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ARTICLE

Overexpression of TP53 protein is associated with the lack of adjuvant chemotherapy benefit in patients with stage III colorectal cancer

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Abstract

TP53 mutations drive colorectal cancer development, with missense mutations frequently leading to accumulation of abnormal TP53 protein. *TP53* alterations have been associated with poor prognosis and chemotherapy resistance, but data remain controversial. Here, we examined the predictive utility of TP53 overexpression in the context of current adjuvant treatment practice for patients with stage III colorectal cancer. A prospective cohort of 264 stage III patients was tested for association of TP53 expression with 5-year disease-free survival, grouped by adjuvant treatment. Findings were validated in an independent retrospective cohort of 274 stage III patients. Overexpression of TP53 protein (TP53+) was found in 53% and 52% of cases from the prospective and retrospective cohorts, respectively. Among patients receiving adjuvant chemotherapy, TP53+ status was associated with shorter disease-free survival ($p \le 0.026$ for both cohorts), while no difference in outcomes between TP53+ and TP53− cases was observed for patients treated with surgery alone. Considering patients with TP53− tumors, those receiving adjuvant treatment had better outcomes compared with those treated with surgery alone ($p \le 0.018$ for both cohorts), while no treatment benefit was apparent for patients with TP53+ tumors. Combined cohort-stratified analysis adjusted for clinicopathological variables and DNA mismatch repair status confirmed a significant interaction between TP53 expression and adjuvant treatment for disease-free survival ($p_{\text{interaction}} = 0.030$). For the combined cohort, the multivariate hazard ratio for TP53 overexpression among patients receiving adjuvant chemotherapy was 2.03 (95% confidence interval 1.41–2.95, $p < 0.001$), while the hazard ratio for adjuvant treatment among patients with TP53− tumors was 0.42 (95% confidence interval 0.24–0.71, $p = 0.001$). Findings were maintained irrespective of tumor location or when restricted to mismatch repair-proficient tumors. Our data suggest that adjuvant chemotherapy benefit in stage III colorectal cancer is restricted to cases with low-level TP53 protein expression. Identifying TP53+ tumors could highlight patients that may benefit from more aggressive treatment or follow-up.

Introduction

5-Fluorouracil-based adjuvant chemotherapy is the standard-of-care for patients with stage III colorectal cancer [1, 2], although some individuals are too frail or decline treatment. Despite being the mainstay of care, the benefit from adjuvant chemotherapy is limited to only 10–15% of individuals and 5-year relapse rates remain at ~60% [3]. Overtreatment with adjuvant chemotherapy (85–90% of patients) is associated with significant toxicities and healthcare costs. As a result, considerable efforts have been invested in the identification of tumor-based molecular markers that can complement standard clinical and pathological staging systems to more accurately predict disease outcome and determine optimal adjuvant treatment approaches. Whilst several markers associated with prognosis have been identified in colorectal cancer, such as DNA mismatch repair status and gene expression profiles [4, 5], there remains a clinical need for biomarkers predictive of which patients will derive a benefit from treatment with chemotherapy.

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Mutation of the *TP53* tumor suppressor is a central driver of colorectal carcinogenesis found in ~50% of sporadic tumors [6]. Wild-type TP53 protein mediates cell-cycle arrest and cell-death checkpoints, which can be triggered by various cellular stress signals [7]. Normal cells under unperturbed conditions express TP53 at low levels, with its degradation mediated by ubiquitin-ligases including MDM2, COP-1, and PIRH-2 [8–10]. In most tumors, both *TP53* alleles are inactivated, usually by a combination of a missense mutation and 17p deletion that eliminates the second *TP53* allele [11]. *TP53* missense mutations frequently lead to accumulation of abnormal TP53 protein with a prolonged half-life in the nucleus, which can be detected by immunohistochemistry [12, 13]. Studies that have examined both TP53 expression and mutation data for colorectal cancer have reported an agreement of immunohistochemistry and mutation detection between 53 and 76% [14–20].

TP53 aberrations are considered a late event in the classic adenoma-to-carcinoma sequence of colorectal tumorigenesis, associated with the transition from adenoma to carcinoma [21]. Some evidence suggest that the frequency of *TP53* aberrations increases with tumor stage [22–31], although this has not been confirmed in other studies [16, 28, 32–40]. There is general agreement that *TP53* aberrations occur less frequently in the proximal colon as compared with the distal colon and rectum [18–20, 30, 34, 39, 41–48].

The significance of *TP53* aberrations as a prognostic marker for colorectal cancer remains a matter of controversy. For investigations of TP53 protein expression which included at least 100 patients, overexpression of TP53 has been associated with inferior outcomes in univariate or multivariate survival analyses in multiple reports [19, 20, 26–28, 31–33, 35, 39, 40, 49–58]. However, other studies have found no association [18, 22, 25, 30, 37, 41, 45, 46, 48, 59–65] or reported the opposite finding [34, 38, 66–68]. A systematic review of TP53 expression data performed by Munro et al. some time ago, which encompassed data for 12,257 patients across all tumor stages, concluded that individuals with abnormal TP53 were at increased risk of death (relative risk 1.32, 95% confidence interval 1.23–1.42), with similar results for assessment of mutation data (relative risk 1.31, 95% confidence interval 1.19–1.45) [69]. However, both publication bias and heterogeneity of results were noted.

The lack of a consensus in the literature on the prognostic significance of TP53 aberrations may be due to the use of heterogeneous study populations such as tumor stages included, differences in immunohistochemical methods, limited cohort sizes and duration of follow-up. A major issue is absence of a standardized immunohistochemistry scoring scheme to optimally correlate TP53 protein expression with *TP53* mutation status. It has further been suggested that the prognostic significance of TP53 aberrations may depend on the ethnic group, body-mass index, tumor location, or stage of disease [34, 39, 42, 43, 52]. TP53 aberrations are also negatively associated with tumor microsatellite instability status [20, 39, 44, 70–72], found in ~15% of colorectal cancers, an established marker of good prognosis for early-stage colorectal cancer [4]. Microsatellite instability is characterized by increased insertion or deletion mutations at simple repeat sequences due to a defect in DNA mismatch repair [73], and may be associated with a lack of benefit from 5-fluorouracil-based adjuvant chemotherapy [74–77].

Furthermore, there are data from colorectal cancer cell lines studies to suggest that the benefit of adjuvant chemotherapy may be limited to patients with *TP53* wild-type cancers. Specifically, colorectal cancer cells with wild-type *TP53* have consistently been found to be more sensitive to 5-fluorouracil and oxaliplatin treatment as compared with cells with mutated *TP53* [78–83]. Accordingly, some patient cohort studies have reported inferior outcomes for individuals receiving chemotherapy if their tumors had TP53 overexpression [20, 38, 84, 85], but this was not observed by others [30, 55, 86–88]. The previous systematic review of Munro at el. did not find a relationship between TP53 aberrations and adjuvant chemotherapy benefit, although it identified a relationship with neoadjuvant chemoradiation for rectal cancer [69]. Results of the TP53 Colorectal Cancer International Collaborative Study, which combined *TP53* mutation data from 25 different research groups in 17 countries, indicated a potential interaction between *TP53* mutation status and adjuvant treatment benefit for Dukes' C patients with distal tumors [89].

To clarify the conflicting data regarding the prognostic and predictive value of TP53 protein expression in early-stage colorectal cancer, we evaluated TP53 expression in a homogenous prospective community-based population of 264 stage III patients. Specifically, the present study sought to clarify the clinical potential of TP53 overexpression as a predictor of outcomes in the context of current adjuvant treatment practice. An optimal cutoff for identifying TP53 overexpression (TP53+ vs TP53−) was determined using the Allred scoring system [90], which considers both proportion of stained cells and stain intensity, based on a set of 66 colorectal cancer cell lines with existing *TP53* mutation data. Findings were validated in an independent retrospective community-based cohort of 274 stage III patients. Hazard ratios and recurrence rates were estimated for patient subgroups by TP53 expression status and adjuvant treatment for a cohort-stratified analysis of the combined 538 patient population.

Materials and methods

Prospective community series of colorectal cancer patients

A total of 264 patients with resected stage III colorectal adenocarcinoma were recruited at the Royal Melbourne Hospital and Western Hospital, Footscray in Australia between 1999 and 2011. Individuals with hereditary polyposis colorectal cancer syndromes or who had received neoadjuvant chemoradiation were excluded. Formalin-fixed paraffin-embedded tumor and matched normal tissue specimens were obtained at surgery, and tissue microarrays consisting of 1-mm-diameter tissue cores were constructed. For each patient, four tumor and two normal tissue cores were embedded, with tumor cores taken from areas of the densest tumor cell percentage. A total of 189 (72%) patients received 5-fluorouracil-based adjuvant chemotherapy. All patients were prospectively followed according to the standard protocols, with 3 monthly clinic visits and testing for carcinoembryonic antigen levels, 12 monthly CT scans of the chest, abdomen and pelvis for 2 years after diagnosis and then 6 monthly clinic visits and carcinoembryonic antigen testing until 5 years from diagnosis. Patient characteristics are summarized in Supplementary Table S1. All participants gave informed consent, and this study was approved by the medical ethics committees of all sites (HREC 12/19).

TP53 protein expression

The DO-7 mouse monoclonal anti-TP53 antibody (Novocastra) was used for immunohistochemistry. Tissue staining was performed on tissue microarray sections as per standard protocol on a BenchMark ULTRA platform (Ventana). Briefly, heat-induced antigen retrieval was performed using CC1 (EDTA) buffer at 95 °C for 36 min. Tissue sections were incubated at 36 °C with the primary TP53 antibody at a 1:100 dilution for 32 min, followed by signal detection using an enzyme-conjugated multimer secondary antibody (UltraView Universal DAB detection kit, Ventana). Sections were counterstained with hematoxylin.

Evaluation of TP53 staining was carried out by a gastrointestinal pathologist (DSW) blinded to all clinical information. We considered only tumor cells with distinct nuclear immunostaining for TP53 as positive (Supplementary Fig. S1). Considering all tumor cores, the Allred score was calculated by adding a score reflecting the percentage of positive tumor cells (0 for none, 1 for <1%, 2 for 1–10%, 3 for 11–33%, 4 for 34–66%, and 5 for 67–100%) and a score reflecting the intensity of immunoreactivity (1 for weak, 2 for moderate, and 3 for strong), with a maximum score of 8 [90]. Based on a set of colorectal cancer cell lines ($n = 66$) with known *TP53* mutation status [91], an Allred score of 6 or greater was selected, reflecting the cutoff value identifying *TP53* missense mutated cases with maximum sensitivity and specificity (Supplementary Methods, Supplementary Fig. S2). To assess the accuracy of our colorectal cancer cell line determined cut-off in primary tumor tissue, we integrated immunohistochemistry scores with *TP53* mutation data available from Sanger sequencing on a subset of 51 tumor samples [92]. A total of 25 of 26 (96%) samples with *TP53* missense mutation exhibited an Allred score of >6, while 19 of 25 (76%) samples without *TP53* missense mutation exhibited an Allred score of ≤6. Reproducibility of TP53 expression scores was verified by re-examination of a random selection of 454 cases across both our study cohorts by a second observer (CB), blinded to all prior data. Interobserver reproducibility for TP53 scoring showed high concordance (κ statistic = 0.85; 95% confidence interval = 0.80–0.90).

DNA mismatch repair status

Whole-tissue sections were cut from tumor and matched normal formalin-fixed paraffin-embedded blocks. For tumor samples, macrodissection was performed to enrich for areas with >60% neoplastic cells guided by hematoxylin and eosin stained tissue sections. DNA was extracted using standard protocols and polymerase chain reaction amplified for the Bethesda consensus panel of microsatellite markers (BAT25, BAT26, D2S123, D5S346, and D17S250) using fluorescently labeled primers [93]. Polymerase chain reaction products were analyzed on a 3130xl Genetic Analyzer (Applied Biosystems). Mismatch repair-deficient status was diagnosed if instability was detected at two or more markers, and mismatch repair-proficient status was diagnosed if instability was detected at fewer than two markers.

Retrospective community series of colorectal cancer patients

To validate findings, we examined an independent cohort of 274 patients with stage III colorectal cancer recruited at Austin Health, Australia, between 1998 and 2015, for whom clinical, treatment and follow-up data were retrospectively assembled with human research ethics approval (HREC H2013/05077). Among these patients, 181 (66%) had received 5-fluorouracil-based adjuvant chemotherapy. Individuals with hereditary polyposis colorectal cancer syndromes or who had received neoadjuvant chemoradiation were excluded. Patient characteristics are summarized in Supplementary Table S1. Archival formalin-fixed paraffin-embedded tumor blocks were retrieved, and tissue microarrays prepared with sampling of three 1-mm-diameter cores per patient. Immunohistochemistry for

TP53 expression was performed on the same platform as for the prospective community series. For this cohort, tumor DNA mismatch repair status was assigned based on standard immunohistochemistry diagnostic assays for mismatch repair proteins (MLH1, MSH2, PMS2, and MSH6) by pathologist DSW [94]. Mismatch repair-deficient was defined as absence of nuclear staining in tumor cells but positive nuclear staining in stromal cells and lymphocytes for at least one mismatch repair protein.

Statistical methods

Statistical analyses were conducted using the statistical computing software R (R Development Core Team, 2011). Receiver operator curves were used to determine the optimal Allred score cutoff for TP53 immunohistochemistry to detect *TP53* missense mutations (see Supplementary Methods). Interobserver reproducibility for determination of TP53 expression status between reviewers was assessed using Cohen's kappa statistic. For univariate analyses, differences between groups were assessed using the Fisher's exact test for categorical variables and the Kruskal–Wallis test for continuous variables. Outcome analyses were conducted for 5-year disease-free survival. Disease-free survival was defined as time from surgery to the first confirmed relapse, with censoring done when a patient died or was alive without recurrence at last contact. Survival curves were generated according to the method of Kaplan and Meier. Cox proportional hazard models were used to assess the associations of TP53 expression status with disease-free survival in the context of patient characteristics, DNA mismatch repair status and adjuvant treatment. Hazard ratios and 95% confidence intervals were calculated. All statistical analyses were two-sided and considered significant if $p < 0.050$.

Results

Patient characteristics of the prospective community-based cohort

Of the 264 patients with stage III adenocarcinoma, 102 (39%) were female, and the median patient age was 70 (range 39–91) years (Supplementary Table S1). A total of 106 (40%) cancers were from the proximal colon and 158 (60%) from the distal colon or rectum; 168 (64%) exhibited low and 96 (36%) high grade. All patients underwent surgical resection, and 189 (72%) patients received 5-fluorouracil-based adjuvant chemotherapy. A total of 91 (34%) patients experienced a disease recurrence. The median follow-up duration was 26.6 (range 3.4–60.0) months for individuals with recurrence and 60.0 (range 0.3–60.0) months for individuals without recurrence.

Overexpression of TP53 was scored based on the intensity and percentage of stained tumor nuclei using a predetermined Allred score cutoff of ≥6, optimized to identify *TP53* missense mutations in a set of 66 colorectal cancer cell lines (Supplementary Methods, Supplementary Figs. S1 and S2). Nonneoplastic colonic mucosa, inflammatory, and stromal cells exhibited weak staining and served as positive internal controls. Overall, 53% (140 of 264) of the tumors exhibited TP53 overexpression, termed TP53+. For mismatch repair status 20% (52 of 264) of the tumors were mismatch repair deficient.

Compared with TP53− tumors, the TP53+ tumors were associated with distal location (odds ratio 1.7, 95% confidence interval 1.0–2.9, $p = 0.044$) and mismatch repair-proficient status (odds ratio 2.1, 95% confidence interval 1.1–4.1, $p = 0.021$) (Table 1). There was no association of TP53 expression status with gender, age at presentation or grade. Mismatch repair-deficient status was positively correlated with proximal tumor location (odds ratio 6.4, 95% confidence interval 3.1–14.1, $p < 0.001$) and high grade (odds ratio 3.0, 95% confidence interval 1.6–5.9, $p < 0.001$).

TP53 expression status and disease-free survival

Overall, patients with TP53+ tumors had inferior 5-year disease-free survival rates than patients with TP53− tumors (54.5% vs 68.9%). The univariate hazard ratio for disease-free survival for the TP53+ group versus the TP53 − group was 1.64 (95% confidence interval 1.08–2.52; $p = 0.020$). In addition, proximal tumor location and high grade were associated with reduced disease-free survival while adjuvant treatment was associated with improved disease-free survival ($p < 0.020$ for all univariate analyses). Patient gender, age at diagnosis, and mismatch repair status were not associated with outcome in our cohort. In multivariate analysis adjusting for clinicopathological variables significant in the univariate models, TP53 expression remained an independent predictor of poor prognosis (hazard ratio 1.93, 95% confidence interval 1.25–2.97, $p = 0.003$).

Adjuvant treatment benefit by TP53 expression status

We next examined whether tumor TP53 expression status was associated with differential benefit from adjuvant treatment. Notably, patients who had received adjuvant treatment were significantly younger than individuals who had not received adjuvant treatment (66 vs 82 years; $p < 0.001$); this trend was similar across the TP53 subgroups (data not shown). The duration of follow-up according to adjuvant treatment was similar between TP53 subgroups ($p > 0.066$).

Table 1 Clinicopathologic and molecular characteristics of patients with stage III colorectal cancer according to TP53 expression status

	Prospective cohort			Retrospective cohort		
	TP53− $n = 124$	TP53+ $n = 140$	p	TP53− $n = 132$	TP53+ $n = 142$	p
Age (years)						
Mean ± SD	69.5 ± 10.9	68.8 ± 11.8	0.626	69.2 ± 3.2	68.3 ± 1.8	0.572
Median	70	69		70.5	71	
Range	39–90	40–91		28–94	42–94	
Gender						
Male	78 (63)	84 (60)	0.704	78 (59)	68 (48)	0.070
Female	46 (37)	56 (40)		54 (41)	74 (52)	
Site						
Proximal	58 (47)	48 (34)	0.044*	92 (70)	63 (44)	<0.001*
Distal	66 (53)	92 (66)		40 (30)	79 (56)	
Grade						
Low grade	76 (61)	92 (66)	0.522	80 (61)	91 (64)	0.618
High grade	48 (39)	48 (34)		52 (39)	51 (36)	
Adjuvant						
No	34 (27)	41 (29)	0.785	50 (38)	43 (30)	0.203
Yes	90 (73)	99 (72)		82 (62)	99 (70)	
Mismatch repair status						
Proficient	92 (74)	120 (86)	0.021*	97 (73)	133 (94)	<0.001*
Deficient	32 (26)	20 (14)		35 (27)	9 (6)	

Data are for the prospective cohort ($n = 264$) and retrospective cohort ($n = 274$). Percentages for columns are shown in round brackets.

*$p < 0.05$

Considering patients grouped by adjuvant treatment, association of TP53 expression status with outcome appeared restricted to individuals receiving chemotherapy. Among adjuvant therapy treated patients, TP53+ tumors showed significantly poorer outcomes than TP53− tumors with a univariate hazard ratio for disease-free survival of 2.09 (95% confidence interval 1.23–3.56, $p = 0.005$) and 5-year disease-free survival rates of 55.7% (95% confidence interval 46.5–66.7%) and 76.3% (95% confidence interval 67.8–86.0%), respectively (Fig. 1a). In multivariate analysis adjusting for clinicopathological variables significant in univariate analysis, the hazard ratio for TP53+ tumors in the adjuvant treatment group was 2.59 (95% confidence interval 1.50–4.47; $p < 0.001$). No difference in outcome by TP53 expression status was apparent for patients treated with surgery alone (univariate hazard ratio 1.02, 95% confidence interval 0.49–2.12, $p = 0.956$, Fig. 1b).

For groups of patients by TP53 expression status, adjuvant treatment benefit was evident for TP53− tumors, but not for TP53+ tumors. Among patients with TP53− tumors, the hazard ratio for disease-free survival for adjuvant treatment as compared with surgery alone, adjusting for variables significant in univariate models, was 0.41 (95% confidence interval 0.19–0.75; $p = 0.008$) (Fig. 2a). In contrast, among TP53+ patients, the univariate hazard ratio for disease-free survival for adjuvant treatment was 0.79 (95% confidence interval, 0.44–1.42; $p = 0.434$) (Fig. 2b).

Independent retrospective community cohort

We validated the differential outcome associations for TP53 expression by adjuvant treatment in an independent retrospective population of 274 patients with stage III colorectal cancer, 66% (181 of 274) of whom had received adjuvant chemotherapy (Supplementary Table S1). A total of 108 (39%) of patients in this cohort had experienced relapse. The median follow-up duration was 36.0 (range, 4.3–60.0) months for individuals with recurrence and 59.0 (range, 0.7–60.0) months for individuals without recurrence. Patients in this cohort showed a similar proportion of TP53+ tumors (52%, 142 of 274) as compared with our prospective cohort; again, TP53 overexpression was inversely associated with mismatch repair-deficient status and proximal tumor location ($p < 0.001$ for both comparisons, Table 1).

Outcomes among the treatment groups mirrored the findings from the prospective cohort: TP53+ tumors exhibited inferior disease-free survival than TP53− tumors for patients receiving adjuvant chemotherapy (multivariate hazard ratio 1.73, 95% confidence interval 1.07–2.79,

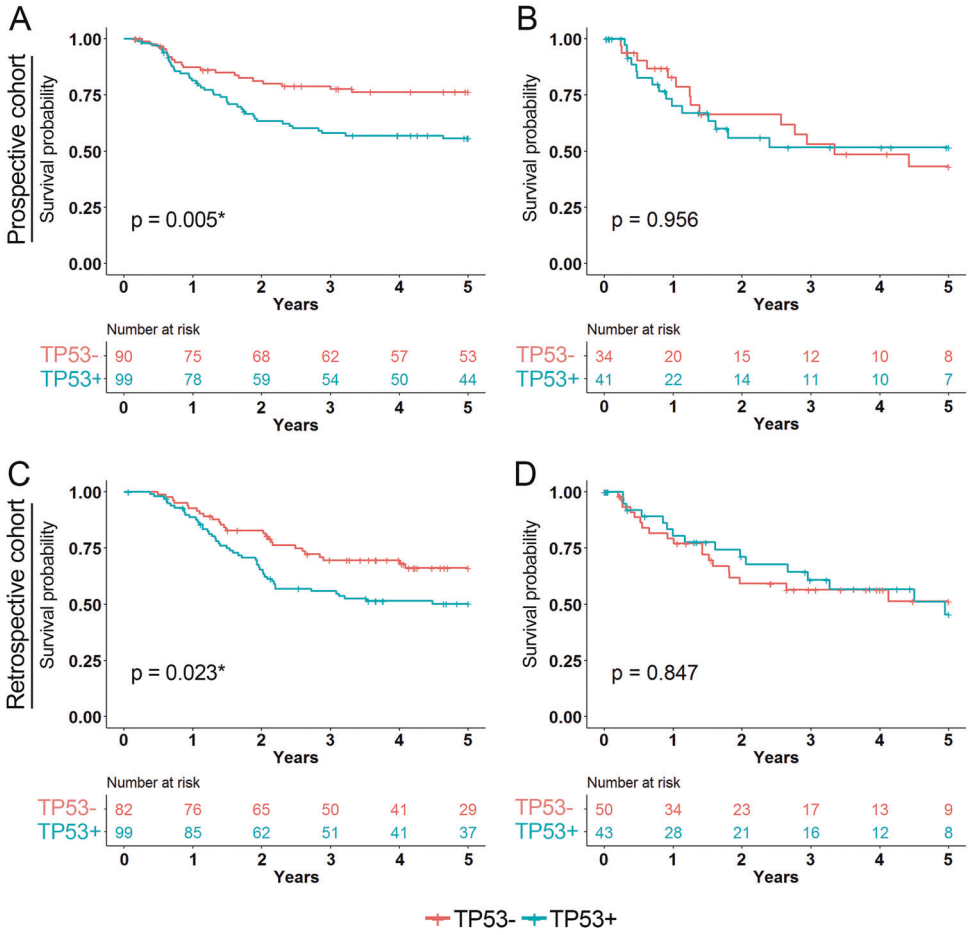

Fig. 1 Kaplan–Meier plot for disease-free survival in patients with stage III colorectal cancer according to TP53 expression status for **a** individuals treated with adjuvant therapy from the prospective cohort ($n = 189$), **b** individuals treated with surgery alone from the prospective cohort ($n = 75$), **c** individuals treated with adjuvant therapy from the retrospective cohort ($n = 181$), and **d** individuals treated with surgery alone from the retrospective cohort ($n = 93$)

$p = 0.026$, Fig. 1c), while similar outcomes were observed for patients treated with surgery alone (multivariate hazard ratio 0.94, 95% confidence interval 0.48–1.82, $p = 0.847$, Fig. 1d). Again, adjuvant treatment benefit appeared limited to patients with TP53− tumors (multivariate hazard ratio 0.48, 95% confidence interval 0.27–0.88, $p = 0.018$, Fig. 2c), with no apparent benefit in TP53+ patients (multivariate hazard ratio 1.08, 95% confidence interval 0.61–1.90, $p = 0.798$, Fig. 2d).

In combined cohort-stratified analysis, multivariate analysis adjusted for all clinicopathological variables and mismatch repair status confirmed a significant interaction between TP53 expression status and adjuvant treatment for disease-free survival ($p_{\text{interaction}} = 0.030$). The 5-year disease-free survival rate was highest for patients with TP53-/adjuvant treated tumors (71.6%, 95% confidence interval 64.9–78.9%), while disease-free survival rates were significantly lower for patients with TP53+/adjuvant treated tumors (52.9%, 95% confidence interval 46.3–60.6%), patients with TP53+/surgery alone tumors (48.1%, 95% confidence interval 36.1–64.0%) and patients with TP53-/surgery alone tumors (47.5%, 95% confidence interval 36.1–62.7%) (Fig. 3). For the combined cohort, the multivariate hazard ratio for TP53 overexpression among patients receiving adjuvant chemotherapy was 2.03 (95% confidence interval 1.41–2.95, $p < 0.001$, Table 2), while the multivariate hazard ratio for adjuvant treatment among patients with TP53− tumors was 0.42 (95% confidence interval 0.24–0.71, $p = 0.001$, Table 3). The relationship between TP53 overexpression and inferior outcome among patients receiving adjuvant chemotherapy was maintained when extending the multivariate analysis to include further available National Comprehensive Cancer Network high-risk features, T4 stage and extramural venous invasion (Supplementary Table S2). Improved survival rates for patients with TP53-/adjuvant treated tumors were found when separately analyzing patients with proximal and distal cancers, or when restricting the analysis to patients with mismatch repair-proficient tumors (Supplementary Table S3).

Discussion

The present study sought to clarify the clinical potential of TP53 overexpression as a predictor of outcomes for patients

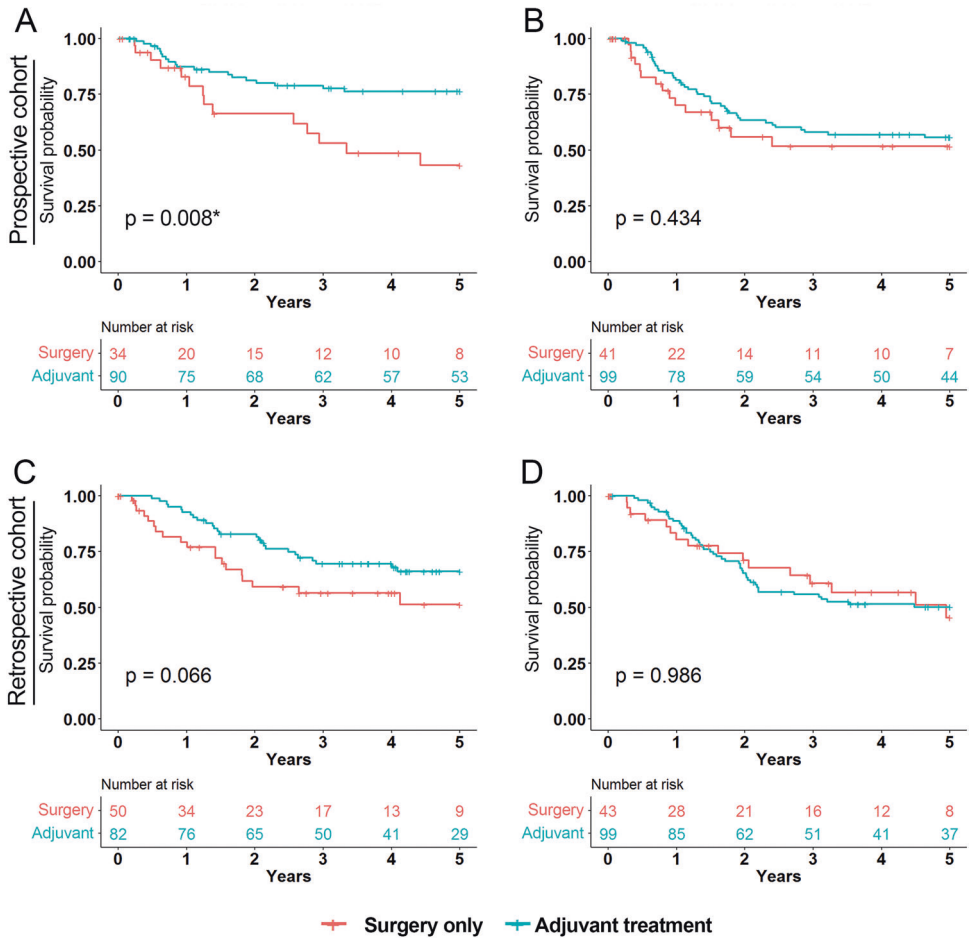

Fig. 2 Kaplan–Meier plot for disease-free survival in patients with stage III colorectal cancer according to adjuvant treatment for **a** individuals with TP53− tumors from the prospective cohort ($n = 124$), **b** individuals with TP53+ tumors from the prospective cohort ($n = 140$), **c** individuals with TP53− tumors from the retrospective cohort ($n = 132$), and **d** individuals with TP53+ tumors from the retrospective cohort ($n = 142$)

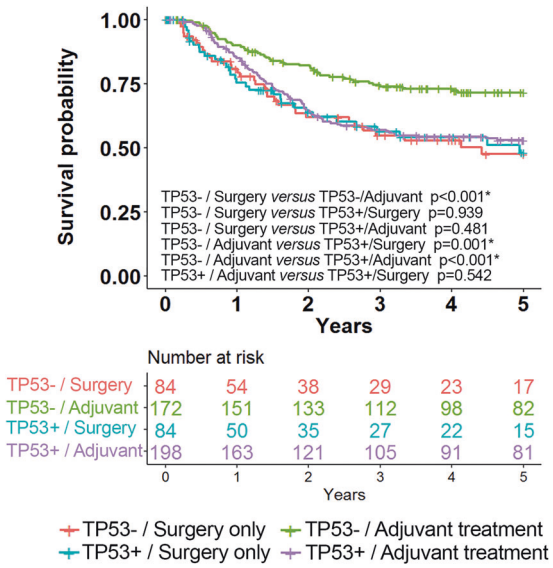

Fig. 3 Kaplan–Meier plot for disease-free survival in patients with stage III colorectal cancer according to TP53 expression status and adjuvant treatment for the combined patient cohort ($n = 538$)

with stage III colorectal cancer in the context of current adjuvant treatment practice. We examined a total of 538 patients across two independent cohorts drawn from prospective and retrospective community-based patient populations. Among patients receiving adjuvant chemotherapy, TP53+status was associated with shorter disease-free survival for both cohorts, while no difference in outcomes between TP53+ and TP53− cases was observed for patients treated with surgery alone. Considering patients with TP53- tumors, those receiving adjuvant treatment had better outcomes as compared those treated with surgery alone, while no significant benefit from adjuvant treatment was apparent for patients with TP53+ tumors. Combined cohort-stratified analysis adjusted for clinicopathological variables and mismatch repair status confirmed a significant interaction between tumor TP53 expression status and adjuvant treatment for disease-free survival. Taken together, these data are consistent with TP53 overexpression-related resistance to 5-fluorouracil-based adjuvant chemotherapy, highlighting TP53+ patients as a subset to consider for more aggressive treatment or follow-up. Activation of

Table 2 TP53 expression status and disease-free survival in patients with stage III colorectal cancer according to adjuvant treatment for the combined patient cohort ($n = 538$)

	Surgery only			Adjuvant therapy		
	$n = 168$			$n = 370$		
	Hazard ratio	95% confidence interval	p	Hazard ratio	95% confidence interval	p
TP53 status (TP53+ vs TP53−)	0.88	0.51–1.51	0.639	2.03	1.41–2.95	<0.001*
Age (decades)	0.99	0.76–1.29	0.926	1.00	0.85–1.19	0.973
Gender (male vs female)	1.31	0.74–2.32	0.349	1.32	0.94–1.86	0.113
Site (proximal vs distal)	1.05	0.59–1.86	0.863	1.48	1.03–2.13	0.034*
Grade (high vs low)	1.31	0.78–2.20	0.301	2.14	1.51–3.01	<0.001*
Mismatch repair status (deficient vs proficient)	0.69	0.34–1.40	0.303	0.80	0.47–1.37	0.418

Data are for multivariate cohort-stratified analysis

*$p < 0.05$

Table 3 Adjuvant treatment and disease-free survival in patients with stage III colorectal cancer according to TP53 expression for the combined patient cohort ($n = 538$)

	TP53-			TP53+		
	$n = 256$			$n = 282$		
	Hazard ratio	95% confidence interval	p	Hazard ratio	95% confidence interval	p
Adjuvant (yes vs no)	0.42	0.24–0.71	0.001*	0.99	0.61–1.63	0.983
Age (decades)	1.00	0.98–1.02	0.699	1.00	0.98–1.02	0.966
Gender (male vs female)	1.79	1.14–2.81	0.011*	1.03	0.71–1.49	0.867
Site (proximal vs distal)	1.27	0.78–2.08	0.331	1.31	0.89–1.93	0.168
Grade (high vs low)	1.10	0.68–1.77	0.694	2.48	1.72–3.57	<0.001*
Mismatch repair status (Deficient vs Proficient)	0.51	0.27–0.97	0.039*	1.40	0.80–2.44	0.239

Data are for multivariate cohort-stratified analysis

*$p < 0.05$

wild-type TP53 plays a pivotal role in triggering apoptosis in response to chemotherapeutic agents, and the predictive value of TP53 overexpression may be related to mutated protein impairing downstream transcriptional activity and abrogating TP53 interaction with pro-survival BCL-2 family proteins [95–98].

Missense mutations in *TP53* are common in sporadic colorectal cancer, and often result in accumulation of TP53 protein. However, there is presently no standardized clinical scoring system for evaluating TP53 status by immunohistochemistry. Studies using the DO-7 antibody report TP53 overexpression in between 30 and 63% of colorectal cancers [18–20, 22, 30, 34–36, 38, 42, 45, 47, 54, 57, 61, 64, 65, 88, 99]. Using a separate training cohort of 66 colorectal cancer cell lines with known *TP53* mutation status, we determined that an Allred score of ≥6, which considers both proportion of stained cells and stain intensity, optimally identified cases with *TP53* missense mutations. Applying this cutoff to our prospective and retrospective patient cohorts, we identified TP53 overexpression in 53% and 52% of cases, respectively. Multiple reports have discussed the relationship of TP53 aberrations with location of the tumor [18–20, 30, 34, 39, 41–48]. Consistent with these studies, we found that the frequency of TP53 overexpression was reduced in tumors from the proximal colon as compared with the distal colon and rectum. We also observed the well-established negative association between TP53 aberrations and mismatch repair deficiency [39, 44, 70–72].

Data regarding the prognostic role of TP53 expression in colorectal cancer are heterogeneous. Several studies have reported that TP53 overexpression is an adverse prognostic factor [19, 20, 26–28, 31–33, 35, 39, 40, 49–58], and this overall trend was evident in our study for combined cohort-stratified multivariate analysis with baseline variables (Supplementary Table S4) and with additional inclusion of

T4 stage and extramural venous invasion (Supplementary Table S5). Nonetheless, other studies found no relationship with outcome [18, 22, 25, 30, 37, 41, 45, 46, 48, 59–65] and a few reports observed the opposite association [34, 38, 66–68].

Besides differences in TP53 assay methodologies, scoring schemes, cohort heterogeneity, study sizes and duration of follow-up, in vitro studies have highlighted TP53 aberration-associated resistance to 5-fluorouracil and oxaliplatin as a potential major confounding factor of colorectal cancer prognostic studies [78–83]. In agreement with these observations, we found that for patients receiving adjuvant chemotherapy, individuals with TP53+ tumors had significantly poorer outcomes than individuals with TP53− tumors. For patients grouped by TP53 expression status, adjuvant treatment was associated with improved outcomes for individuals with TP53− tumors, but not for individuals with TP53+ tumors. Improved survival rates for patients with TP53-/adjuvant treated tumors were observed irrespective of tumor location and when restricting the analysis to patients with mismatch repair-proficient tumors. However, these results should be interpreted with caution because of the non-randomized use of adjuvant chemotherapy. In addition, we considered all FU-based treatment regimens as one group, although TP53 aberrations may show different predictive values according to the exact type of treatment used [47]. Bearing in mind these caveats, our results support the contention that the benefit from adjuvant chemotherapy depends on TP53 expression status. Consistent with our data, several cohort studies have reported an apparent lack of benefit from chemotherapy for early-stage and metastatic tumors with TP53 overexpression [20, 38, 84, 85]. However, other reports of patients with early-stage colorectal cancer did not reproduce this observation [30, 55, 86–88], although these differed in methods or cut-offs for scoring TP53 status and included patients with stage II tumors. These studies lacked validation cohorts, and in one study inspection of the Kaplan–Meier curves shows a trend towards chemotherapy benefit in the TP53-ve group [87]. Another study of adjuvant chemotherapy treated patients identified poorer outcomes for TP53+ tumors only if these were also high for BAX expression [72].

In our study, we ensured technical quality by performing the immunohistochemistry detection in an accredited diagnostic laboratory, using the Allred scoring system with a pre-determined TP53 overexpression cutoff optimized to detect *TP53* missense mutations. Reproducibility of TP53 immune-staining scores was confirmed by two reviewers blinded to all clinical data. Clinicopathologic and outcome associations with TP53 status were validated across two independent cohorts. Immunohistochemistry is an attractive approach for detecting biomarkers due to its applicability to routine archival clinical specimens, relatively low cost, rapid test turn-around time, straightforward methodology and analysis. Immunohistochemistry can be readily incorporated into existing panels of biomarkers for specific cancer subtypes.

Limitations of this study include that our cohorts were derived from population-based series treated according to standard-practice for adjuvant therapy, not a randomized clinical trial. As a result of this study design, there is potential bias for poorer outcomes in the untreated population group, likely to be older, frailer or subject to more comorbidities. For this reason, disease-free survival was chosen as the preferred measure for response to therapy rather than overall survival, since disease-free survival is less likely to be impacted by age, frailty, or comorbidities. The study was not standardized for adjuvant treatment regimen, with single-agent 5-fluorouracil and 5-fluorouracil/oxaliplatin combination treatments used as per routine care. As stage III patients cannot ethically be randomized to adjuvant chemotherapy versus surgery alone, analysis of tumor samples from previous randomized clinical trials would be the most appropriate approach for validation of our findings and further examination of TP53 predictive value by treatment regimen. Another limitation of our study is that TP53 immunohistochemistry assessment was based on tissue microarray cores, which may not be representative of the entire tumor due to heterogeneity. Sampling of at least three cores from different regions of tumor should have addressed this issue to some extent. Consistent with most previous reports, our study focused on scoring TP53 overexpression, associated with missense mutations; this excludes loss events due to nonsense, frameshift or splice-site mutations (bi-allelic or with loss of heterozygosity) which account for a subset of ~6% of *TP53* mutated colorectal cancers as estimated from The Cancer Genome Atlas whole-exome sequencing data on colorectal cancers [11]. Conversely, posttranscriptional stabilization processes such as those induced by DNA damage can lead to accumulation of wild-type TP53 protein [100].

In conclusion, our examination of the predictive value of TP53 expression in the context of current adjuvant treatment practice suggests that overexpression of TP53 protein is associated with minimal adjuvant chemotherapy benefit in patients with stage III colorectal cancer. Notably the disease-free survival rate for patients with TP53+ tumors receiving adjuvant treatment was similar to the disease-free survival rates in patients with TP53+ or TP53− tumors treated with surgery alone. Our findings indicate patients with TP53+ tumors as a subset to consider for more aggressive treatment or follow-up. Further evaluation of TP53 expression status as a predictive biomarker for adjuvant therapy appears warranted, utilizing tumor samples from previous randomized clinical trials to determine an optimal diagnostic approach using standardized immunohistochemistry scoring methods

and comparison with sequencing-based testing. Allred scores of 7–8 have very high specificity for *TP53* mutation, and immunohistochemistry can serve as a cost-effective primary screen to identify these cases. Sequencing might optimally be used to identify non-responders to chemotherapy in equivocal (Allred 6) cases, or for tumors lacking TP53 expression which may harbor inactivating *TP53* mutations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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