

## Article

# Prognostic Impact of TP53 Mutations and Tumor Mutational Load in Colorectal Cancer

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**Abstract:** The DNA damage response (DDR) is critical for maintaining genome stability, and abnormal DDR—resulting from mutations in DNA damage-sensing and repair proteins—is a hallmark of cancer. Here, we aimed to investigate the predictive power of DDR gene mutations and the tumor mutational load (TML) for survival outcomes in a cohort of 22 rectal cancer patients who received pre-operative neoadjuvant therapy. Univariate analysis revealed that TML-high and *TP53* mutations were significantly associated with worse overall survival (OS) with TML-high retaining significance in multivariate analyses. Kaplan–Meier survival analyses further showed TML-high was associated with worse disease-free ( $p = 0.036$ ) and OS ( $p = 0.024$ ) results in our patient cohort. A total of 53 somatic mutations were identified in 22 samples with eight (36%) containing mutations in DDR genes, including *ATM*, *ATR*, *CHEK2*, *MRE11A*, *RAD50*, *NBN*, *ERCC2* and *TP53*. *TP53* was the most frequently mutated gene, and *TP53* mutations were significantly associated with worse OS ( $p = 0.023$ ) in Kaplan–Meier survival analyses. Thus, our data indicate that TML and *TP53* mutations have prognostic value for rectal cancer patients and may be important independent biomarkers for patient management. This suggests that prognostic determination for rectal cancer patients receiving pre-operative neoadjuvant therapy should include consideration of the initial TML and tumor genetic status.

**Keywords:** DNA damage response (DDR); colorectal cancer (CRC); mismatch repair (MMR); neoadjuvant chemo/radiotherapy; next-generation sequencing (NGS); tumor mutational load (TML)

## 1. Introduction

Colorectal cancer (CRC) is the third-most common malignancy in both men and women and the second leading cause of cancer-related death worldwide [1], with over 1.8 million new cases and 900,000 deaths in 2020 [2]. Although the overall rates of CRC

have decreased in the past several years, both incidence and mortality are rising in adults under 50 years of age [1,2].

Surgical resection remains the most effective treatment for CRC; however, full resection with clear margins can be difficult due to limited tumor accessibility, particularly within the rectum, resulting in high rates of recurrence for rectal cancer patients [3,4]. Consequently, although colon cancer patients generally receive only postoperative chemotherapy, as needed, based on the risk level and disease severity, for many years, the standard of care for rectal cancer patients involved neoadjuvant chemo/radiotherapy prior to surgery followed by postoperative chemotherapy [3,5,6].

Critically, this treatment paradigm has reduced the recurrence rates and improved the survival outcomes for rectal cancer patients [7]. However, individual patient response is highly variable, with a complete response observed in less than 30% of patients and 40% displaying no significant clinical response [8–10]. A more recent approach in which both neoadjuvant chemo/radiotherapy and adjuvant chemotherapy are administered prior to surgery, known as total neoadjuvant chemotherapy, has shown promise and is becoming increasingly common [11,12].

In addition, nonoperative management of rectal cancer for patients who display a clinical complete response after neoadjuvant therapy, referred to as the Watch and Wait approach, is gaining in popularity [13,14]. However, many unanswered questions remain, and there is an urgent need for biomarkers that can be leveraged to identify the individuals most likely to respond well to preoperative neoadjuvant chemo/radiotherapy, while avoiding potential for treatment-associated toxicity in those unlikely to benefit from these therapies. To this end, various molecular biomarkers for predicting CRC prognosis and response to treatment have been proposed; however, none are currently in clinical use [15–21].

One potential group of molecular biomarkers for CRC are proteins that function in the DNA damage response (DDR). This system detects DNA double-strand breaks (DSBs) and other forms of DNA injury, and in response, it induces both DNA repair pathways and expression of checkpoint proteins, which halt cell cycle progression until the damage can be repaired or apoptosis initiated, thereby, maintaining genome integrity [22]. Thus, mutations in key DDR proteins, such as p53, a mediator of this pathway, often play key roles in cancer development [23].

DDR functionality can further affect the sensitivity to radiotherapy-based cancer treatment, which utilizes ionizing radiation (IR) to generate a wide range of DNA lesions, including DSBs, single-strand breaks (SSBs) and base modifications, leading to cancer cell death. Similarly, chemotherapeutic drugs administered in neoadjuvant therapy, such as 5-fluorouracil (5-FU), induce DNA damage and activate cellular checkpoint proteins, and thus, the drug response is affected by DDR function and DNA repair capabilities [24].

DSBs—the most severe form of DNA damage—are detected by poly(ADP-ribose) polymerase 1 (PARP1) and the ku70–ku80 heterodimer and are repaired via two main pathways: non-homologous end joining (NHEJ) and homologous recombination (HR) [22,25,26]. NHEJ, which is initiated by ku70–ku80, does not require sequence homology and is an error-prone process. In contrast, HR is mediated by the RAD51 recombinase and is homology based, utilizing the undamaged sister chromatid as a template and, thus, is relatively accurate.

These repair processes largely proceed through distinct protein subsets. However, a heterotrimeric complex composed of meiotic recombination 11 (MRE11), DNA repair protein Rad50 (RAD50) and Nijmegen breakage syndrome 1 (NBS1), referred to as the MRN complex, is involved in both NHEJ and HR repair, and mutations in these proteins are associated with numerous cancers, including CRC [27].

A third pathway, known as mismatch repair (MMR), identifies and corrects mismatched base pairs resulting from errors in replication or repair. Mismatch detection is primarily mediated by the MutS homolog (MSH)2–MSH6 heterodimer, known as MutS $\alpha$ , as well as by the MSH2–MSH3 heterodimer (MutS $\beta$ ) [28]. Other MMR proteins, including MutL homolog (MLH)1, MLH3 and post-meiotic segregation (PMS)2, increased and then

form higher order complexes with replication factors and function to remove the lesion and repair the DNA [28]. Critically, as with other DDR pathways, disruptions in MMR lead to genomic instability and increased mutation rates, which can both contribute to cancer development and affect therapeutic outcomes [28].

A number of recent studies have utilized targeted next-generation sequencing (NGS) to assess the prognostic value of specific mutations, as well as the total tumor mutational load (TML), an indicator of genomic instability, for predicting survival and therapeutic outcomes in cancer patients [29–33]. However, to our knowledge, none have used this technology to assess the value of DDR mutations for predicting therapeutic outcomes in CRC. Here, we used the OncoPrint™ Tumor Mutation Load Assay to determine whether mutations in DDR/MMR proteins and/or TML status may represent possible biomarkers for predicting the response to chemo/radiotherapy treatment in rectal cancer patients who received pre-operative neoadjuvant therapy.

## 2. Results

### 2.1. Patient Characteristics

A total of 22 patients (15 males (66.5%) and 7 females (33.5%)) were included in this study. The patient demographic characteristics are listed in Table 1. The median age was 63 years (range: 35–82 years). All 22 patients received preoperative therapy. Patients were followed for a median period of 3.1 years (range: 0–10.6 years), and the median time to death was 2.1 years after surgery (range: 0–9.2 years).

**Table 1.** Patient characteristics.

	Total <i>n</i> = 22	TML-High (%) * <i>n</i> = 7	TML-Low/Med (%) * <i>n</i> = 15	<i>p</i> -Value
Sex	Male	5 (71.4)	10 (66.7)	0.83
	Female	2 (28.6)	5 (33.3)	
Age	≤63	4 (57.1)	10 (66.7)	0.67
	>63	3 (42.9)	5 (33.3)	
Tumor stage	T1–2	2 (28.6)	2 (13.3)	0.40
	T3–4	5 (71.4)	13 (86.7)	
Node stage	Negative	5 (71.4)	7 (46.7)	0.29
	Positive	2 (28.6)	8 (53.3)	
Grade	1–2	7 (100)	14 (93.3)	0.50
	3	0	1 (6.7)	
Vascular invasion	Absent	6 (85.7)	11 (73.3)	0.53
	Present	1 (14.3)	4 (26.7)	
Perineural invasion	Absent	6 (85.7)	12 (80.0)	0.75
	Present	1 (14.3)	3 (20.0)	
Adjuvant therapy	No	4 (66.7) #	7 (63.6) #	0.90
	Yes	2 (33.3) #	4 (36.4) #	
TRG	Good response	0	1 (6.7)	0.84
	Poor response	7 (100)	14 (93.3)	

\* TML-high (>49 Mut/Mb); TML-low/med (<48 Mut/Mb). # Available cases. **Abbreviations:** TML, tumor mutational load; and TRG, tumor regression grade.

We first performed TML calculations for all patients using the default Ion Reporter™ TML analysis workflow, in which the initial variant caller considers not only non-synonymous variants but also synonymous mutations, for TML calculation. To further reduce noise in TML estimations, variants were then filtered to eliminate germline variants and select for the highest quality somatic variants using a specifically designed filter (OncoPrint™ Variants v5.16).

We found that, in our cohort of 22 samples, the median TML was 24 mutations (Mut)/Mb, with TML values ranging from 2 to 241. Based on previous publications, TML can be categorized as low (<11 Mut/Mb), intermediate (12–48 Mut/Mb) and high (>49 Mut/Mb) TML [30,34]. Here, due to our small sample size ( $n = 22$ ), we combined the low and intermediate groups into a low/med-TML low group to achieve statistically meaningful data analysis.

Investigation of the association between the TML level and clinicopathological characteristics (Table 1) revealed no significant differences in age, sex, tumor stage, lymph node involvement, metastasis, vascular invasion, perineural invasion, or tumor regression grade (TRG) in patients with low vs. high TML. We further assessed the mutational frequency in known DDR and MMR genes in patients with low/med and high TML status (Table 2).

**Table 2.** DDR/MMR mutations in CRC patients.

Gene	Subgroups $n = 22$	TML-High (%) * $n = 7$	TML-Low/Med (%) * $n = 15$	$p$ -Value
TP53	Mutant	5 (71.4)	10 (66.7)	0.83
	WT	2 (28.6)	5 (33.3)	
KRAS	Mutant	1 (14.3)	5 (33.3)	0.36
	WT	6 (85.7)	10 (66.7)	
RAD50	Mutant	1 (14.3)	3 (20.0)	0.75
	WT	6 (85.7)	12 (80.0)	
MLH1	Mutant	1 (14.3)	2 (13.3)	0.95
	WT	6 (85.7)	13 (86.7)	
MSH2	Mutant	0 (0)	3 (20.0)	0.21
	WT	7 (100)	12 (80.0)	
MSH6	Mutant	1 (14.3)	1 (6.7)	0.52
	WT	6 (85.7)	14 (93.3)	
PMS2	Mutant	1 (14.3)	0 (0)	0.14
	WT	6 (85.7)	15 (100)	

\* TML-high (>49 Mut/Mb); TML-low/med (<48 Mut/Mb). **Abbreviations:** TML, tumor mutational load; and WT, wild type.

From this analysis, we found that *TP53* was more frequently mutated in TML-high tumors compared to TML-low/med tumors (71.4% vs. 66.7%), although the difference was not significant, likely due to small sample size ( $p = 0.83$ ). Overall, mutations in *TP53*, *KRAS* and *RAD50* were detected in 68.2% (15/22), 27.3% (6/22) and 18.2% (4/22), respectively, of patient samples. A total of nine mutations in four MMR genes, *MLH1* ( $n = 3$ ), *MSH2* ( $n = 3$ ), *MSH6* ( $n = 2$ ), *PMS2* ( $n = 1$ ), were detected in 27.3% (6/22) of samples with TML-low/med and 3/22 (13.6%) samples with TML-high.

## 2.2. TML Is Significantly Associated with Worse Prognosis for Rectal Cancer Patients

We next performed univariate Cox regression analysis to compare OS in CRC patients with differing clinical features and gene mutations, as well as low/med- vs. high-TML. Among all variables tested, including sex, age at diagnosis, tumor stage, lymph node involvement and DDR gene mutations, we found that TML-high was significantly associated with reduced OS (HR = 0.219, 95% confidence interval [CI]: 0.052–0.924,  $p = 0.039$ ; Table 3).

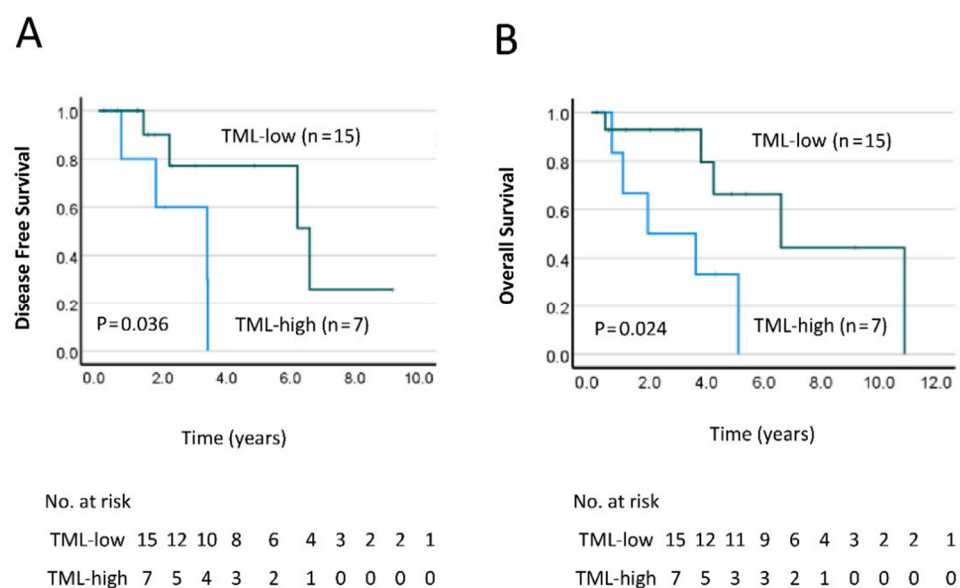
Of the different gene mutations assessed, mutant *TP53* was also significantly associated with a worse OS. However, in a multivariate Cox regression model, only TML-high (HR = 0.167, 95% CI: 1.030–0.936,  $p = 0.042$ ) remained significantly associated with a worse OS, suggesting that TML may be an independent predictive variable for OS of CRC patients. In contrast, MMR gene mutations were not associated with the patient outcome.

We further evaluated TML as a potential prognostic biomarker for poor outcomes in CRC using Kaplan–Meier survival analysis. We found that, in our CRC patient cohort, TML-high was significantly associated with worse DFS ( $p = 0.036$ , Figure 1A) and OS ( $p = 0.024$ ; Figure 1B). This further suggests that TML may be a positive predictive factor for determining the prognosis of patients with CRC.

**Table 3.** Cox regression analyses of TML and DDR gene mutants with the overall survival. Significant values are in bold.

Feature		HR	Univariate 95% CI	p-Value	HR	Multivariate 95% CI	p-Value
TML	High *	0.219	0.052–0.924	<b>0.039</b> #	0.167	0.030–0.936	<b>0.042</b> #
	Low/med *						
Sex	Male	2.261	0.600–8.514	0.228	3.910	1.452–9.663	0.226
	Female						
Age	≤63	1.449	0.387–5.435	0.582	4.459	0.450–4.126	0.201
	>63						
Tumor stage	T1–2	0.229	0.038–1.381	0.108	0.339	0.029–3.952	0.388
	T3–4						
Node stage	Negative	0.568	0.146–2.206	0.414	0.364	0.045–2.928	0.342
	Positive						
Vascular invasion	Absent	0.542	0.066–4.421	0.567	0.821	0.015–4.318	0.922
	Present						
Perineural invasion	Absent	0.985	0.117–8.267	0.989	0.929	0.048–17.798	0.608
	Present						
Adjuvant therapy	No	0.290	0.032–2.663	0.274	0.340	0.036–3.177	0.344
	Yes						
<b>Mutation</b>							
<i>TP53</i>	WT	8.666	1.002–74.297	<b>0.046</b> #	4.927	0.567–4.278	0.148
	Mutant						
<i>RAD50</i>	WT	3.893	0.148–12.256	0.415	1.101	0.612–10.135	0.932
	Mutant						
<i>MLH1</i>	WT	0.431	0.052–3.555	0.434	0.923	0.119–10.394	0.949
	Mutant						
<i>MSH2</i>	WT	0.031	0.000–24.248	0.306	0.004	0.077–10.974	0.942
	Mutant						
<i>MSH6</i>	WT	0.586	0.069–4.992	0.625	0.341	0.023–4.261	0.348
	Mutant						
<i>PMS2</i>	WT	1.359	0.162–11.429	0.778	0.371	0.026–5.189	0.461
	Mutant						

\* TML-high (>49 Mut/Mb); TML-low/med (<48 Mut/Mb). # Significant values are shown in bold. **Abbreviations:** CI, confidence interval; DDR, DNA damage repair; HR, hazard ratio; and TML, tumor mutational load.



**Figure 1.** Kaplan–Meier curves comparing the (A) disease-free survival (DFS) and (B) overall survival (OS) in patients with a low/med- vs. high-tumor mutation load (TML). Differences between the groups were analyzed by the log-rank test, and p-values are shown.



### 2.3. Mutational Profile of Rectal Cancer

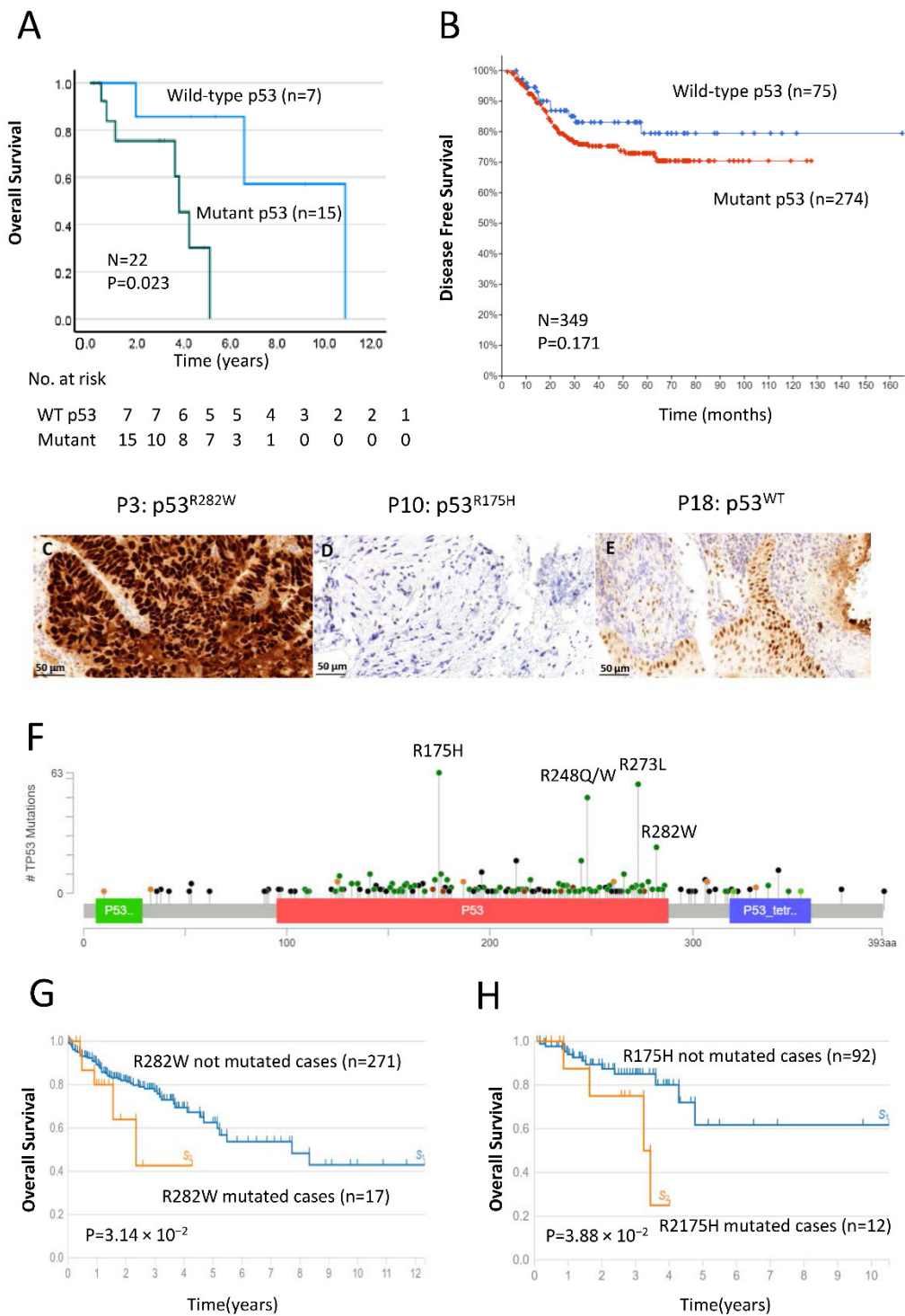
Overall, targeted NGS analysis of tumor tissue from the 22 patients in our CRC cohort revealed 242 unique variants in 53 genes (Table 4). The most commonly mutated gene in our cohort was found to be *TP53* (15/22; 68.2%), followed by *HNF1A* (12/22; 54.5%), *ARID1A* (10/22; 45.5%) and *KRAS* (5/22; 22.7%). Eight samples (36.3%) contained mutations in HR-DDR genes, including *ATM*, *ATR*, *CHEK2*, *CDK12*, *FANCA*, *MRE11A*, *RAD50*, *NBN*, *ERCC2* and *TP53*, and six samples (27%) harbored mutations in the MMR genes *MLH1*, *MSH2*, *MSH6* and *PMS2*.

**Table 4.** Somatic mutations in CRC patients treated with neoadjuvant therapy.

Patient ID	Genes with Mutations Identified by NGS Analysis
P1	<i>ARID1A</i> , <i>NRAS</i> , <i>CDKN2A</i> , <i>PTCH1</i> , <i>ABL1</i> , <i>TSC1</i> , <i>HNF1A</i> , <i>CREBBP</i> , <i>TP53</i> , <i>CHEK2</i> , <i>ATR</i>
P2	<i>NOTCH2</i> , <i>MSH2</i> , <i>NPM1</i> , <i>KRAS</i> , <i>HNF1A</i> , <i>CREBBP</i> , <i>TP53</i>
P3	<i>TP53</i>
P4	<i>ARID1A</i> , <i>NOTCH2</i> , <i>CDKN2B</i> , <i>NOTCH1</i> , <i>KRAS</i> , <i>CREBBP</i> , <i>TP53</i> , <i>MIR4733</i> , <i>STK11</i> , <i>SMARCA4</i> , <i>ERCC2</i>
P5	<i>ARID1A</i> , <i>NOTCH2</i> , <i>BRAF</i> , <i>HNF1A</i>
P6	<i>ARID1A</i> , <i>NOTCH2</i> , <i>DNMT3A</i> , <i>MLH1</i> , <i>FGFR3</i> , <i>FBXW7</i> , <i>NPM1</i> , <i>FGFR1</i> , <i>CDKN2A</i> , <i>NOTCH1</i> , <i>WT1</i> , <i>KRAS</i> , <i>HNF1A</i> , <i>TSC2</i> , <i>CREBBP</i> , <i>SMARCA4</i> , <i>ERCC2</i>
P7	<i>ARID1A</i> , <i>NRAS</i> , <i>NOTCH2</i> , <i>NTRK1</i> , <i>DNMT3A</i> , <i>ALK</i> , <i>MSH6</i> , <i>ATR</i> , <i>TET2</i> , <i>PIK3R1</i> , <i>RAD50</i> , <i>PMS2</i> , <i>EGFR</i> , <i>JAK2</i> , <i>PTCH1</i> , <i>NOTCH1</i> , <i>RET</i>
P8	<i>MLH1</i> , <i>PIK3CA</i> , <i>HNF1A</i> , <i>TP53</i> , <i>CDK12</i> , <i>ASXL1</i>
P9	<i>KRAS</i> , <i>HNF1A</i>
P10	<i>ATM</i> , <i>HNF1A</i> , <i>RB1</i> , <i>TP53</i> , <i>MRE11A</i>
P11	<i>ARID1A</i> , <i>NOTCH1</i> , <i>CREBBP</i> , <i>TP53</i>
P12	<i>ARID1A</i> , <i>NOTCH2</i> , <i>FTR</i>
P13	<i>MTOR</i> , <i>ARID1A</i> , <i>NOTCH1</i> , <i>DNMT3A</i> , <i>ALK</i> , <i>MSH2</i> , <i>MSH6</i> , <i>SF3B1</i> , <i>FANCD2</i> , <i>MLH1</i> , <i>SETD2</i> , <i>BAP1</i> , <i>ATR</i> , <i>FGFR3</i> , <i>TET</i> , <i>FBXW7</i> , <i>PIK3R</i> , <i>RAD50</i> , <i>NPM1</i> , <i>ESR1</i>
P14	<i>FBXW7</i> , <i>HNF1A</i> , <i>CREBBP</i> , <i>TP53</i>
P15	<i>FGFR2</i> , <i>TP53</i>
P16	<i>ARID1A</i> , <i>NOTCH2</i> , <i>DNMT3A</i> , <i>TET</i> , <i>CDKN2B</i> , <i>HNF1A</i> , <i>TSC2</i> , <i>CREBBP</i> , <i>FANCA</i> , <i>TP53</i> , <i>ERCC2</i> , <i>ATR</i>
P17	<i>KRAS</i> , <i>TP53</i>
P18	<i>NOTCH1</i> , <i>RAD50</i> , <i>NBN</i> , <i>FANCA</i> , <i>NF1</i> , <i>CHEK2</i>
P19	<i>ARID1A</i> , <i>ATR</i> , <i>FBXW7</i> , <i>HNF1A</i> , <i>TP53</i>
P20	<i>CDKN2B</i> , <i>HNF1A</i> , <i>TP53</i>
P21	<i>NOTCH1</i> , <i>DNMT3A</i> , <i>MSH2</i> , <i>FBXW7</i> , <i>RAD50</i> , <i>TSC2</i> , <i>CREBBP</i> , <i>PALB2</i> , <i>TP53</i> , <i>SMARCA4</i> , <i>ERCC2</i> , <i>CHEK2</i> , <i>ATR</i>
P22	<i>PIK3CA</i> , <i>HNF1A</i> , <i>TP53</i>

As *TP53* was the most commonly mutated gene in our cohort, we further examined the p53 mutation to determine whether the genetic changes affected a specific protein function. *TP53* mutations were found to be predominantly localized in the DNA-binding domain (amino acid residues 94–290), effectively blocking wild-type (WT) p53 from binding to its transcription response elements and transactivating downstream target genes.

In total, we found the following seven amino acid substitutions in p53 mutational hotspots: R175H (p.Arg175His), H179Y (p.His179Tyr), R248W (p.Arg248Trp), R248Q (p.Arg248Gln), R273L (p.Arg273Leu), P278S (p.Pro278Ser) and R282W (p.Arg282Trp), all of which are localized in the DNA-binding domain. Notably, using Kaplan–Meier survival analysis, we found that patients with mutant p53 displayed worse OS ( $p = 0.023$ ; Figure 2A) than those with WT p53, suggesting that the *TP53* mutational status may also have prognostic value for CRC patients.



**Figure 2.** Survival analysis of patients with *TP53* mutations, as determined by next-generation sequencing (NGS), validation of p53 prognostic impact via Cancer Genomics database (<http://www.cBioPortal.org>, accessed on 15 July 2022) and immunohistochemical staining of the p53 protein in patient samples. (A) Kaplan–Meier curves comparing OS in patients with wild-type (WT) and mutant p53. Differences between the groups were analyzed by the log–rank test, and *p*-values are shown. (B) Kaplan–Meier survival analysis of DFS in rectal cancer patient groups with wild-type (*n* = 75, blue line) and mutant p53 (*n* = 274, red line). Total cases and *p*-values are shown. (C–E) Representative examples of typical nuclear staining for p53 in CRC patients with (C) TP53 mutant–overexpression, (D) TP53 mutant–reduced expression and (E) TP53 WT–normal expression (40× magnification, scale bar = 50 μm). (F) Diagram represents the protein domains of p53 encoded by *TP53* genes. Plot was

generated via cBioPortol tools based on a recent rectal cancer study (MSKCC, Nature Medicine 2022), and the p53 DNA-binding domain is shown in a red box. The presence of a mutation is shown on the  $x$ -axis, and the frequency of mutations is shown on the  $y$ -axis. Missense mutations are shown in green circles, and deleterious mutations (truncating, in frame and splice) are shown in black and brown circles. (G,H) Kaplan–Meier curves comparing p53<sup>R282W</sup> (G) and p53<sup>R175H</sup> (H) mutational statuses in patients with not-mutated cases and mutated cases provided by The Cancer Genome Atlas (TCGA-READ and TCGA-COAD).

#### 2.4. Validation by IHC and Confirmation of TP53 Status by Cancer Genomics Databases

Mutation of the p53 gene is a common phenomenon in numerous human tumors, including CRC [35]. This often leads to an accumulation of nonfunctional p53 protein in the cell nuclei or loss of p53 protein, which can be detected by immunohistochemical techniques. Previous studies have further shown that p53 staining in tissue by IHC accurately reflects *TP53* mutational status with high specificity, and the patterns of *TP53* nucleotide variants correlate with the expression of p53 in cancers [36,37].

Here, to validate our findings by NGS, we performed immunohistochemical analysis to examine the expression of p53 protein in CRC tissue (Figure 2C–E). The results show a typical nuclear staining pattern in CRC tissue with WT *TP53* (Figure 2E; patient ID, P18) and aberrant staining patterns in samples with mutant *TP53*, consistent with accumulation of non-functional p53 (Figure 2C; patient ID, P3) or loss of expression (Figure 2D; patient ID, P10), indicating that abnormal p53 levels are present in CRC tissue containing mutations in the *TP53* gene.

Both patients with mutant *TP53* (P3 and P10) were treated with neoadjuvant radiotherapy, followed by additional 5-FU-based adjuvant chemotherapy. By NGS, we found that the p53<sup>R282W</sup> mutation was present in P3, who displayed p53 overexpression (Figure 2C), whereas P10, which showed reduced p53 expression (Figure 2D), contained the p53<sup>R175H</sup> mutation, which harbors other mutations, including *ATM*, *HNF1A*, *RB1*, *TP53* and *MRE11A*. This suggests that these mutations have distinct effects on the expression and possibly function of the p53 protein.

To confirm our findings in this study, we also performed the validation experiments using Cancer Genomics database (<http://www.cBioPortol.org>, accessed on 15 July 2022) to validate our results and we compared them with a recent rectal cancer study (MSKCC, Nature Medicine 2022). The Kaplan–Meier curve revealed that *TP53* mutational status correlates with worse DFS ( $n = 349$ , Figure 2B), indicating potential prognostic role of p53 for CRC patients. In addition, a lollipop plot was generated using the same dataset via cBioPortol. Figure 2F represents the protein domains of p53 encoded by *TP53* genes and shows the DNA-binding domain (DBD) of p53 covering from 94 to 290 amino acids.

Mutations in the DBD either interrupt the DNA binding directly or bring local/global change in the p53 structure. Both p53<sup>R282W</sup> and p53<sup>R175H</sup> mutations are classified as part of the structure mutations. To explore potential prognostic features for p53<sup>R282W</sup> and p53<sup>R175H</sup> mutations, Kaplan–Meier survival analysis was performed using The Cancer Genome Atlas (TCGA) database ( $n = 300$ , TCGA-READ and TCGA-COAD). The outcome results revealed both p53<sup>R282W</sup> and p53<sup>R175H</sup> mutational statuses are significantly associated with poor OS ( $p = 3.14 \times 10^{-2}$ , Figure 2G) and ( $p = 3.88 \times 10^{-2}$ , Figure 2H), respectively.

### 3. Discussion

Despite declining incidence rates in recent years, CRC remains among the top three most prevalent cancers and a leading cause of cancer-related death worldwide [1,2]. Challenges associated with complete tumor removal, particularly for rectal cancer patients, contribute to high recurrence and mortality rates and necessitate the use of more aggressive preoperative neoadjuvant therapies, which can improve outcomes for those with this CRC subtype [3–7]. However, therapeutic efficacy is highly variable, producing only partial or



no response in most patients treated and highlighting the need for molecular biomarkers to identify patients most likely to benefit from such therapies [8–10].

Here, to address this need, we used targeted NGS technology to assess the prognostic value of specific DDR/MMR mutations, as well as total TML, for predicting survival and therapeutic outcomes in a cohort of 22 rectal cancer patients who received pre-operative neoadjuvant therapy. We found that both TML-high (>49 Mut/Mb) and mutations in *TP53* were significantly associated with worse survival outcomes in our patient cohort, suggesting these molecular features may have prognostic value as biomarkers to determine the optimal therapeutic course for rectal cancer patients.

In total, 53 somatic mutations were identified in 22 patient samples; seven (32%) samples contained mutations in DDR genes, and *TP53* was the most frequently mutated gene. We note, however, that our mutation counts likely represent underestimates, as the OncoPrint™ NGS panel used in this study does not cover the full length of all DNA damage genes assessed but rather targets known mutational hotspots to estimate mutational burden. Regardless of this limitation, the results from univariate and Kaplan–Meier survival analyses indicate that both TML-high (>49 Mut/Mb) and mutations in *TP53* were significantly associated with worse survival outcomes in our patient cohort. Thus, our findings suggest that TML and *TP53* mutation have prognostic value as molecular biomarkers for determining the therapeutic course of rectal cancer patients.

Numerous studies have highlighted the prognostic value of tumor TML for predicting response to immune checkpoint inhibitor therapies, with a high TML associated with improved outcomes in patients with a diverse array of malignancies, including melanoma [38], non-small cell lung cancer [30] and CRC [34]. It is hypothesized that an elevated TML leads to production of a broader array of neoantigens for induction of immunogenicity [39,40]. Similarly, higher levels of microsatellite instability (MSI), resulting from MMR deficiency (features present in approximately 15% of CRC tumors [41,42]) are associated with increased neoantigens and better response to immunotherapy in CRC [43,44].

Less is known, however, about the value of TML for predicting prognosis of cancer patients receiving standard chemotherapy or radiotherapy. One study found that TML did not have prognostic value in a large cohort of patients with a diverse array of solid tumors who did not receive immunotherapy [45]. In contrast, findings from a meta-analysis suggested that standard platinum-based chemotherapy may produce better outcomes relative to immunotherapy for patients with low TML [46]. Notably, studies have shown that defective MMR and high MSI are associated with decreased efficacy of 5-FU therapy for CRC patients, although the prognostic value of TML was not assessed [47,48].

Here, we found that for our cohort of rectal cancer patients who underwent preoperative chemo/radiotherapy, TML-high was associated with worse OS in univariate and multivariate analyses. Further, subjects with a higher TML displayed worse DFS and OS in Kaplan–Meier survival analyses. These findings suggest that elevated genomic instability associated with a high TML may promote increased levels of cancer growth and metastasis and resistance to DNA-damaging treatments, possibly via the overexpression of DNA repair proteins and leading to worse outcomes with such therapies.

Thus, TML may represent a valuable molecular biomarker for predicting response to standard chemo/radiotherapy, with low TML patients most likely to benefit from such treatments. As high TML is associated with improved response to immunotherapy, this feature may be leveraged to help to determine the optimal therapeutic course for patients with CRC.

Chemo/radiotherapy kills cancer cells by producing high levels of DNA damage that cannot be repaired, ultimately triggering apoptosis. Treatment efficacy is therefore dependent on the DNA repair capacity of cancer cells, such that malignancies proficient in DNA repair will be more likely to display resistance, whereas those defective for repair are more sensitive [24,49,50]. Moreover, enhanced DNA repair may inhibit anti-tumor immunity, which is induced in response to DNA damage via the production of type I interferon [51].

Accordingly, numerous studies have reported that DDR defects promote enhanced sensitivity to chemo/radiotherapy [50,52], and mutations in DDR/MMR repair genes and/or aberrant expression of these proteins have been proposed as possible biomarkers for various malignancies, including CRC [17–21]. Consistent with this possibility, in a previous study, we found that elevated expression of the DNA repair proteins MRE11 and ATM was predictive for worse outcomes in CRC patients treated with neoadjuvant radiotherapy [16].

Similarly, overexpression of the HR repair protein RAD51 was found to be associated with worse outcomes in a large cohort of CRC patients, including the subset of rectal cancer patients treated with radiotherapy [15]. Qin et al. further reported that expression of the DNA repair protein X-ray repair complementing defective repair in Chinese hamster cells 2 (XRCC2) is linked to enhanced radioresistance and worse survival outcomes of rectal cancer patients, consistent with findings from other cancer types [53].

Paradoxically, however, for other DNA repair proteins, including RAD52 and RAD50, lower levels of expression are associated with worse outcomes for rectal cancer patients treated with neoadjuvant chemo/radiotherapy [54,55], implying that these proteins may have unique functions that contribute to resistance via unknown mechanisms. This is supported by our finding that, unlike RAD50 alone, elevated expression of the MRN complex, which contains RAD50 and plays a role in both NHEJ and HR, is associated with poorer prognosis of chemo/radiotherapy-treated rectal cancer patients [56].

Here, of the 53 somatic mutations identified in our 22-patient cohort, 13 mutations in seven patient samples were located in DDR/MMR genes. Of these, *TP53* was the most frequently mutated gene (68.2%; 15/22). This is consistent with findings from previous studies and the central role of p53 in CRC development [35,50,57]. Most *TP53* mutations were found within the DNA-binding domain and would thus be expected to disrupt the activation of p53 target genes.

IHC analyses further revealed aberrant patterns of p53 staining in representative *TP53* mutant CRC tissue, confirming that the observed mutations also disrupt p53 protein expression. The results from the Kaplan–Meier survival analyses and univariate Cox regression further indicate that *TP53* mutation was associated with worse survival outcomes in our rectal cancer patient cohort, although in this case, a significant association was not present in multivariate analyses.

We did not, however, observe significant association between other individual DDR/MMR mutations and patient outcomes in our univariate analysis. Previous studies have shown that the relationship between MMR gene mutations and patient outcome in CRC is complex and largely affected by features such as tumor stage and concurrent *BRAF* mutations [58–61]. Here, it is likely that we were unable to detect a positive relationship between MMR gene mutations and patient outcome due to our small patient cohort ( $n = 22$  subjects)—a limitation of this study.

Thus, although our current findings suggest *TP53* mutation may have prognostic value for rectal cancer patients who undergo neoadjuvant chemo/radiotherapy, future studies with larger patient populations will be needed to determine the clinical significance of other individual DDR/MMR gene mutations for this subset of CRC patients. Our small sample size may also explain why mutations in *KRAS* were detected at a slightly lower frequency than what has been reported in previous studies (27.3% vs. 30–50%, respectively) [57,62,63].

As the study focused on known MRN complex and MMR mutations in the DDR/MMR mutation panel we did not assess the mutational frequency of ERCC2/XPD. Lastly, we note that this study was limited by our lack of access to biopsy samples; as a result, all samples were obtained post-treatment, and we were unable to include complete responders in our patient cohort.

## 4. Materials and Methods

### 4.1. Patients

This study was approved by the South Western Sydney Local Health District Human Research Ethics Committee (HREC Reference: HREC/14/LPOOL/186; project number 14/103). Specimens were collected from 22 patients who underwent surgery and chemoradiotherapy for CRC between 2000 and 2011. All patients were treated with neoadjuvant chemo/radiotherapy prior to total mesorectal excision and anterior or abdominoperineal resection. Patients were treated with either a 25-Gy dose of radiotherapy administered in five treatment fractions or a 50.4-Gy dose administered in 28 fractions; both treatment groups received 5-FU-based chemotherapy. Follow-up included clinic visits, blood tests, colonoscopy and imaging based on the recommendations of the treating specialist.

### 4.2. FFPE Tissue Processing and DNA Extraction

Each formalin-fixed paraffin-embedded (FFPE) tissue section ranging in size from 5 to 10  $\mu\text{m}$  in thickness, with no more than 2.25  $\text{cm}^2$  of tissue area, was deparaffinized by submerging in xylene and vortexing vigorously 10 sec, followed by centrifugation at  $20,000 \times g$  for 2 min at room temperature. DNA was then extracted from deparaffinized tissue sections using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany). All procedures were performed in accordance with the manufacturer's instructions.

### 4.3. Qubit Assay

DNA derived from FFPE tissue was quantified using the Qubit dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). To prepare samples for analysis, 2  $\mu\text{L}$  of purified DNA was added to 198  $\mu\text{L}$  of Qubit working solution, for final volume of 200  $\mu\text{L}$ . This mixture was then incubated at room temperature for at least 2 min before quantitation was performed with the Qubit 3.0 fluorometer (Thermo Fisher Scientific).

### 4.4. NGS Library Preparation and TaqMan qPCR

Library preparation was performed for the OncoPrint™ Tumor Mutation Load Assay, according to the manufacturer's protocol (Thermo Fisher Scientific). This targeted NGS panel covers portions of 409 genes that are known to be mutational hotspots in cancer and detects variants within their coding regions. Genomic DNA libraries were prepared using the Ion AmpliSeq™ Library Kit Plus (Thermo Fisher Scientific), starting with 20 ng DNA for each sample.

After combining the target amplification reactions and performing digestion of amplicons, ligation and purification, the purified libraries were quantified using the Ion Library TaqMan® Quantitation Kit (Thermo Fisher Scientific) and run on the QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific), according to the manufacturer's instructions. The final concentration of each library after dilution was determined (50 pM).

### 4.5. Sequencing and Data Analysis

The quantified libraries were clonally amplified by emulsion PCR and enriched on the Ion Chef instrument for automated template preparation using the Ion 540™ Kit-Chef, according to the manufacturer's protocol (Thermo Fisher Scientific). Templates were loaded onto an Ion 540™ chip (four samples per chip, two chips per run) and sequenced with the Ion GeneStudio™ S5 sequencer system (Thermo Fisher Scientific), according to the manufacturer's protocols.

NGS results were analyzed using the default settings of the integrated OncoPrint™ Tumor Mutation Load-w3.1-DNA-Single Sample analysis workflow pipeline, available on the Ion Reporter software v.5.18 (Thermo Fisher Scientific). In our analysis workflow, the sequenced reads were aligned to the human reference genome hg19, and the resulting BAM files were transferred to Ion Reporter software for variant calling. Variants were identified

by filtering with the OncoPrint™ Variant Annotator plugin in Ion Reporter v.5.18, with default workflow settings.

#### 4.6. Immunohistochemistry (IHC)

Immunohistochemical staining was performed according to the standard protocol used by the Anatomical Pathology Laboratory, Liverpool Hospital. Prior to staining, the tissue sections were incubated at 60 °C for at least 1 h. Deparaffinization was performed by immersing samples in xylene three times, followed by rehydration through graded, decreasing concentrations of ethanol, ending in running water. The sections were then incubated in pre-heated 98 °C citrate buffer, pH 6.0, in a hot water bath for 20 min, followed by cooling at room temperature for 20 min.

Endogenous peroxidase was blocked with hydrogen peroxidase for 20 min, and then slides were incubated with primary antibody for 20 min at room temperature in a moist chamber (p53, 1:800 dilution; Invitrogen, Thermo Fisher Scientific). Goat secondary antibody (Invitrogen, Thermo Fisher Scientific) for detection of the primary antibody–antigen complex, and high-sensitivity 3, 3'-diaminobenzidine tetrahydrochloride was then added for 15 min, followed by hematoxylin counterstaining and mounting.

#### 4.7. Statistical Analysis

Statistical analysis was performed with SPSS for Windows 27.0 (IBM Corporation, Armonk, NY, USA). The overall survival (OS) and disease-free survival (DFS) were assessed by performing Kaplan–Meier analysis, and the significance of the differences between groups was determined by the log–rank test, with  $p < 0.05$  considered to be statistically significant. Univariate and multivariate analyses to compare survival in CRC patients with different clinical features and DDR/MMR gene mutational status were performed using Kaplan–Meier curves and Cox's proportional hazard ratio (HR) survival modeling. Covariates included sex; age; Tumor, Node and Metastasis (TNM) stage; histological grade; vascular invasion; perineural invasion; chemotherapy; and radiotherapy. Significance for univariate and multivariate analyses was assessed using the Mann–Whitney U test, and in all cases,  $p < 0.05$  was considered statistically significant.

## 5. Conclusions

In summary, our results indicate that both TML-high (>49 Mut/Mb) and *TP53* mutation were associated with worse survival outcomes for rectal cancer patients who received neoadjuvant therapy prior to surgery. Overall, the targeted OncoPrint™ Tumor Mutation Load Assay NGS panel uncovered 53 somatic mutations in our 22-patient cohort, with seven (32%) patients containing mutations in 13 DDR/MMR genes and *TP53* representing the most frequently mutated gene. Collectively, our findings indicate that TML and *TP53* mutation may have prognostic value for CRC patients, suggesting that these features can potentially be leveraged as independent biomarkers for patient management and therapeutic course determination.

**Author Contributions:** V.H., L.C. and C.S.L. conceived and designed the research; L.C., Y.M., B.W., V.L., A.A. and V.H. collected and did preliminary analysis on the data; V.H., L.C., S.H.L., Y.M., B.W., W.N., M.L., T.L.R., W.C. and C.S.L. further analyzed and interpreted the data; V.H. and L.C. wrote the draft manuscript; S.H.L., Y.M., B.W., W.N., M.L., T.L.R., W.C. and C.S.L. provided critical material. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available due to data size and privacy.

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