Targeted Next-Generation Sequencing-Based Multiple Gene Mutation Profiling of Patients with Rectal Adenocarcinoma Receiving or Not Receiving Neoadjuvant Chemoradiotherapy

You-Kang Chang 1,2,†, Hui-Hwa Tseng 3,†, Chung-Man Leung 4, Kuo-Cheng Lu 5,6 © and Kuo-Wang Tsai 7,* ©

1 Department of Radiation Oncology, Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Taipei 23142, Taiwan
2 College of Medicine, Tzu Chi University, Hualien City 97004, Taiwan
3 Department of Anatomic Pathology, Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, New Taipei City 97004, Taiwan
4 Department of Radiation Oncology, Kaohsiung Veterans General Hospital, Kaohsiung 81341, Taiwan
5 Division of Nephrology, Department of Medicine, Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, New Taipei City 97004, Taiwan
6 Division of Nephrology, Department of Medicine, Fu-Jen Catholic University Hospital, School of Medicine, Fu-Jen Catholic University, New Taipei City 24205, Taiwan
7 Department of Research, Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, New Taipei City 23142, Taiwan
* Correspondence: tch33225@tzuchi.com.tw; Tel.: +886-2-26623277 (ext. 5796); Fax: +886-2-66281258
† These authors contributed equally to this work.

Abstract: This study investigated whether oncogenic and tumor-suppressive gene mutations are involved in the differential outcomes of patients with rectal carcinoma receiving neoadjuvant chemoradiotherapy (nCRT). Genomic DNA was obtained from formalin-fixed paraffin-embedded (FFPE) specimens of patients with rectal carcinoma who received a complete nCRT course. Gene mutation status was examined in specimens from patients before and after nCRT by using the AmpliSeq platform. Our data revealed that the nonsynonymous p53, APC, KRAS, CDKN2A, and EGFR mutations were observed in 93.1%, 65.5%, 48.6%, and 31% of the patients with rectal adenocarcinoma, respectively. BRAF, FBXW7, PTEN, and SMAD4 mutations were observed in 20.7% of patients with rectal carcinoma. The following 12 gene mutations were observed more frequently in the patients exhibiting a complete response than in those demonstrating a poor response before nCRT: ATM, BRAF, CDKN2A, EGFR, FLT3, GNA11, KDR, KIT, PIK3CA, PTEN, PTPN11, SMAD4, and TP53. In addition, APC, BRAF, FBXW7, KRAS, SMAD4, and TP53 mutations were retained after nCRT. Our results indicate a complex mutational profile in rectal carcinoma, suggesting the involvement of BRAF, SMAD4, and TP53 genetic variants in the outcomes of patients with nCRT.

Keywords: rectal carcinoma; cancer panel; next-generation sequencing; chemoradiotherapy

1. Introduction

Rectal carcinoma is a common cause of cancer deaths worldwide. More than 0.73 million new rectal cancer cases and 339,022 deaths were estimated to occur in 2020, representing about 3.4% of cancer cases and deaths [1]. Preoperative neoadjuvant chemoradiotherapy (nCRT) is a general therapeutic modality for patients with rectal adenocarcinoma and locally advanced rectal cancer, and has been demonstrated to significantly reduce local recurrence and prolong survival [2,3]. However, many patients who receive nCRT experience no benefit but severe side effects [4,5]. Until now, no precision biomarkers for predicting patients who would have a complete response following nCRT have been identified. DNA alterations, abnormal gene expression, and epigenetic changes can be used as biomarkers for predicting the tumor response to radiotherapy in patients with rectal carcinoma [6–8].
Genetic mutations were identified as the predictors of the tumor response to nCRT in rectal cancer, including TP53, KRAS, and EGFR [9–11]. Although many genetic mutations have been identified as the biomarkers of the rectal tumor response to nCRT in rectal cancer, some studies have reported opposite results or indicated that genetic mutations cannot be used as predictors [12–14].

The Sanger sequencing method is a gold standard for identifying gene variants in cancer; however, it has low throughput and poor sensitivity [15]. A powerful method, next-generation sequencing (NGS) with high throughput, was developed to comprehensively identify gene mutations in the whole genome in patients with cancer [16–18]. Whole-genome sequencing is expensive because only 3–4% of the genome comprises the protein-coding region [19]. The exon capture method for targeted sequencing, or targeting a subset of genes of interest for sequencing, has been widely used and can reduce costs and time. Until now, targeted amplicon-based multigene mutational screening has been widely applied for the detection of gene variants in patients with cancer; this method can use a minimum amount of gDNA from a formalin-fixed paraffin-embedded (FFPE) sample [16,20–22]. In this study, we examined 55 samples from 29 patients with rectal carcinoma. All the 29 patients received preoperative chemoradiotherapy, and 26 of the 29 patients received postoperative chemoradiotherapy. Identification of gene mutations in patients receiving preoperative nCRT may help determine mutations driving rectal carcinoma progression. Moreover, identification of gene mutations in patients receiving postoperative nCRT may help identify those involved in resistance to nCRT. Our findings provide new insights to evaluate the response of advanced rectal cancer to nCRT.

2. Results

This study included 29 patients with rectal carcinoma who received a complete nCRT course before surgery. Before nCRT, 29 FFPE rectal carcinoma specimens were collected from the biopsy samples of the patients. Following complete nCRT, we collected the corresponding post-nCRT FFPE surgical specimens from these patients. Table 1 summarizes the clinicopathological features of the patients. Among the 29 patients, 9, 11, and 9 exhibited a complete, partial, and poor response to nCRT (tumor regression grades 0, 1, and 2 or 3), respectively (Figure 1). To identify whether gene variant profiles differ between the patients exhibiting a complete response and those exhibiting a poor response to nCRT, we performed the NGS of 50 genes in the 55 specimens obtained from the 29 patients with rectal carcinoma. We extracted genomic DNA from the FFPE specimens and amplified them using 207 primer pairs to amplify the hotspot regions of 50 genes (Figure 1B). Amplicons were identified through NGS, and gene variants were analyzed by performing a bioinformatics analysis. Three types of surgical specimens were collected from 21, 26, and 28 patients after nCTR failed to meet quality control requirements (Supplementary Table S1). Furthermore, an average of 1,379,426 mapped sequence reads were obtained for the 55 samples, and the 500× coverage was 98.6% (Supplementary Table S1).

Through sequencing, we identified 648 variants (frequency > 5%) in 50 genes, including 297 nonsynonymous and 351 synonymous variants (Supplementary Tables S2–S4). Furthermore, we determined that the number of single nucleotide polymorphism (SNP) did not significantly differ between the pre-nCRT and post-nCRT specimens (Figure 2A). The number of gene mutations was lower in the post-CRT specimens than in the pre-CRT specimens (Figure 2B).

To identify gene mutations involved in nCRT resistance, we excluded SNPs by using the SNP database. As presented in Supplementary Table S5, a total of 160 mutations were identified with a frequency of >5% in the 55 rectal carcinoma specimens, including 30 synonymous and 130 nonsynonymous variants. Because nonsynonymous variants considerably affect gene function, we included rare mutations with frequencies ranging from 2% to 5%. As presented in Supplementary Table S4, we identified 394 mutations with a frequency of 2–5% from all the samples. These gene candidates with nonsynonymous mutations (>2%) were included in the subsequent analysis.
Table 1. Clinicopathological characteristics of 29 patients with rectal carcinoma.

| Tumor Regression | Grade 0 (n = 9) | Grade 1 (n = 11) | Grade 2–3 (n = 9) | p Value |
|------------------|----------------|------------------|-------------------|---------|
|                  | n (%)          | n (%)            | n (%)             |         |
| Age              |                |                  |                   | 0.870   |
| <65 years        | 4 (44.4)       | 6 (54.5)         | 5 (55.6)          |         |
| ≥65 years        | 5 (55.6)       | 5 (45.5)         | 4 (44.4)          |         |
| Sex              |                |                  |                   | 0.568   |
| Female           | 4 (44.4)       | 3 (27.3)         | 2 (22.2)          |         |
| Male             | 5 (55.6)       | 8 (72.7)         | 7 (77.8)          |         |
| Clinical stage   |                |                  |                   | 0.402   |
| I-II             | 2 (22.2)       | 4 (36.4)         | 1 (11.1)          |         |
| III-IV           | 7 (77.8)       | 7 (63.6)         | 8 (88.9)          |         |
| pT stage         |                |                  |                   | 0.192   |
| I-II             | 0 (0.0)        | 2 (18.2)         | 2 (22.2)          |         |
| III-IV           | 9 (100.0)      | 9 (81.8)         | 7 (77.8)          |         |
| pN stage         |                |                  |                   | 0.367   |
| N0               | 3 (33.3)       | 4 (36.4)         | 1 (11.1)          |         |
| >N1              | 6 (66.7)       | 7 (63.6)         | 8 (88.9)          |         |
| pM stage         |                |                  |                   | 0.081   |
| M0               | 7 (77.8)       | 11 (100.0)       | 9 (100.0)         |         |
| M1               | 2 (22.2)       | 0 (0.0)          | 0 (0.0)           |         |
| Lymph node metastasis |          |                  |                   | 0.096   |
| No               | 8 (88.9)       | 5 (45.5)         | 5 (55.6)          |         |
| Yes              | 1 (11.1)       | 6 (54.5)         | 4 (44.4)          |         |

Figure 1. Identification of gene variants in rectal carcinoma through NGS. (A) Histopathological examination of biopsy specimens collected from patients with rectal carcinoma before nCRT (left panels). Histopathological examination of surgery specimens collected from patients with rectal carcinoma after nCRT (right panels). The black triangle indicates tumor cells, and the white triangle indicates the normal tissue. The response to nCRT was determined on the basis of the tumor regression grade. The image of specimens was determined through the microscope (magnification with 40× lens). (B) Targeted NGS workflow. Ten nanograms of genomic DNA was used for AmpliSeq cancer panel library preparation. Ion and high-throughput sequencing was performed using ion Torrent.
As presented in Figure 3 and Table 2, the top 10 high-frequency nonsynonymous gene mutations, TP53 (27/29; 93.1%), APC (19/29; 65.5%), KRAS (14/29; 48.3%), CDKN2A (9/29; 31%), EGFR (7/29; 24.1%), FBXW7 (6/29; 20.7%), BRAF (6/29; 20.7%), SMAD4 (6/29; 20.7%), PIK3CA (6/29; 20.7%), and PTEN (6/29; 20.7%), were identified in the pre-nCRT biopsy specimens. These variants resulted in nonsynonymous changes in protein sequences. We compared the mutation frequency between the patients exhibiting a complete response (tumor regression grade 0) and those demonstrating a poor response (tumor regression grades 1–3). We identified a higher mutation frequency of the following 12 genes in the complete response group than in the poor response group after nCRT treatment: ATM (grade 0: 11.1%; grade 1–3: 20%), BRAF (grade 0: 11.1%; grade 1–3: 25%), CDKN2A (grade 0: 22.2%; grade 1–3: 35%), EGFR (grade 0: 22.2%; grade 1–3: 25%), FLT3 (grade 0: 11.1%; grade 1–3: 15%), GNA11 (grade 0: 0%; grade 1–3: 10%), KDR (grade 0: 11.1%; grade 1–3: 15%), KIT (grade 0: 11.1%; grade 1–3: 20%), PIK3CA (grade 0: 11.1%; grade 1–3: 25%), PTEN (grade 0: 11.1%; grade 1–3: 25%), PTPN11 (grade 0: 11.1%; grade 1–3: 25%), SMAD4 (grade 0: 11.1%; grade 1–3: 25%), and TP53 (grade 0: 88.9%; grade 1–3: 95%); Figure 3 and Table 2. Due to the small sample size, these gene mutations were not significantly different in poor CRT response compared to complete response by chi-square test (Supplementary Table S6).

After the excision of the tumor, gene mutations should be gradually decreased during the nCRT process, especially in patients exhibiting a complete response. If a gene mutation contributes to nCRT resistance in rectal carcinoma, it should be retained after nCRT. Our data revealed that most of the mutations disappeared after the completion of nCRT in the patients exhibiting a complete response (Figure 4A). We compared the mutation status between the pre-nCRT and post-nCRT specimens in the patients exhibiting a poor response and observed that some of the gene mutations were retained, namely APC, BRAF, FBXW7, FLT3, KIT, KRAS, PTPN11, SMAD4, STK11, and TP53 (Figure 4B,C and Table 3). Among them, three gene mutations, BRAF, SMAD4, and TP53, were more frequently observed in the poor response group than in the complete response group (Figure 3 and Table 2). On the basis of these complex mutational profiles in rectal carcinoma, we speculated about the involvement of BRAF, SMAD4, and TP53 gene mutations in the outcomes of nCRT in patients with rectal carcinoma.
Int. J. Mol. Sci. 2022, 23, x FOR PEER REVIEW 5 of 14

Complete response (tumor regression grade 0) and those demonstrating a poor response (tumor regression grades 1–3). We identified a higher mutation frequency of the following 12 genes in the complete response group than in the poor response group after nCRT treatment: **ATM** (grade 0: 11.1%; grade 1–3: 20%), **BRAF** (grade 0: 11.1%; grade 1–3: 25%), **CDKN2A** (grade 0: 22.2%; grade 1–3: 35%), **EGFR** (grade 0: 22.2%; grade 1–3: 25%), **FLT3** (grade 0: 11.1%; grade 1–3: 15%), **GNA11** (grade 0: 0%; grade 1–3: 10%), **KDR** (grade 0: 11.1%; grade 1–3: 15%), **KIT** (grade 0: 11.1%; grade 1–3: 20%), **PIK3CA** (grade 0: 11.1%; grade 1–3: 25%), **PTEN** (grade 0: 11.1%; grade 1–3: 25%), **PTPN11** (grade 0: 11.1%; grade 1–3: 20%), **SMAD4** (grade 0: 11.1%; grade 1–3: 25%), and **TP53** (grade 0: 88.9%; grade 1–3: 95%); Figure 3 and Table 2. Due to the small sample size, these gene mutations were not significantly different in poor CRT response compared to complete response by chi-square test (Supplementary Table S6).

**Figure 3.** Gene mutation status was examined in biopsy samples from patients with rectal carcinoma. Mutation profiles of biopsy specimens collected from 29 patients with rectal carcinoma before CRT, including 9 patients with grade 0, 11 patients with grade 1, and 9 patients with grade 2 or 3. A green square indicates that a mutation with a frequency of >5% was detected in the gene, a yellow square indicates that a mutation with a frequency of 2–5% was observed in the gene, and an empty square indicates that no relevant mutation was observed for the gene.

**Table 2.** Percentage of individual genes with mutations in patients with rectal carcinoma before and after nCRT.

| Gene     | Total n = 29 | Grade 0 n = 9 | Grade 1–3 n = 20 |
|----------|--------------|---------------|------------------|
| ALK      | 2            | 6.9%          | 11.1%            |
| APC      | 19           | 65.5%         | 77.8%            |
| ATM      | 5            | 17.2%         | 11.1%            |
| BRAF     | 6            | 20.7%         | 11.1%            |
| CDKN2A   | 9            | 31.0%         | 22.2%            |
| CTNNB1   | 3            | 10.3%         | 11.1%            |
| EGFR     | 7            | 24.1%         | 11.1%            |
| ERBB4    | 3            | 10.3%         | 11.1%            |
| FBXW7    | 6            | 20.7%         | 33.3%            |
| FGR1     | 5            | 17.2%         | 22.2%            |
| FLT3     | 4            | 13.8%         | 11.1%            |
| GNA11    | 2            | 6.9%          | 0.0%             |
| GNAQ     | 1            | 3.4%          | 11.1%            |
| KDR      | 4            | 13.8%         | 11.1%            |
| KIT      | 5            | 17.2%         | 11.1%            |
| KRAS     | 14           | 48.3%         | 66.7%            |
| MET      | 3            | 10.3%         | 22.2%            |
| PIK3CA   | 6            | 20.7%         | 11.1%            |
| PTEN     | 6            | 20.7%         | 11.1%            |
| PTPN11   | 4            | 13.8%         | 11.1%            |
| RB1      | 3            | 10.3%         | 11.1%            |
| RET      | 4            | 13.8%         | 11.1%            |
| SMAD4    | 6            | 20.7%         | 11.1%            |
| STK11    | 3            | 10.3%         | 11.1%            |
| TP53     | 27           | 93.1%         | 88.9%            |

5 of 14
Figure 4. Gene mutation status was examined in specimens from patients with rectal carcinoma before and after nCRT. (A) Mutation profiles of biopsy-collected specimens (pre-nCRT) and surgery-collected specimens (post-nCRT) from nine patients with rectal carcinoma with tumor regression grade 0. (B) Mutation profiles of biopsy-collected specimens (pre-nCRT) and surgery-collected specimens (post-nCRT) from 11 patients with rectal carcinoma with tumor regression grade 1. (C) Mutation profiles of biopsy-collected specimens (pre-nCRT) and surgery-collected specimens (post-nCRT) from nine patients with rectal carcinoma with tumor regression grade 2–3. A green square indicates that a mutation with a frequency of >5% was detected in a gene, a yellow square indicates that a mutation with a frequency of 2–5% was observed in a gene, and an empty square indicates that no relevant mutation was observed for the gene.
Table 3. Gene mutations retained in rectal carcinoma after nCRT.

| Patients No. | Genes | Protein Change | Mutation Type | Nucleotide Change | Pre-CRT% | Post-CRT% |
|--------------|-------|----------------|---------------|-------------------|----------|-----------|
| 23           | APC   | p.M1383fs      | Frameshift    | c.4146_4147insA   | 38.3     | 8.1       |
| 29           | APC   | p.L1488fs      | Frameshift    | c.4461delT        | 11.7     | 8.0       |
| 18           | APC   | p.E1309 *      | Stop gained   | c.3925G > T       | 16.6     | 6.6       |
| 18           | APC   | p.E1353 *      | Stop gained   | c.4057G > T       | 19.2     | 6.4       |
| 25           | BRAF  | p.V600E        | Missense      | c.1799T > A       | 15.1     | 11.9      |
| 7            | FBXW7 | p.G459E        | Missense      | c.1376G > A       | 0.0      | 5.2       |
| 29           | FBXW7 | p.R505C        | Missense      | c.1513C > T       | 12.2     | 9.6       |
| 23           | KRAS  | p.A146T        | Missense      | c.436G > A        | 21.7     | 5.1       |
| 18           | SMAD4 | p.A118V        | Missense      | c.353C > T        | 0.0      | 6.1       |
| 23           | TP53  | p.M237I        | Missense      | c.711G > A        | 37.6     | 6.9       |
| 25           | TP53  | p.R342 *       | Stop gained   | c.1024C > T       | 39.7     | 22.7      |
| 20           | TP53  | p.R213fs       | Frameshift    | c.636dupT         | 15.6     | 7.1       |
| 29           | TP53  | p.Q104 *       | Stop gained   | c.310C > T        | 23.6     | 12.0      |
| 18           | TP53  | p.V173M        | Missense      | c.517G > A        | 26.3     | 7.2       |

* indicates stop codon.

We investigated the effects of BRAF, SMAD4, and TP53 mutations on colorectal carcinoma progression by using cBioPortal for Cancer Genomics (http://cbioportal.org, accessed on 3 March 2021) [23,24]. The mutation profiles of 3083 patients with colorectal carcinoma were downloaded from five studies [25–28]. The mutation rates of BRAF, SMAD4, and TP53 were 12.4% (384 patients), 16.6% (514 patients), and 65.6% (2025 patients) in the colorectal carcinoma cohort, respectively (Figure 5A–E). Furthermore, mutations in BRAF, TP53, or SMAD4 significantly reduced the progression-free survival of patients with colorectal carcinoma ($p = 4.24 \times 10^{-4}$) but were not correlated with overall survival ($p = 0.27$; Figure 5F,G). The simultaneous occurrence of BRAF, TP53, and SMAD4 mutations significantly reduced the progression-free survival ($p = 4.67 \times 10^{-3}$) and overall survival ($p = 4.98 \times 10^{-4}$) of patients with colorectal cancer (Figure 5H,I). Mutations in BRAF, SMAD4, or TP53 were significantly correlated with the poor progression-free survival (BRAF: $p = 3.62 \times 10^{-3}$; SMAD4: $p = 6.28 \times 10^{-4}$; and TP53: $p = 6.33 \times 10^{-5}$) and overall survival (BRAF: $p = 9.96 \times 10^{-9}$; SMAD4: $p = 5.96 \times 10^{-5}$) of patients with colorectal cancer. However, the TP53 mutation alone was not correlated with poor overall survival (Supplementary Figure S1). In general, patients with advanced rectal cancer will be treated with nCRT before surgery. Therefore, a cohort of metastatic colorectal cancer has further analyzed the prognosis of BRAF, SMAD4, and TP53 variants. Similar results revealed that mutations in BRAF and SMAD4 were significantly correlated with the overall survival (Supplementary Figure S2; BRAF: $p = 4.47 \times 10^{-6}$ and SMAD4: $p = 0.048$) of patients with metastatic colorectal cancer. However, the TP53 mutation alone was not correlated with poor overall survival of patients with metastatic colorectal carcinoma.

Taken together, our results indicate that nCRT might result in physiological selective pressure to accumulate gene mutations in residual rectal cancer regions. BRAF, SMAD4, and TP53 genetic mutations might be involved in resistance to nCRT and poor prognosis in colorectal carcinoma. However, these findings must be confirmed.
We investigated the effects of \textit{BRAF}, \textit{SMAD4}, and \textit{TP53} mutations on colorectal carcinoma progression by using cBioPortal for Cancer Genomics (http://cbioportal.org, accessed on 3 March 2021) [23,24]. The mutation profiles of 3083 patients with colorectal carcinoma were downloaded from five studies [25–28]. The mutation rates of \textit{BRAF}, \textit{SMAD4}, and \textit{TP53} were 12.4% (384 patients), 16.6% (514 patients), and 65.6% (2025 patients) in the colorectal carcinoma cohort, respectively (Figure 5A–E). Furthermore, mutations in \textit{BRAF}, \textit{TP53}, or \textit{SMAD4} significantly reduced the progression-free survival of patients with colorectal carcinoma ($p = 4.24 \times 10^{-4}$) but were not correlated with overall survival ($p = 0.27$; Figure 5F,G). The simultaneous occurrence of \textit{BRAF}, \textit{TP53}, and \textit{SMAD4} mutations significantly reduced the progression-free survival ($p = 4.67 \times 10^{-3}$) and overall survival ($p = 4.98 \times 10^{-4}$) of patients with colorectal cancer (Figure 5H,I). Mutations in \textit{BRAF}, \textit{SMAD4}, or \textit{TP53} were significantly correlated with the poor progression-free survival ($\textit{BRAF}: p = 3.62 \times 10^{-3}$; \textit{SMAD4}: $p = 6.28 \times 10^{-4}$; \textit{TP53}: $p = 6.33 \times 10^{-5}$) and overall survival ($\textit{BRAF}: p = 9.96 \times 10^{-9}$; \textit{SMAD4}: $p = 5.96 \times 10^{-3}$) of patients with colorectal cancer. However, the \textit{TP53} mutation alone was not correlated with poor overall survival (Supplementary Figure S1). In general, patients with advanced rectal cancer will be treated with nCRT before surgery. Therefore, a cohort of metastatic colorectal cancer has further analyzed the prognosis of \textit{BRAF}, \textit{SMAD4}, and \textit{TP53} variants. Similar results revealed that mutations in \textit{BRAF} and \textit{SMAD4} were significantly correlated with the overall survival (Supplementary Figure S2; \textit{BRAF}: $p = 4.47 \times 10^{-6}$ and \textit{SMAD4}: $p = 0.048$) of patients with metastatic colorectal cancer. However, the \textit{TP53} mutation alone was not correlated with poor overall survival of patients with metastatic colorectal carcinoma.

Figure 5. BRAF, SMAD4, and TP53 genetic variants in patients with colorectal carcinoma. (A) Oncoprint indicates genetic alterations in \textit{BRAF}, \textit{SMAD4}, and \textit{TP53} in patients with colorectal carcinoma, respectively. Colors indicate the type of genetic alterations (green: mutation; purple: structure variation; red: amplification, and gray: multiple alterations) and different cohorts below the oncoprint. (B–D) Alteration frequency of \textit{BRAF}, \textit{SMAD4}, and \textit{TP53} in colorectal carcinoma in five cohorts. CAN = copy number alterations. (E) Distribution of patients with \textit{BRAF}, \textit{SMAD4}, and \textit{TP53} genetic variants. (F, G) The effect of variants in \textit{BRAF}, \textit{SMAD4}, or \textit{TP53} on progression-free survival and overall survival were analyzed from five colorectal cancer databases. (H, I) The effects of the simultaneous occurrence of \textit{BRAF}, \textit{TP53}, and \textit{SMAD4} mutations on progression-free survival and overall survival were analyzed from five rectal cancer databases. Taken together, our results indicate that nCRT might result in physiological selective pressure to accumulate gene mutations in residual rectal cancer regions. \textit{BRAF}, \textit{SMAD4}, and \textit{TP53} genetic mutations might be involved in resistance to nCRT and poor prognosis in colorectal carcinoma. However, these findings must be confirmed.
13 mutation. In addition, tumors with the response to cetuximab-based chemoradiotherapy [31]. Davies et al. reported that KRAS (43%), APC (17%), BRAF (4%), NRAS (4%), PIK3CA (4%), and TP53 (11%) in rectal cancer, and these mutations were well associated with a complete response to nCRT, especially in patients with BRAF, NRAS, APC, or TP53 mutations [33]. These findings indicate that TP53, KRAS, and BRAF mutations might be used as biomarkers for the prediction of the response to nCRT.

**Figure 5.** BRAF, SMAD4, and TP53 genetic variants in patients with colorectal carcinoma. (A) Oncoprint indicates genetic alterations in BRAF, SMAD4, and TP53 in patients with colorectal carcinoma, respectively. Colors indicate the type of genetic alterations (green: mutation; purple: structure variation; red: amplification, and gray: multiple alterations) and different cohorts below the oncoprint. (B–D) Alteration frequency of BRAF, SMAD4, and TP53 in colorectal carcinoma in five cohorts. CAN = copy number alterations. (E) Distribution of patients with BRAF, SMAD4, and TP53 genetic variants. (F,G) The effect of variants in BRAF, SMAD4, or TP53 on progression-free survival and overall survival were analyzed from five colorectal cancer databases. (H,I) The effects of the simultaneous occurrence of BRAF, TP53, and SMAD4 mutations on progression-free survival and overall survival were analyzed from five rectal cancer databases.

3. Discussion

Preoperative nCRT followed by tumor resection has become the standard treatment guideline for patients with locally advanced rectal cancer. Preoperative nCRT can downstage the tumor and increase the possibility of sphincter preservation [29]. Such treatments can enable patients to have a better quality of life after surgery. Although numerous studies have reported that several genetic mutations can serve as the biomarkers of the response of the rectal tumor to nCRT, some studies have indicated opposite results or that genetic mutations cannot be used as predictors. Yang et al. performed the whole-genome sequencing of 28 paired advanced rectal cancer specimens before and after CRT and observed that mutations in CTDSPL2, APC, KRAS, TP53, and NFKB1Z confer selective pressure on cancer cells, resulting in resistance to CRT [12]. Concurrent KRAS and TP53 mutations contributed to resistance to CRT and metastasis in rectal cancer [13]. A study examining the genetic profiles of 229 pretreatment specimens from patients with stage II or III rectal cancer reported that KRAS and combined KRAS/TP53 mutations acted as independent biomarkers for a poor response to nCRT [14]. Garcia-Aguilar et al. reported that three individual genetic mutations, KRAS, CCND1, and MTHFR, were well correlated with a complete response (grade 0) to CRT in rectal carcinoma [30]. However, BRAF mutations were not detected, and KRAS and PTEN mutations were reported to not be associated with a response to cetuximab-based chemoradiotherapy [31]. Davies et al. reported that ERK and AKT signaling activation but not KRAS mutations were well correlated with the response to nCRT in rectal carcinoma [32]. Compared with wild-type KRAS, KRAS mutations were associated with a poor response to nCRT, especially the KRAS codon 13 mutation. In addition, tumors with the KRAS codon 13 mutation are often accompanied by TP53 mutations [8]. Russo et al. observed a high frequency of mutations in KRAS (43%), APC (17%), BRAF (4%), NRAS (4%), PIK3CA (4%), and TP53 (11%) in rectal cancer, and these mutations were well associated with a complete response to nCRT, especially in patients with BRAF, NRAS, APC, or TP53 mutations [33]. These findings indicate that TP53, KRAS, and BRAF mutations might be used as biomarkers for the prediction of the response to nCRT.
Russo et al. did not observe differences in individual gene mutation rates between pre- and post-CRT samples [33]. We used the pre-nCRT and post-nCRT paired samples to identify gene mutation biomarkers for predicting the response to nCRT. Our findings are consistent with those reported by Russo. We observed that most of the gene mutations disappeared after nCRT (Figure 4). Some genetic mutations were retained after nCRT, suggesting that these gene mutations might contribute to resistance to nCRT. Finally, three genetic mutations, \(BRAF\), \(SMAD4\), and \(TP53\), were successfully identified as biomarkers for predicting the response to nCRT. By examining the mutation status of \(BRAF\), \(SMAD4\), and \(TP53\), we can identify patients who would benefit from nCRT and select alternative therapeutic strategies for those who would not benefit from nCRT. In addition, we noted that \(BRAF\) genetic mutations exhibited mutual exclusivity with \(TP53\) mutations in rectal cancer but significantly cooccurred (\(p < 0.001\)) with \(SMAD4\) genetic mutations (\(p = 0.024\)).

We identified that mutations in \(BRAF\), \(SMAD4\), and \(TP53\) might contribute to the response to nCRT in patients with rectal carcinoma. Among them, \(SMAD4\) mutations have been rarely identified in other studies through high-throughput sequencing to identify biomarkers of the response to nCRT. A study reported that \(SMAD4\) mutations or deletions frequently occurred in late-stage colon cancer [34]. Another study reported that the loss of expression of \(SMAD4\)-induced \(RICTOR/AKT\) signaling activation was correlated with the poor survival of patients with colorectal carcinoma [35]. Furthermore, the depletion of \(SMAD4\) expression or the activation of \(RICTOR/AKT\) signaling contributed to resistance to irinotecan in colon cancer cells. In addition, some studies have reported that the loss of \(SMAD4\) expression contributed to resistance to 5-fluorouracil in colon cancer [36,37]. \(SMAD4\) expression causes radioresistance in pancreatic cancer through the induction of reactive oxygen species and autophagy [38]. A meta-analysis revealed that \(SMAD4\) mutations were well associated with overall, progression-free, and relapse-free survival and several clinicopathological parameters, including lymph node metastasis [39]. These findings suggest that \(SMAD4\) mutations are involved in sensitivity to a chemotherapeutic drug and cancer progression. \(TP53\) is a crucial tumor suppressor involved in maintaining genome stability and regulating the cell cycle and apoptosis. Park et al. reported that the loss of \(SMAD4\) and \(TP53\) synergistically occurs in intestinal carcinogenesis by inhibiting \(p21\) and increasing \(Wnt/\beta\)-catenin signaling activity [40]. Although oncogenic \(BRAF\) mutations play a crucial role in tumorigenesis, only the \(BRAF\) mutant was inefficient in generating tumors in vivo. Loss of pro-differentiation transcription factors, such as \(CDX2\) and \(SMAD4\), can accelerate tumorigenesis [41,42]. Taken together, these findings indicate that \(SMAD4\) is a crucial tumor suppressor and that its mutation might be involved in several cancer cell biological functions, including maintaining cancer genome stability and regulating the cell cycle and apoptosis. The results demonstrate that \(SMAD4\) mutations might serve as a biomarker for predicting the response of rectal cancer to nCRT. However, more data need to be collected prospectively to further demonstrate these new findings.

Although our study identified three genetic variant genes (\(BRAF\), \(SMAD4\), and \(TP53\)) that may act as biomarkers for predicting nCRT response, there were some shortcomings in our study. First, the small sample size resulted in low statistical power. In addition, the study relied on retrospective data and specimens. It might have missed some important information, and the nCRT process might be slightly different for each patient. These shortcomings resulted in most gene variants showing no significant difference between patients with good and poor responses to nCRT (Supplementary Table S6). In order to overcome the small sample size, we attempted to evaluate the clinical impacts of \(BRAF\), \(SMAD4\), and \(TP53\) mutation by analyzing publicly available datasets. Combined with public datasets, it was further confirmed that patients with \(BRAF\), \(SMAD4\), and \(TP53\) gene variants were significantly associated with poorer progression-free survival in colorectal cancer. Although combining data from various databases can improve statistical power, differences in genetic backgrounds by race and lack of standard treatment procedures for each patient remain issues to overcome.
In summary, we observed that the patients whose tumors harbored \textit{BRAF}, \textit{SMAD4}, and \textit{TP53} genetic mutations had significantly poorer disease-free survival, and the detection of these mutations can help identify patients who would benefit from nCRT. Detection of these genetic biomarkers can enable the selection of optimal treatment strategies and improvement in patients’ quality of life after surgery.

4. Methods and Materials

4.1. Clinical Samples

Patients were diagnosed with histologically confirmed rectal cancer between 2014 and 2018. Before any treatment, all cases underwent endorectal ultrasonography and biopsy. All patients needed to conduct a complete nCRT therapy course before surgery. FFPE rectal cancer specimens from 29 patients were collected in this study, including pre-nCRT and corresponding post-nCRT FFPE specimens. These pre-nCRT and post-nCRT FFPE blocks were cut into 4-µm-thick sections for DNA extraction. Among them, the DNA extraction of post-nCRT FFPE specimens from 3 patients with grade 0 (complete response) failed due to too few tumor cells. Our study protocol was independently reviewed and approved by the Institutional Review Board of Kaohsiung Veterans General Hospital and Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, (IRB approval number: VGHKS11-CT12-08 and 10-X-018).

4.2. DNA Extraction

Each FFPE block was cut into 4-µm-thick sections by using standard techniques. After excess wax was removed, pure tumor tissues were collected from three to four paraffin sections. Genomic DNA was extracted using the NucleoSpin FFPE DNA kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany). The quantity and integrity of the extracted genomic DNA were determined using Qubit (Thermo Fisher Scientific Inc., San Jose, CA, USA) and Fragment Analyzer (Advanced Analytical Technologies, Inc., Ankeny, IA, USA), respectively.

4.3. Cancer Hotspot Panel v2 Sequencing

Ten nanograms of genomic DNA was amplified using 207 primer pairs (Ion AmpliSeq Cancer Hotspot Panel v2, Thermo Fisher Scientific Inc., San Jose, CA, USA) to target the hotspot regions of 50 genes. Amplicons were ligated with barcoded adaptors by using the Ion Ampliton Library Kit (Thermo Fisher Scientific Inc., San Jose, CA, USA). Barcoded libraries were subsequently conjugated with sequencing beads through emulsion polymerase chain reaction and enriched using IonChef (Thermo Fisher Scientific Inc., San Jose, CA, USA) in accordance with the Ion Torrent protocol (Thermo Fisher Scientific Inc., San Jose, CA, USA). The quality and quantity of the amplified library were determined using the fragment analyzer (AATI) and Qubit (Thermo Fisher Scientific Inc., San Jose, CA, USA), respectively. Sequencing was performed on the Ion Proton sequencer by using the Ion PI chip (Thermo Fisher Scientific Inc., San Jose, CA, USA) in accordance with the manufacturer’s protocol.

4.4. Data Analysis

Raw reads generated by the sequencer were mapped to the hg19 reference genome by using the Ion Torrent Suite (v. 4.4). The coverage depth was calculated using the Torrent Coverage Analysis plug-in, and the result is presented in Supplementary Table S1. Single-nucleotide variants (SNVs) and short insertion/deletions were identified using the Torrent Variant Caller plug-in (version 4.4). The Variant Effect Predictor was used to annotate every variant with a database from COSMIC: v.70; dbSNP 138 and 1000 Genomes: phase 1. We filtered out variants with a coverage of lower than 50 or a frequency of <2%. Supplementary Tables S2–S4 list the results of gene variants detected through NGS.
4.5. Statistical Analysis

The variants numbers between pre-CRT and post-CRT were analyzed using Student’s t-tests. The demographics of patients with rectal cancer are presented as numbers and percentages. The chi-squared test was used to test the association between the categorical descriptive variables. Cumulative overall survival or progression free survival curves were estimated using the Kaplan–Meier method. The difference was considered significant when \( p < 0.05 \). All statistical analyses were performed using SPSS version 20.0 for Windows (SPSS Inc., Armonk, NY, USA).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms231810353/s1.

Author Contributions: Y.-K.C. and H.-H.T. executed the study and drafted the manuscript. C.-M.L. and K.-C.L. edited the manuscript. K.-W.T. supervised the study and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation (TCRD-TPE-111-36, TCRD-TPE-111-04, TCRD-TPE-MOST-109-17, TCRD-TPE-110-RT-1 and TCMF-CM3-111-01).

Institutional Review Board Statement: Our study protocol was independently reviewed and approved by the Institutional Review Board of Kaohsiung Veterans General Hospital and Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, (IRB approval number: VGHKS11-CT12-08 and 10-X-018).

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare that they have no competing interests.

References
1. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J. Clin. 2021, 71, 209–249. [CrossRef] [PubMed]
2. De Caluwe, L.; Van Nieuwenhove, Y.; Ceelen, W.P. Preoperative chemoradiation versus radiation alone for stage II and III resectable rectal cancer. Cochrane Database Syst. Rev. 2013, 28, CD006041. [CrossRef] [PubMed]
3. Bosset, J.F.; Collette, L.; Calais, G.; Mineur, L.; Maingon, P.; Radosovic-Jelic, L.; Daban, A.; Bardet, E.; Beny, A.; Ollier, J.C.; et al. Chemotherapy with preoperative radiotherapy in rectal cancer. N. Engl. J. Med. 2006, 355, 1114–1123. [CrossRef] [PubMed]
4. Nahas, S.C.; Rizkallah Nahas, C.S.; Sparapan Marques, C.F.; Ribeiro, U., Jr.; Cotti, G.C.; Imperiale, A.R.; Capareli, F.C.; Chi Chih Chen, A.T.; Hoff, P.M.; Cecconello, I. Pathologic Complete Response in Rectal Cancer: Can We Detect It? Lessons Learned From a Proposed Randomized Trial of Watch-and-Wait Treatment of Rectal Cancers. Dis. Colon Rectum 2016, 59, 255–263. [CrossRef] [PubMed]
5. Glynne-Jones, R.; Grainger, J.; Harrison, M.; Ostler, P.; Makris, A. Neoadjuvant chemotherapy prior to preoperative chemoradiation or radiation in rectal cancer: Should we be more cautious? Br. J. Cancer 2006, 94, 363–371. [CrossRef] [PubMed]
6. Molinari, C.; Matteucci, F.; Caroli, P.; Passardi, A. Biomarkers and Molecular Imaging as Predictors of Response to Neoadjuvant Chemoradiotherapy in Patients With Locally Advanced Rectal Cancer. Clin. Colorectal Cancer 2015, 14, 227–238. [CrossRef] [PubMed]
7. Chen, M.B.; Wu, X.Y.; Yu, R.; Li, C.; Wang, L.Q.; Shen, W.; Lu, P.H. P53 status as a predictive biomarker for patients receiving neoadjuvant radiation-based treatment: A meta-analysis in rectal cancer. PLoS ONE 2012, 7, e45388. [CrossRef]
8. Duldulao, M.P.; Lee, W.; Nelson, R.A.; Li, W.; Chen, Z.; Kim, J.; Garcia-Aguilar, J. Mutations in specific codons of the KRAS oncogene are associated with variable resistance to neoadjuvant chemoradiation therapy in patients with rectal adenocarcinoma. Ann. Surg. Oncol. 2013, 20, 2166–2171. [CrossRef]
9. Aghagolzadeh, P.; Radpour, R. New trends in molecular and cellular biomarker discovery for colorectal cancer. World J. Gastroenterol. 2016, 22, 5678–5693. [CrossRef]
10. Gonzalez-Pons, M.; Cruz-Corra, M. Colorectal Cancer Biomarkers: Where Are We Now? BioMed Res. Int. 2015, 2015, 149014. [CrossRef]
11. Sveen, A.; Kopetz, S.; Lothe, R.A. Biomarker-guided therapy for colorectal cancer: Strength in complexity. Nat. Rev. Clin. Oncol. 2020, 17, 11–32. [CrossRef] [PubMed]
12. Yang, J.; Lin, Y.; Huang, Y.; Jin, J.; Zou, S.; Zhang, X.; Li, H.; Feng, T.; Chen, J.; Zuo, Z.; et al. Genome landscapes of rectal cancer before and after preoperative chemoradiotherapy. Theranostics 2019, 9, 6856–6866. [CrossRef] [PubMed]
13. Kamran, S.C.; Lennerz, J.K.; Margolis, C.A.; Liu, D.; Reardon, B.; Wankowicz, S.A.; Van Seventer, E.E.; Tracy, A.; Wo, J.Y.; Carter, S.L.; et al. Endoscopic ultrasound fine-needle aspiration cytology mutation profiling using targeted next-generation sequencing: Personalized care for rectal cancer. *Am. J. Clin. Pathol.* 2015, 143, 879–888. [CrossRef] [PubMed]

14. Chow, O.S.; Kuk, D.; Keskin, M.; Smith, J.J.; Camacho, N.; Pelosof, R.; Chen, C.T.; Chen, Z.; Avila, K.; Weiser, M.R.; et al. KRAS and Combined KRAS/TP53 Mutations in Locally Advanced Rectal Cancer are Independently Associated with Decreased Response to Neoadjuvant Therapy. *Ann. Surg. Oncol.* 2016, 23, 2548–2555. [CrossRef]

15. Sanger, F.; Nicklen, S.; Coulston, A.R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 1977, 74, 5463–5467. [CrossRef]

16. Crumley, S.M.; Pepper, K.L.; Phan, A.T.; Olsen, R.J.; Schwartz, M.R.; Portier, B.P. Next-Generation Sequencing of Matched Primary and Metastatic Rectal Adenocarcinomas Demonstrates Minimal Mutation Gain and Concordance to Colonic Adenocarcinomas. *Arch. Pathol. Lab. Med.* 2015, 140, 529–535. [CrossRef]

17. Gleeson, F.C.; Kipp, B.R.; Voss, J.S.; Campion, M.B.; Minot, D.M.; Tu, Z.J.; Klee, E.W.; Sciallis, A.P.; Graham, R.P.; Lazaridis, K.N.; et al. Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. *Proc. Natl. Acad. Sci. USA* 2009, 106, 19096–19101. [CrossRef] [PubMed]

18. McCourt, C.M.; McArt, D.G.; Mills, K.; Catherwood, M.A.; Maxwell, P.; Waugh, D.J.; Hamilton, P.; O’Sullivan, J.M.; Salto-Tellez, M. Validation of next generation sequencing technologies in comparison to current diagnostic gold standards for BRAF, EGFR and KRAS mutational analysis. *PLoS ONE* 2013, 8, e69604. [CrossRef] [PubMed]

19. Choi, M.; Scholl, U.I.; Ji, W.; Liu, T.; Tikhonova, I.R.; Zumbo, P.; Nayir, A.; Bakkaloglu, A.; Ozen, S.; Sanjad, S.; et al. Genetic profiling of thymic carcinoma using targeted next-generation sequencing. *Lung Cancer* 2014, 86, 174–179. [CrossRef] [PubMed]

20. Zhang, L.; Chen, L.; Sah, S.; Latham, G.J.; Patel, R.; Song, Q.; Koeppen, H.; Tam, R.; Schleifman, E.; Mashhedi, H.; et al. Validation of targeted next generation sequencing technologies in comparison to current diagnostic gold standards for BRAF, EGFR and KRAS mutational analysis. *PLoS ONE* 2013, 8, e69604. [CrossRef] [PubMed]

21. Shitara, M.; Okuda, K.; Suzuki, A.; Tatematsu, T.; Hikosaka, Y.; Moriyama, S.; Sasaki, H.; Fujii, Y.; Yano, M. Genetic profiling of thymic carcinoma using targeted next-generation sequencing. *Lung Cancer* 2014, 86, 174–179. [CrossRef] [PubMed]

22. Hoadley, K.A.; Yau, C.; Hinoue, T.; Wolf, D.M.; Lazar, A.J.; Drill, E.; Shen, R.; Taylor, A.M.; Cherniack, A.D.; Thorsson, V.; et al. Cell-of-Origin Patterns Dominate the Molecular Classification of 10,000 Tumors from 33 Types of Cancer. *Cell* 2018, 173, 301–314.e6. [CrossRef] [PubMed]

23. Donoghue, M.T.A.; et al. Clinical Sequencing Defines the Genomic Landscape of Metastatic Colorectal Cancer. *Oncologist* 2014, 19, 336–343. [CrossRef]

24. Hoadley, K.A.; Yau, C.; Hinoue, T.; Wolf, D.M.; Lazar, A.J.; Drill, E.; Shen, R.; Taylor, A.M.; Cherniack, A.D.; Thorsson, V.; et al. Cell-of-Origin Patterns Dominate the Molecular Classification of 10,000 Tumors from 33 Types of Cancer. *Cell* 2018, 173, 301–314.e6. [CrossRef] [PubMed]

25. Shitara, M.; Okuda, K.; Suzuki, A.; Tatematsu, T.; Hikosaka, Y.; Moriyama, S.; Sasaki, H.; Fujii, Y.; Yano, M. Genetic profiling of thymic carcinoma using targeted next-generation sequencing. *Lung Cancer* 2014, 86, 174–179. [CrossRef] [PubMed]

26. Hoadley, K.A.; Yau, C.; Hinoue, T.; Wolf, D.M.; Lazar, A.J.; Drill, E.; Shen, R.; Taylor, A.M.; Cherniack, A.D.; Thorsson, V.; et al. Cell-of-Origin Patterns Dominate the Molecular Classification of 10,000 Tumors from 33 Types of Cancer. *Cell* 2018, 173, 301–314.e6. [CrossRef] [PubMed]

27. Aker, T.; Chatila, W.K.; Lipsyc, M.D.; Hechtman, J.F.; Cercek, A.; Sanchez-Vega, F.; Jayakumaran, G.; Siddha, S.; Zehir, A.; Donoghue, M.T.A.; et al. Clinical Sequencing Defines the Genomic Landscape of Metastatic Colorectal Cancer. *Cancer Cell* 2018, 33, 125–136.e3. [CrossRef] [PubMed]

28. Mondaca, S.; Walch, H.; Nandakumar, S.; Chatila, W.K.; Schultz, N.; Yaeoger, R. Specific Mutations in APC, but Not Alterations in DNA Damage Response, Associate with Outcomes of Patients with Metastatic Colorectal Cancer. *Gastroenterology* 2020, 159, 1975–1978.e4. [CrossRef] [PubMed]

29. Sauer, R.; Becker, H.; Hohenberger, W.; Rodel, C.; Wittekind, C.; Fietkau, R.; Martus, P.; Hager, E.; Hess, C.F.; et al. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N. Engl. J. Med.* 2004, 351, 1731–1740. [CrossRef]

30. Garcia-Aguilar, J.; Chen, Z.; Smith, D.D.; Li, W.; Madoff, R.D.; Cataldo, P.; Marcet, J.; Pastor, C. Identification of a biomarker profile associated with resistance to neoadjuvant chemoradiation therapy in rectal cancer. *Ann. Surg.* 2011, 254, 486–492, discussion 492–483. [CrossRef]

31. Erben, P.; Strobel, P.; Horisberger, K.; Popa, J.; Bohn, B.; Hanfstein, B.; Kahler, G.; Kienle, P.; Post, S.; Wenz, F.; et al. KRAS and BRAF mutations and PTEN expression do not predict efficacy of cetuximab-based chemoradiotherapy in locally advanced rectal cancer. *Int. J. Radiat. Oncol. Biol. Phys.* 2011, 81, 1032–1038. [CrossRef]

32. Davies, J.M.; Trembath, D.; Deal, A.M.; Funkhouser, W.K.; Calvo, B.F.; Finnegan, T.; Weck, K.E.; Tepper, J.E.; O’Neill, B.H. Phospho-ERK and AKT status, but not KRAS mutation status, are associated with outcomes in rectal cancer treated with chemoradiotherapy. *Radiat. Oncol.* 2011, 6, 114. [CrossRef] [PubMed]

33. Russo, A.L.; Ryan, D.P.; Borger, D.R.; Wo, J.Y.; Szymonifka, J.; Liang, W.Y.; Kvak, E.L.; Blaszkowski, L.S.; Clark, J.W.; Allen, J.N.; et al. Integrated and clinical predictors of pathologic complete response in the treatment of locally advanced rectal cancer. *J. Gastrointest. Cancer* 2014, 45, 34–39. [CrossRef] [PubMed]
34. Cancer Genome Atlas, N. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012, 487, 330–337. [CrossRef] [PubMed]

35. Wong, C.K.; Lambert, A.W.; Ozturk, S.; Papageorgis, P.; Lopez, D.; Shen, N.; Sen, Z.; Abdolmaleky, H.M.; Gyorffy, B.; Feng, H.; et al. Targeting RICTOR Sensitizes SMAD4-Negative Colon Cancer to Irinotecan. *Mol. Cancer Res.* 2020, 18, 414–423. [CrossRef]

36. Boulay, J.L.; Mild, G.; Lowy, A.; Reuter, J.; Lagrange, M.; Terracciano, L.; Laffer, U.; Herrmann, R.; Rochlitz, C. SMAD4 is a predictive marker for 5-fluorouracil-based chemotherapy in patients with colorectal cancer. *Br. J. Cancer* 2002, 87, 630–634. [CrossRef] [PubMed]

37. Alhopuro, P.; Alazzouzi, H.; Sambalkorpi, H.; Davalos, V.; Salovaara, R.; Hemminki, A.; Jarvinen, H.; Mecklin, J.P.; Schwartz, S., Jr.; Aaltonen, L.A.; et al. SMAD4 levels and response to 5-fluorouracil in colorectal cancer. *Clin. Cancer Res.* 2005, 11, 6311–6316. [CrossRef] [PubMed]

38. Wang, F.; Xia, X.; Yang, C.; Shen, J.; Mai, J.; Kim, H.C.; Kirui, D.; Kang, Y.; Fleming, J.B.; Koay, E.J.; et al. SMAD4 Gene Mutation Renders Pancreatic Cancer Resistance to Radiotherapy through Promotion of Autophagy. *Clin. Cancer Res.* 2018, 24, 3176–3185. [CrossRef]

39. Fang, T.; Liang, T.; Wang, Y.; Wu, H.; Liu, S.; Xie, L.; Liang, J.; Wang, C.; Tan, Y. Prognostic role and clinicopathological features of SMAD4 gene mutation in colorectal cancer: A systematic review and meta-analysis. *BMC Gastroenterol.* 2021, 21, 297. [CrossRef] [PubMed]

40. Park, J.W.; Seo, M.J.; Cho, K.S.; Kook, M.C.; Jeong, J.M.; Roh, S.G.; Cho, S.Y.; Cheon, J.H.; Kim, H.K. Smad4 and p53 synergize in suppressing autochthonous intestinal cancer. *Cancer Med.* 2022, 11, 1925–1936. [CrossRef] [PubMed]

41. Sakamoto, N.; Feng, Y.; Stolfi, C.; Kurosu, Y.; Green, M.; Lin, J.; Green, M.E.; Sentani, K.; Yasui, W.; McMahon, M.; et al. BRAF(V600E) cooperates with CDX2 inactivation to promote serrated colorectal tumorigenesis. *Elife* 2017, 6, e20331. [CrossRef] [PubMed]

42. Tong, K.; Pellon-Cardenas, O.; Sirihorachai, V.R.; Warder, B.N.; Kothari, O.A.; Perekatt, A.O.; Fokas, E.E.; Fullem, R.L.; Zhou, A.; Thackray, J.K.; et al. Degree of Tissue Differentiation Dictates Susceptibility to BRAF-Driven Colorectal Cancer. *Cell Rep.* 2017, 21, 3833–3845. [CrossRef] [PubMed]