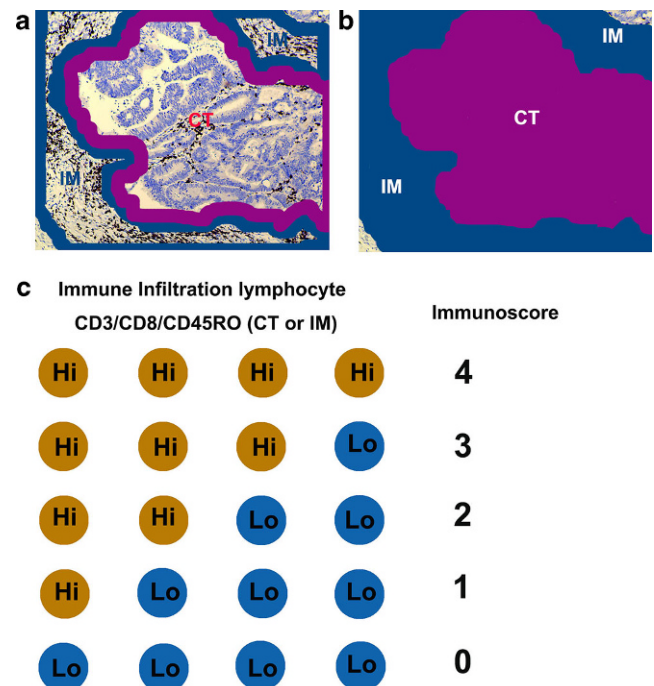


Fig. 2 The definition and method for calculating the immunoscore **a** One representative image of a tumor sample from a rectal cancer patient after immunostaining is shown. The area inside the purple line is the center of the tumor (CT). The area inside the blue line is the invasion margin (IM), which borders the CT and normal tissues. **b** A sketch of the CT and IM. **c** The method for calculating the immunoscore. The pretreatment expression of CD3, CD8, and CD45RO was evaluated in CT and IM area by immunohistochemistry, as 1 point for high expression and 0 points for low expression for each marker. The scores of CD3/CD8, CD3/CD45RO, and CD8/CD45RO in two regions were summarized as 0–4 points, among which 0–2 points for lower immune group, 3–4 points for a high immune score group

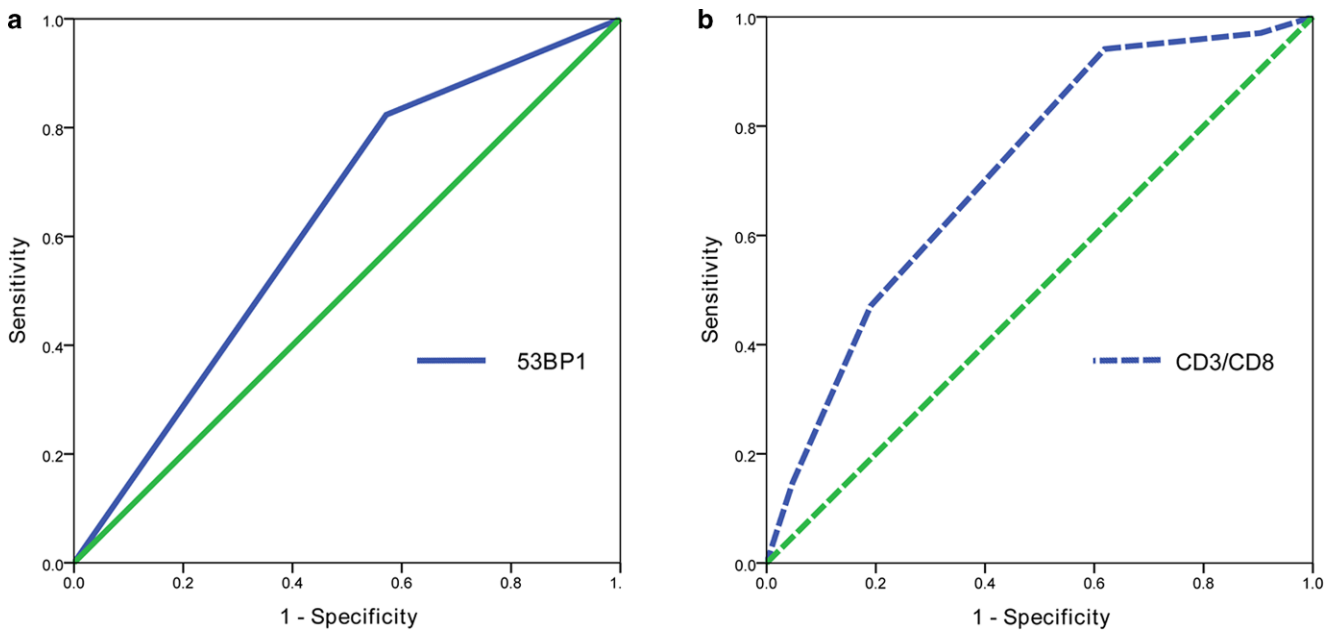


Fig. 3 Receiver operating curves for 53BP1 and CD3/CD8; 53BP1 and CD3/CD8 could predict the response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer patients. **a** The area under the curve (AUC) for 53BP1 was 0.626 (accuracy 67.2%). **b** The AUC for CD3/CD8 was 0.717 (accuracy 72.3%)

to further evaluate their value in predicting the response to neoadjuvant therapy. The sensitivity, accuracy, and AUC of 53BP1 expression in predicting the therapeutic response were 82.3%, 67.2%, and 0.626, respectively, and the corresponding values for the CD3/CD8 immunoscore were 94.1%, 72.3% and 0.717, respectively. Our results suggested that both the 53BP1 expression level and the CD3/CD8 immunoscore could be used to predict the neoadjuvant therapy response (Supplementary Table 2 and Fig. 3).

Pretreatment 53BP1 expression and immunoscore status of the tumor tissue are closely correlated with DFS after CRT

All 55 enrolled patients completed subsequent follow-up to obtain DFS and OS data. After the median follow-up time of 29 months (ranging from 3 to 48 months), four patients developed local recurrence and nine patients had distant organ metastases during follow-up. Based on the survival analysis, the impact of pretreatment 53BP1 expression and CD3/CD8 immunoscore on DFS and OS were evaluated. We found that although both 53BP1 and CD3/CD8 immunoscore had no effect on 2-year OS (53BP1 high expression vs. low expression: 100% vs. 97.5%; CD3/CD8 high immunoscore vs. low immunoscore: 100% vs. 97.1%; Supplementary Figures 1a, b). In the DFS analysis, the high 53BP1 expression group demonstrated a higher 2-year DFS rate than the low expression group (87.5% [95%CI

77.3–97.7] vs. 53.3% [95%CI 28.1–78.6]; $p=0.018$) for all patients (Fig. 4a). Moreover, in a subset analysis, the influence of 53BP1 expression levels on DFS was not affected by CD3/CD8 immunoscore status (Fig. 4e, f). It seemed there was no significant difference in 2-year DFS between the high and low immunoscore groups for all patients ($p>0.05$; Fig. 4b). In a subset analysis, among patients with a CD3/CD8 immunoscore ≤ 2 , 2-year DFS rate was shorter with low 53BP1 expression than in those with high 53BP1 expression (53.8% [95%CI 40.6–67.7] vs. 86.4% [95%CI 79.1–93.7], $p=0.048$; Fig. 4c). By contrast, a CD3/CD8 immunoscore greater than 2 resulted in no difference between the high and low 53BP1 expression groups (Fig. 4d).

Furthermore, univariate and multivariate Cox regression analyses were employed to assess the influence of clinical factors on DFS for neoadjuvant treatment in rectal cancer, which confirmed that 53BP1 expression and CRM status were independent factors affecting prognosis (Table 2).

Discussion

The DDR pathway plays a pivotal role in influencing tumor cells' radiosensitivity. *53BP1* is a key component of the DDR pathway and acts as an important early response mediator in the DNA damage repair. Different degrees of *53BP1* deletions have been found during the occurrence and development of multiple tumors, which are closely

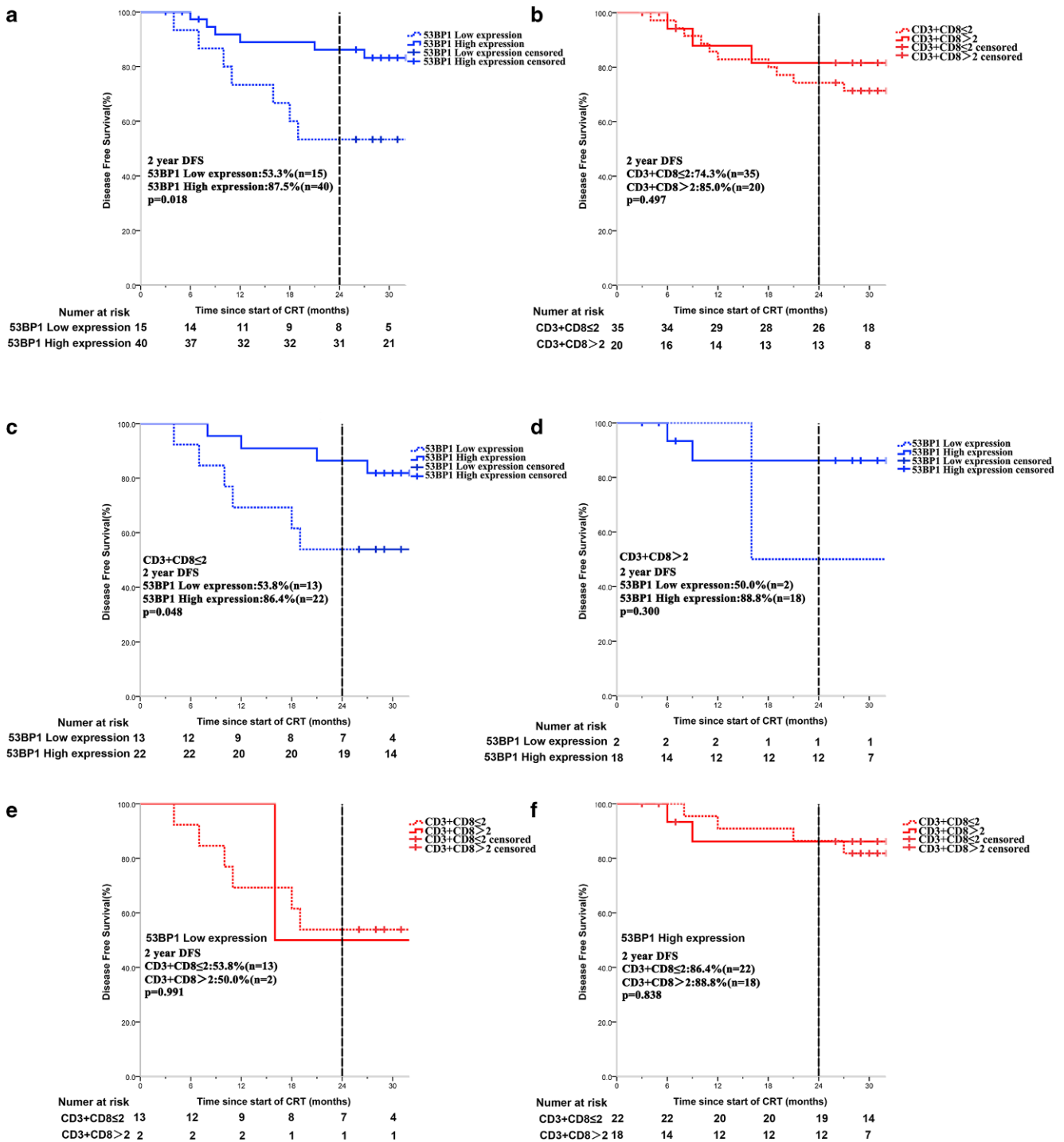


Fig. 4 The Kaplan–Meier method was applied to assess disease-free survival (DFS). **a** 2-year DFS rate was higher in patients with low 53BP1 expression than in those with high 53BP1 expression ($p < 0.05$). **b** There was no significant difference in 2-year DFS rate between patients with a CD3/CD8 immunoscore ≤ 2 and those with a CD3/CD8 immunoscore > 2 ($p > 0.05$). **c** Among patients with a CD3/CD8 immunoscore ≤ 2 , 2-year DFS rate was lower with low 53BP1 expression than in those with high 53BP1 expression ($p < 0.05$). **d** Among patients with a CD3/CD8 immunoscore > 2 , there was no significant difference in 2-year DFS rate between patients with low and high 53BP1 expression ($p > 0.05$). **e, f** Among patients with 53BP1 high expression or 53BP1 low expression, there was no significant difference in 2-year DFS rate between patients with different immunoscores ($p > 0.05$)

Table 2 Univariate and multivariate Cox regression analyses of prognostic factors for neoadjuvant treatment in rectal cancer patients

	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
Gender	1.446	0.320–6.526	0.632	–	–	–
Age	0.698	0.155–3.152	0.641	–	–	–
Tumor location	1.529	0.514–4.552	0.446	–	–	–
Tumor grade	0.993	0.334–2.957	0.990	–	–	–
cT stage	3.397	1.217–9.481	0.020	2.414	0.531–10.983	0.254
cN stage	0.199	0.006–6.093	0.355	–	–	–
Clinical stage	4.803	1.600–14.415	0.005	2.238	0.481–10.419	0.305
CRM	0.399	0.221–0.721	0.002	0.240	0.067–0.866	0.029
EMVI	0.702	0.368–1.340	0.284	–	–	–
CEA	2.323	0.714–7.554	0.161	–	–	–
TRG	2.904	0.948–8.894	0.062	–	–	–
53BP1	0.289	0.097–0.862	0.026	0.268	0.075–0.957	0.043
CD3/CD8	0.642	0.177–2.332	0.500	–	–	–
CD45RO/CD8	0.524	0.144–1.904	0.326	–	–	–
CD3/CD45RO	0.294	0.081–1.069	0.063	–	–	–

CRM circumferential resection margin, CRT chemoradiation therapy, EMVI extramural vascular invasion, TRG tumor regression grade, HR hazard ratio, CI confidence interval

related to tumor stage, malignant grade, and even treatment resistance. We previously confirmed that the expression of 53BP1 was absent in colorectal cancer tissues and that this deficiency was closely related to a poor prognosis. Subsequent in vitro and animal studies revealed that the loss of 53BP1 expression leads to resistance to treatment (chemotherapy and radiotherapy) [7, 8]. In this study, we further verified the effect of 53BP1 on the efficacy of neoadjuvant CRT by collecting expression information of 53BP1 in clinical samples. The expression of 53BP1 was significantly correlated with the treatment response rate. The 53BP1 high expression group showed a high response rate, while the low expression group showed a significant decrease in the response to CRT treatment; this outcome is consistent with our previous studies and suggests that 53BP1 is an effective biomarker for predicting the efficacy of colorectal cancer CRT [7, 8].

In addition to some genetic abnormalities, immunoscore based on the density of CD3/CD8, CD3/CD45RO, and CD8/CD45RO cells in the CT and IM regions has been recognized as another important biomarker for prognosis [14, 18–20]. A series of clinical trials have confirmed close connections of immunoscore with clinical outcomes, as low immunoscore (I0-2) was always accompanied by poor therapeutic effects, while high immunoscore (I3-4) was often associated with better therapeutic effects [9, 19]. Based on the consideration that most previous studies only focused on the influence of TIL level on chemotherapy efficacy—ignoring radiotherapy—or emphasized the role of CD3/CD8 score in therapeutic efficacy—ignoring the role of CD45RO⁺ T cells in immunoscore [14, 21]—we evaluated in detail the relationship between the three scores and

therapeutic efficacy. Our results revealed the TIL level in the tumor area was positively correlated with the therapeutic efficacy, and the CD3/CD8 score demonstrated the greatest relevance to the TRG, with an AUC for predicting the response to CRT of 0.717 among the three immunoscores. This result is consistent with the expert consensus regarding the CD3/CD8 score as the most suitable for clinical application.

Mounting evidence from recent studies has revealed the intimate intrinsic correlation between the immune microenvironment and the DNA damage repair pathway in tumor [5, 22–24]. Some gene mutations or deletions in the DDR pathway, such as the breast cancer susceptibility gene 1 (*BRCA1*) and *Ku70/Ku80*, have been found to induce tumor cells upregulating the expression of programmed death-ligand 1 (PD-L1), which can effectively inhibit the activity of TILs in the tumor immune microenvironment, such as CD8 cells, and then lead to tumor escape [25]. Our previous study has confirmed the close relationship among the 53BP1 expression, tumor radiosensitivity, and proliferation, as that the loss of 53BP1 could significantly enhance the proliferation of tumors and lead to radiation resistance. Some studies have shown that 53BP1 also plays an important role in the normal development of the immune system, participating in the development and normal functioning of T lymphocytes. The loss of 53BP1 expression can lead to a decreased peripheral lymphocyte number and corresponding immune function defects [26]. Therefore, we speculated that 53BP1 loss could decrease colorectal cancer's radiosensitivity not only by enhancing tumor proliferation, also by influencing the immune status of the host.

Thus, we explored the relationship between T cell infiltration density and 53BP1 expression in tumor tissues. The results show that high 53BP1 expression is always accompanied by extensive T cell infiltration, while low 53BP1 expression is accompanied by a lack of T cell infiltration in tissues. The CD3/CD8 immunoscore of the former tissues was significantly higher than that of the latter. Further survival analysis revealed that although the role of 53BP1 in DFS was not affected by the immunoscore, the role of the immunoscore in prognosis was affected by the level of 53BP1 expression; when CD3/CD8 immunoscore was ≤ 2 , the DFS duration of patients with low 53BP1 expression was obviously shorter than that of patients with high 53BP1 expression. These results all suggest a potential synergistic effect between 53BP1 expression and host immune function, but the exact mechanisms still need to be explored in the future.

Taken together, in this study, we confirmed that pretreatment 53BP1 expression and CD3/CD8 immunoscore in tumor tissue could effectively predict CRT efficacy, and the level of 53BP1 expression was closely related to the extent of T cell infiltration in the tumor microenvironment. These results suggest that assessing both the biological properties of a tumor and the immune microenvironment could facilitate a more accurate and objective prediction of the treatment response.

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Author Contribution Ai Huang and Peng chunfen carried out the studies and participated in data collection. Hong Ma and Yong Xiao drafted the manuscript. Tao Liu and Zhenyu Lin performed the statistical analysis and participated in its design. Qin Yang, Jun Liu, and Tao Zhang helped draft the manuscript. All authors read and approved the final manuscript.

Compliance with ethical guidelines

Conflict of interest A. Huang, Y. Xiao, C. Peng, T. Liu, Z. Lin, Q. Yang, T. Zhang, J. Liu, and H. Ma declare that they have no competing interests.

Ethical standards This study was a retrospective observational study approved by the Ethics Committee of Huazhong University of Science and Technology, China. Informed consent was obtained from all patients.

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