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ORIGINAL ARTICLE

Mutation in *BRAF* and *SMAD4* associated with resistance to neoadjuvant chemoradiation therapy in locally advanced rectal cancer

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Abstract

Our study was done in order to identify novel molecular markers to predict which locally advanced rectal cancers (LARCs) might be resistant to neoadjuvant chemoradiotherapy (nCRT). Seventy-four patients with LARCs treated with nCRT were collected. Pathological evaluation after nCRT was performed according to the tumor regression grading (TRG) system. Next-generation sequencing kit including 279 exons of 59 genes was performed on Illumina Miseq Platform. Sanger sequencing was performed to confirm some mutations. Four of the tumors (4/74, 5.4%) had *BRAF* mutation, which presented in one TRG 2 tumor and three TRG 3 tumors but was not observed in TRG 0–1 tumors. Higher mutational frequency of *BRAF* gene in TRG 3 tumors (3/12, 25%) was found in comparison with the TRG 0–2 tumors (1/62, 1.6%; $p = 0.012$). Eight tumors (8/74, 10.8%) harbored *SMAD4* mutations, which was mutated across all TRG groups. However, *SMAD4* mutated more in TRG 3 tumors (4/12, 33.3%) compared with that in TRG 0–2 tumors (4/62, 6.5%; $p = 0.020$). The patients with *BRAF*-mutated LARCs had shorter progression-free survival (PFS) ($p = 0.045$) and shorter overall survival (OS) ($p = 0.000$) than the *BRAF* wild-type (WT) ones. The patients with *SMAD4*-mutated tumors had shorter PFS than the WT cases ($p = 0.008$). *BRAF* and *SMAD4* genetic mutations might be important molecular markers to predict resistance to nCRT and poor prognosis in LARCs. More cases are needed to confirm these findings in the near future.

Keywords Rectal cancer · Neoadjuvant chemoradiotherapy · Predictor · *BRAF* · *SMAD4*

Introduction

Pre-operative neoadjuvant chemoradiation therapy (nCRT), followed by total mesorectal excision (TME) has become the standard treatment for patients with locally advanced rectal cancers (LARCs), because this treatment can downstage tumors, decrease the risk of local recurrence, and increase the possibility of sphincter preservation [1, 2]. However, variable tumor responses can be presented between individuals after nCRT. Predicting which patients will benefit from nCRT would allow identification of the patients who are likely to achieve pathological complete response (pCR), and which patients will not respond to the nCRT who would then select alterative treatment strategies, such as excision, burgeoning immunotherapy, or other strategies, is an important research topic in recent years.

Studies focusing on genetic mutations as predictors of tumor response to nCRT in rectal cancers have been published.

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These genetic predictors included *KRAS* status, specific *KRAS* codons mutation, *TP53* status, and so on; however, some studies had achieved the opposite results and stated that these gene statuses did not have the ability to be predictors [3–10]. To get more information about this topic, we have tested the status of 279 exons of 59 genes in 74 LARCs who received nCRT by using next-generation sequencing (NGS).

Materials and methods

Patients and treatment

This study was approved by the West China Hospital Institutional Review Board. From January 2013 to June 2016, 77 patients with stage II and III rectal cancers treated with pre-operative nCRT at our institution were collected for this retrospective study. Finally, 74 rectal cancer patients were enrolled in this study, as 3 cases did not get genetic testing data because of poor DNA quality. All tumors were pathologically confirmed as adenocarcinoma from the biopsies before nCRT. Pre-therapeutic clinical tumor-node-metastasis (TNM) stage (cT, cN, cM, and cAJCC_Stage) was evaluated by radiography and endoscopic ultrasonography, following by the AJCC 8th edition. The patients received chemotherapy including XELOX and modified FOLFOX-6 (mFOLFOX-6) for 1 to 4 cycles, and subsequently received 45 to 50.4 Gy of radiation (single dose of 1.8 Gy, delivered in 25 to 28 fractions using intensity-modulated radiation therapy (IMRT) or volumetric-modulated arc radiotherapy (VMAT) technique) accompanied by xeloda or 5-Fu. None of the patients received the anti-*EGFR* therapies during the nCRT period. All patients underwent curative resection, including TME at around 4 to 8 weeks after the completion of pre-operative chemoradiotherapy. Post-therapeutic pathological TNM stage (ypT, ypN, ypM, and ypAJCC_Stage) was reviewed following the AJCC 8th edition. T downstage is defined as the tumor which has a lower ypT rather than cT.

Assessment of tumor histological type

All of the tumor slides (including biopsy and surgical samples) of the 74 cases were evaluated for tumor histological types. Following the World Health Organization classification of tumors of the digestive system (2010 edition), the tumors were described as adenocarcinoma in situ, adenocarcinoma, medullary carcinoma, mucinous carcinoma, signet ring cell carcinoma, squamous cell carcinoma, and so on. The adenocarcinomas were graded as well differentiated (G1, > 95% gland formation), moderately differentiated (G2, 50–95% gland formation), and poorly differentiated (G3, < 50% gland formation). Two pathologists (D.J. and D.H.) graded the tissue slides independently. If there were inconsistent cases, the cases were evaluated together and discussed to get a final result.

Assessment of tumor response

All of the 74 cases had surgical samples to be pathologically evaluated for tumor response. Pathological evaluation of tumor response after nCRT was performed according to the National Comprehensive Cancer Network (NCCN) [11] recommended tumor regression grading (TRG) system [12]. TRG 0 (complete response), no remaining viable cancer cells remain, known as pCR. TRG 1 (moderate response), only small clusters or single cancer cells remaining. TRG 2 (minimal response), only residual cancer is remaining, but there is predominant fibrosis. TRG 3 (poor response), minimal or no tumor was killed, extensive residual cancer. Two pathologists (D.J. and D.H.) graded the tissue slides independently. If there were inconsistent cases, the cases were evaluated together and discussed to achieve a final grade. Representative images of tissue sections of different TRG are illustrated in Fig. 1.

Immunohistochemistry and assessment of MMR protein

Tissue sections (4 μm) were cut from the formalin-fixed, paraffin-embedded (FFPE) tumor. The slides had undergone deparaffinization, rehydration, antigen retrieval, and endogenous peroxidase activity was blocked by incubation with 3% H2O2. The sections were then mounted on the BenchMark ULTRA Advanced Staining system and then subjected to the MLH1 (M1), MSH2 (G29-1129), MSH6 (44), PMS2 (A16-4) mouse monoclonal antibodies (Roche, Ventana Medical Systems, Inc.). Finally, the slides were counterstained with hematoxylin.

The tumors were considered to be devoid of MLH1, MSH2, MSH6, and PMS2 expression if no nuclear staining of tumor cells could be detected. The surrounding stromal cells and lymphocytes served as an internal positive control [13]. The tumor that showed at least one of the MMR proteins absent was defined as MMR deficiency (dMMR), otherwise, MMR proficiency (pMMR) (Supplementary Fig. 1).

Mutational analysis

Genomic DNA extraction

A total of 83 FFPE colorectal tumors of the 74 pre-treatment biopsy samples and 9 surgical samples were prepared for mutational test. The blocks harboring tumor area covering more than 20% were chosen by the pathologist (D. J.). Genomic DNA was extracted from a minimum of four 4-μm-thick FFPE sections using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) as per the manufacturer's

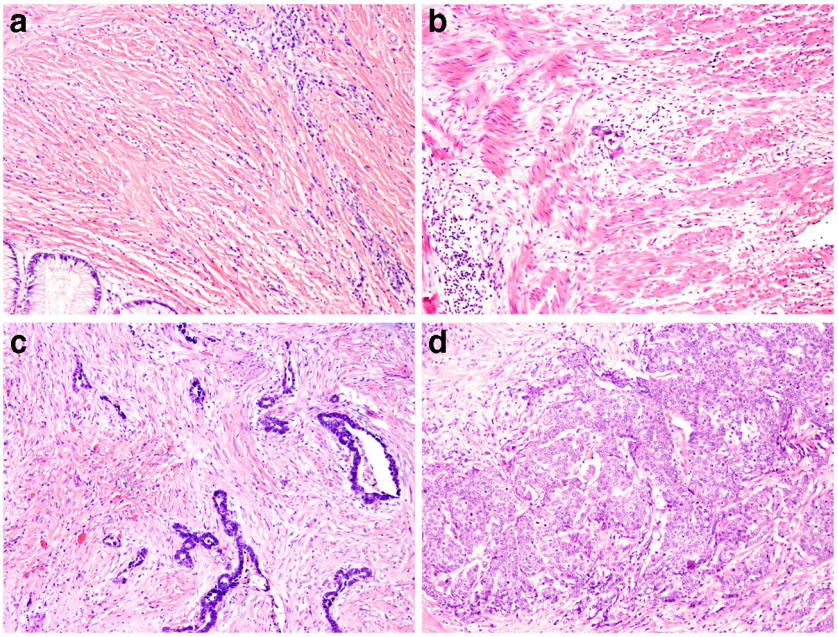

Fig. 1 Presentative images of histopathological assessment of tumor response after neoadjuvant chemoradiotherapy (HE, magnification ×100). **a** TRG (tumor regression grade) 0, complete response, no remaining viable cancer cells, known as pCR (pathological complete response). **b** TRG 1, moderate response, only small clusters or single cancer cells remaining. **c** TRG 2, minimal response, residual cancer remaining, but with predominant fibrosis. **d** TRG 3, poor response, minimal or no tumor killed, extensive residual cancer

instructions. DNA concentration was measured using the Qubit dsDNA HS Assay kit in combination with a Qubit 3.0 fluorimeter (Life Technologies, Eugene, Oregon, USA). DNA samples were stored at − 20 °C before use.

Targeted NGS assay (Illumina Miseq)

A total of 20 ng DNA for each FFPE sample was subjected to library construction using the OncoAim™ DNA Mutation Panel (Singlera Genomics, Shanghai, China) according to the manufacturers' protocols. Briefly, genomic regions of 59 genes (Supplementary Table 1) were amplified using pooled primer pairs, followed by specific excision of primers and ligation with adaptors and barcodes. Following purification of ligation production, the target fragments were amplified. Finally, the indexed sequencing libraries were purified again. Libraries were quantified using the Qubit 3.0 fluorimeter and KAPA SYBR® FAST universal qPCR Kits. Each library was diluted to a concentration of 2 nmol/L. Up to 48 libraries were pooled in equimolar amount and sequenced simultaneously on the same flow cell in an Illumina Miseq sequencer (Illumina, Hayward, CA, USA).

Variant calling

The output data (Sequence FASTQ files) was automatically uploaded for data quality control, sequence alignment, and variant calling with a vendor-supplied bioinformatics pipelines. Single-nucleotide variants (SNPs) or insertions/deletions (INDELs) were identified. In order to eliminate erroneous base calling and generate the final variant calling, sequencing samples with median coverage depth ≥ 500 and somatic variants with frequency ≥ 5% were included for further downstream analysis as the prior study described [14]. Detected variants were manually checked on the Integrative Genomics Viewer (IGV) (https://www.broadinstitute.org/software/igv/home). The process of NGS used in this study was published elsewhere [15].

Sanger sequencing

Polymerase chain reaction (PCR) amplification of 3 regions of *KRAS* (exon 2, 3, 4) and *BRAF* (exon 15) were performed separately. Detailed primer information is shown in Supplementary Table 2. PCR program was set as follows: preheating at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Purified PCR products were sequenced on an ABI 3730xL sequencer using the BigDye 3.1 Terminator sequencing kit (Applied Biosystems, USA) according to the manufacturer's protocol. The sequencing results were interpreted using Chromas software V.1.45 (Technelysium Pty, Helensvale, Australia).

Statistics

Interactions between treatment and genetic mutations were assessed by using Fisher's exact test with 2 sides. Overall survival (OS) was calculated from initial diagnosis to disease-related death or the last date of follow-up. Progression-free survival (PFS) was calculated from initial diagnosis to the first event (local recurrence/progression, distant recurrence, or disease-related death). OS or PFS by *BRAF* and *SMAD4* status was estimated using the Kaplan-Meier method and the differences were compared using log-rank test. Analyses were performed using SPSS 19.0 software. $p < 0.05$ was considered statistically significant.

Results

Patient demographics and clinical characteristics

Clinicopathological features of these 74 patients in our study are summarized in Table 1, and the information in detail is presented in Supplementary Table 3. This group included 52 male and 22 female patients, the median age was 52.5 years (range, 28–76 years). Twenty-five patients (25/74, 33.8%) presented with stage II rectal cancer, 49 patients (49/74, 66.2%) presents with stage III rectal cancers. Histologically, 60 tumors were adenocarcinoma (60/74, 81.1%), 12 tumors were mucinous carcinoma (12/74, 16.2%), and 2 tumors presented as signet ring cell carcinoma (2/74, 2.7%).

After nCRT, 13 of 74 (13/74, 17.6%) patients achieve pCR (TRG 0); 19 (19/74, 25.7%) patients had TRG 1; 30 had (30/74, 40.5%) TRG 2; and 12 (12/74, 16.2%) had TRG 3. After nCRT, 50 patients (50/74, 67.6%) experienced primary tumor downstaging.

After exclusion of 3 patients (2 patients were lost to follow-up, 1 patient dead of cardiovascular disease), the median follow-up time of the 71 patients was 41 months (range 12–67 months). Twenty patients experienced disease progression (local/distant recurrence); median time of recurrence was 20.5 months (range 5–42 months).

Genetic mutations and pathological response to nCRT

Sixty-two pre-therapeutic biopsies samples (62/74, 83.8%) had one or more tested gene mutations. The genetic mutation results are presented in Table 2 and Fig. 2. The genetic results in detail are listed in Supplementary Table 4.

BRAF mutation was not observed in TRG 0, and TRG 1 samples, however, was observed in one TRG 2 sample (1/30, 3.3%) and three TRG 3 samples (3/12, 25%). There was significantly higher frequency of *BRAF* mutation in the TRG 3 group (3/12, 25%) compared with that in the TRG 0–2 group (1/62, 1.6%, $p = 0.012$). However, there was no significance of mutational frequencies between pCR tumors (0/13, 0%) and non-pCR tumors (4/61, 6.6%). For the mutation sites, three samples harbored *BRAF* p.V600E mutation, one sample showed p.D594G mutation. Sanger sequencing confirmed these mutations (Supplementary Fig. 2).

Eight (8/74, 10.8%) tumors harbored *SMAD4* mutation. *SMAD4* gene was mutated in one (1/13, 7.7%) TRG 0 tumor, one (1/19, 5.3%) TRG 1 tumor, two (2/30, 6.7%) TRG 2 tumors, and four (4/12, 33.3%) TRG 3 tumors. There was a significantly higher mutational frequency of *SMAD4* gene in TRG 3 tumors (4/12, 33.3%) compared with that in TRG 0–2 tumors ((4/62, 6.5%; $p = 0.020$). However, there was no significance of mutational frequencies between pCR tumors

Table 1 Clinicopathological features of this cohort of patients

Variable	Subtype	*n*	%
Age (year)			
	> 50	42	56.8%
	≤ 50	32	43.2%
Gender			
	Male	52	70.3%
	Female	22	29.7%
Histologic type			
	Adenocarcinoma	60	81.1%
	G1(well differentiated)	15	20.3%
	G2(moderately differentiated)	37	50%
	G3(poorly differentiated)	8	10.8%
	Mucinous carcinoma	12	16.2%
	Signet ring cell carcinoma	2	2.7%
MMR			
	dMMR	4	5.4%
	pMMR	70	94.6%
TRG			
	0	13	17.6%
	1	19	25.7%
	2	30	40.5%
	3	12	16.2%
cT			
	2	2	2.7%
	3	31	41.9%
	4a	8	10.8%
	4b	33	44.6%
ypT			
	0	13	17.6%
	1	1	1.4%
	2	14	18.9%
	3	38	51.4%
	4a	2	2.7%
	4b	6	8.1%
	Downstage	50	67.6%
cAJCC_Stage			
	II	25	33.8%
	III	49	66.2%
ypAJCC_Stage			
	0	12	16.2%
	I	11	14.9%
	II	24	32.4%
	III	25	33.8%
	Uncertain	2	2.7%

MMR, mismatch repair protein; *dMMR*, mismatch repair protein deficient; *pMMR*, mismatch repair protein proficient; *TRG*, tumor regression grade; *cT*: clinical stage of primary tumor; *ypT*: pathological stage of primary tumor after neoadjuvant therapy; *cAJCC_Stage*: clinical TNM stage following eighth edition of AJCC (American Joint Committee on Cancer) TNM staging system; *ypAJCC_Stage*: pathological TNM stage following eighth edition of AJCC TNM staging system after neoadjuvant therapy

(1/13, 7.7%) and non-pCR tumors (7/61, 11.5%). The most common mutated codon of *SMAD4* gene was codon 361, which was found in three samples.

In this study, 26 (26/74, 35.1%) samples had *KRAS* mutation. There was no significant difference of *KRAS* mutational frequencies neither in TRG 3 samples (4/12, 33.3%) compared with TRG 0–2 samples (22/62, 35.5%), nor in pCR samples (4/13, 30.8%) compared with non-pCR samples (22/61, 36.1%). Seventeen samples had *KRAS* mutation in codon 12, three in codon 146, two in codon 13, two in codon 61, one in codon 22, and one case had mutation both in codon 12 and 13. No tumors with pCR had *KRAS* codon 13 or 146 mutation. The mutations in some extend codons, such as in codon 22, 146 were only found in TRG 2–3 samples. However, all these results have no significances.

Seven (7/74, 9.5%) samples had *PIK3CA* mutation. *PIK3CA* was mutated in four (4/62, 6.5%) TRG 0–2 sample, while, in three (3/12, 25%) TRG 3 samples, and in one (1/13, 7.7%) pCR samples compared with in six (6/61, 9.8%) non-pCR samples. However, there were no significances for all these results. Another gene in the *PI3K*/*AKT* pathway, *PTEN*, was observed, only mutated in one TRG 3 sample.

TP53 had broad spectrum mutations, including point mutations, deletions, and nonsense mutations, and so on. Forty (40/74, 54.1%) samples had *TP53* mutation, the mutational frequencies varied from 50 to 61.5% among the different TRG tumors.

Table 2 Genetic mutations of this cohort of patients

Gene	Total, *n* (%)	Tumor regression grade (TRG), *n* (%)			
		TRG 0	TRG 1	TRG 2	TRG 3
	74	13	19	30	12
BRAF	4 (5.4)	0 (0)	0 (0)	1 (3.3)	3 (25)
RAF1	1 (1.4)	0 (0)	0 (0)	1 (3.3)	0 (0)
KRAS	26 (35.1)	4 (30.8)	6 (31.6)	12 (40)	4 (33.3)
NRAS	1 (1.4)	0 (0)	0 (0)	1 (3.3)	0 (0)
TP53	40 (54.1)	8 (61.5)	11 (57.9)	15 (50)	6 (50)
APC	14 (18.9)	3 (23.1)	4 (21.1)	4 (13.3)	3 (25)
PIK3CA	7 (9.5)	1 (7.7)	2 (10.5)	1 (3.3)	3 (25)
PTEN	1 (1.4)	0 (0)	0 (0)	0 (0)	1 (8.3)
FBXW7	7 (9.5)	2 (15.4)	2 (10.5)	3 (10)	0 (0)
SMAD4	8 (10.8)	1 (7.7)	1 (5.3)	2 (6.7)	4 (33.3)
ERBB2	1 (1.4)	0 (0)	1 (5.3)	0 (0)	0 (0)
ERBB3	3 (4.1)	1 (7.7)	0 (0)	2 (6.7)	0 (0)
ATM	3 (4.1)	1 (7.7)	2 (10.5)	0 (0)	0 (0)
AKT1	2 (2.7)	1 (7.7)	0 (0)	0 (0)	1 (8.3)
RET	1 (1.4)	0 (0)	0 (0)	1 (3.3)	0 (0)
PDGFRA	1 (1.4)	1 (7.7)	0 (0)	0 (0)	0 (0)
SMARCB1	2 (2.7)	0 (0)	0 (0)	2 (6.7)	0 (0)
EZH2	1 (1.4)	1 (7.7)	0 (0)	0 (0)	0 (0)
CDKN2A	1 (1.4)	0 (0)	0 (0)	1 (3.3)	0 (0)
CTNNB1	1 (1.4)	0 (0)	0 (0)	1 (3.3)	0 (0)
EGFR	1 (1.4)	0 (0)	0 (0)	1 (3.3)	0 (0)
FLT3	1 (1.4)	0 (0)	0 (0)	1 (3.3)	0 (0)
JAK1	1 (1.4)	0 (0)	0 (0)	1 (3.3)	0 (0)
MPL	1 (1.4)	0 (0)	0 (0)	1 (3.3)	0 (0)
PTCH1	1 (1.4)	0 (0)	0 (0)	1 (3.3)	0 (0)

Genetic mutations and T downstage to nCRT

No significances were reached for the correlation between the tested genetic mutations and T downstage cases (*BRAF* 3/50, 6%; *SMAD4* 3/50, 6%; *KRAS* 15/50, 30%; *PIK3CA* 4/50, 8%; *TP53* 30/50, 60%) and the T stable group (*BRAF* 1/24, 4.2%; *SMAD4* 5/24, 20.8%; *KRAS* 11/24, 45.8%; *PIK3CA* 3/24, 12.5%; *TP53* 10/24, 41.7%).

MMR status and pathological response/T downstage to nCRT

Four tumors showed dMMR deficiency (4/74, 5.4%), which presented as one TRG 1 tumor and three TRG 2 tumors. No significances were reached between MMR status either with tumor response, or with T downstage.

Mutations in pre- and post-treatment tissues

Of the 12 TRG 3 cases, 9 cases had paired pre- and post-treatment tissues. Six (6/9, 66.7%) cases had identical mutational results between the pre-nCRT biopsy tissues and post-nCRT surgical specimens, while the remaining three (3/9, 33.3%) cases had inconsonant genetic mutations. Of these three cases, two cases were observed to have one more genetic mutation (*AKT1* and *SMAD4*, respectively) in pre-treatment biopsies than in the post-treatment surgical tissues, one case was found to have two more mutations (*KRAS*, *PIK3CA*) in post-treatment tissue, and *TP53* mutation was only found in pre-treatment tissue.

Survival analysis

As *BRAF* and *SMAD4* gene mutation was correlated with poor pathological response, we tried to analyze the correlation between these two genes with survival (Fig. 3). The patients with *BRAF*-mutated LARCs had shorter PFS ($p = 0.045$) and shorter OS ($p = 0.000$) than the patients with *BRAF* wild-type tumors. *SMAD4*-mutated patients had shorter PFS ($p = 0.008$); however, no significance was reached for OS analysis.

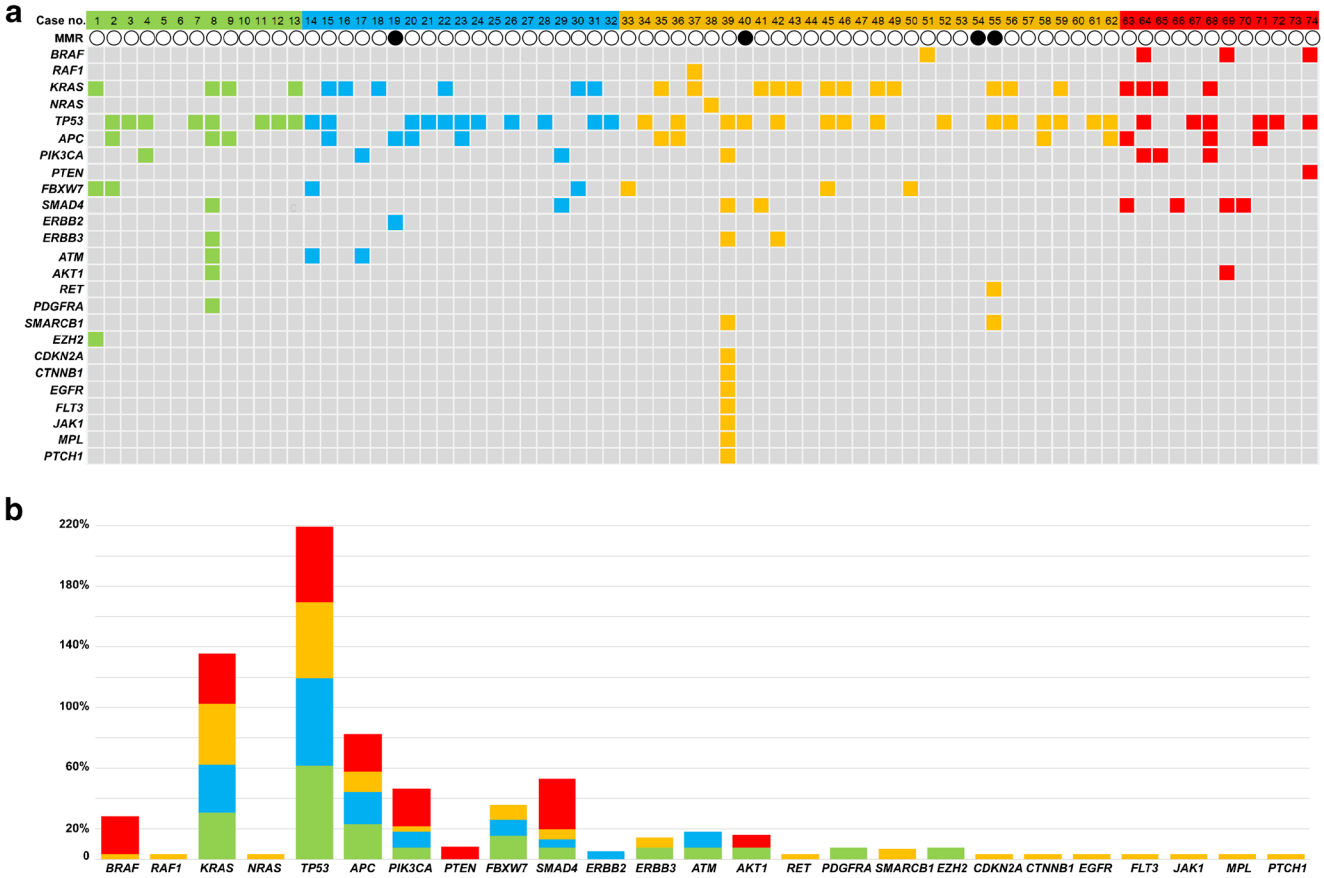

Fig. 2 Genetic mutations in this cohort of patients who received neoadjuvant chemoradiotherapy. **a** The block chart shows the MMR status and genetic mutations for each case. Case number is listed in the first row of this chart, and the names of the tested genes are listed to the left. The solid black circle represents dMMR status (losing one MMR protein expression at least), and the hollow black circles represent pMMR status (the case shows positive for MLH, MSH2, MSH6, and PMS2 proteins). The gray blocks represent the wild-type status, while the mutated genes are represented by colored blocks. Green indicates TRG (tumor regression grade) 0 cases, blue indicates TRG 1 cases, orange indiactesTRG 2 cases, and red indicates TRG 3 cases. **b** Mutation frequencies of the different TRG groups in each gene. Green bars represent the TRG 0 group, blue bars for TRG 1 group, orange for TRG 2, and red for TRG 3. For example, in the bar noted as *TP53*, 61.5% cases had *TP53* mutation in the TRG 0 group, 57.9% in TRG 1, 50% in TRG 2, and 50% in TRG 3 group

Discussion

To the best of our knowledge, this is the first study using next-generation sequencing technique to test the status of dozens of genes, and finally to find that *BRAF* and *SMAD4* gene mutation was associated with variable resistance to nCRT in patients with LARCs. Furthermore, the patients with *BRAF*-mutated tumors showed shorter PFS and OS; the patients with *SMAD4*-mutated cases presented with shorter PFS. However, *KRAS*, *PIK3CA*, *TP53*, and MMR status could not be predictors for tumor response of nCRT.

BRAF, an oncogene located at the downstream of *RAS* pathway, is a poor prognosis marker in colorectal cancer (CRC), and the tumor with mutated *BRAF* is less likely to respond to anti-*EGFR* antibody therapy [16]. *BRAF* was reported to not predict chemoradiation response in previous studies [3–6]. However, our study showed that *BRAF* gene mutation could be a biomarker to predict resistance to nCRT in LARCs. Possible interpretation for this discrepancy might be due to the previous studies comparing the significance between pCR and non-pCR groups, which had no significant result either. However, we found the mutational cases showing variant resistance (TRG 3 versus TRG 0–2), and most importantly, the patients with *BRAF*-mutated LARCs had poor prognosis after nCRT. More such cases are needed to analyze in the future to prove this result.

SMAD4 gene, acts as a tumor suppressor, whose mutation in CRC may correlate with poor outcome and resistance to 5-fluorouracil [17]. No previous study focused on the predictive role of *SMAD4* gene status for nCRT in LARCs. *SMAD4* mutation can be found in the tumors with different therapy response in our study. However, *SMAD4* gene had a significantly higher mutational frequency in TRG 3 tumors than in TRG 0–2 tumors in this group of patients. Moreover, the patients with *SMAD4* gene–mutated tumors had shorter PFS after nCRT. It might be interpreted as the CRC with *SMAD4* mutation being resistant to 5-fluorouracil which cause the poor tumor response for pre-operative therapy, and

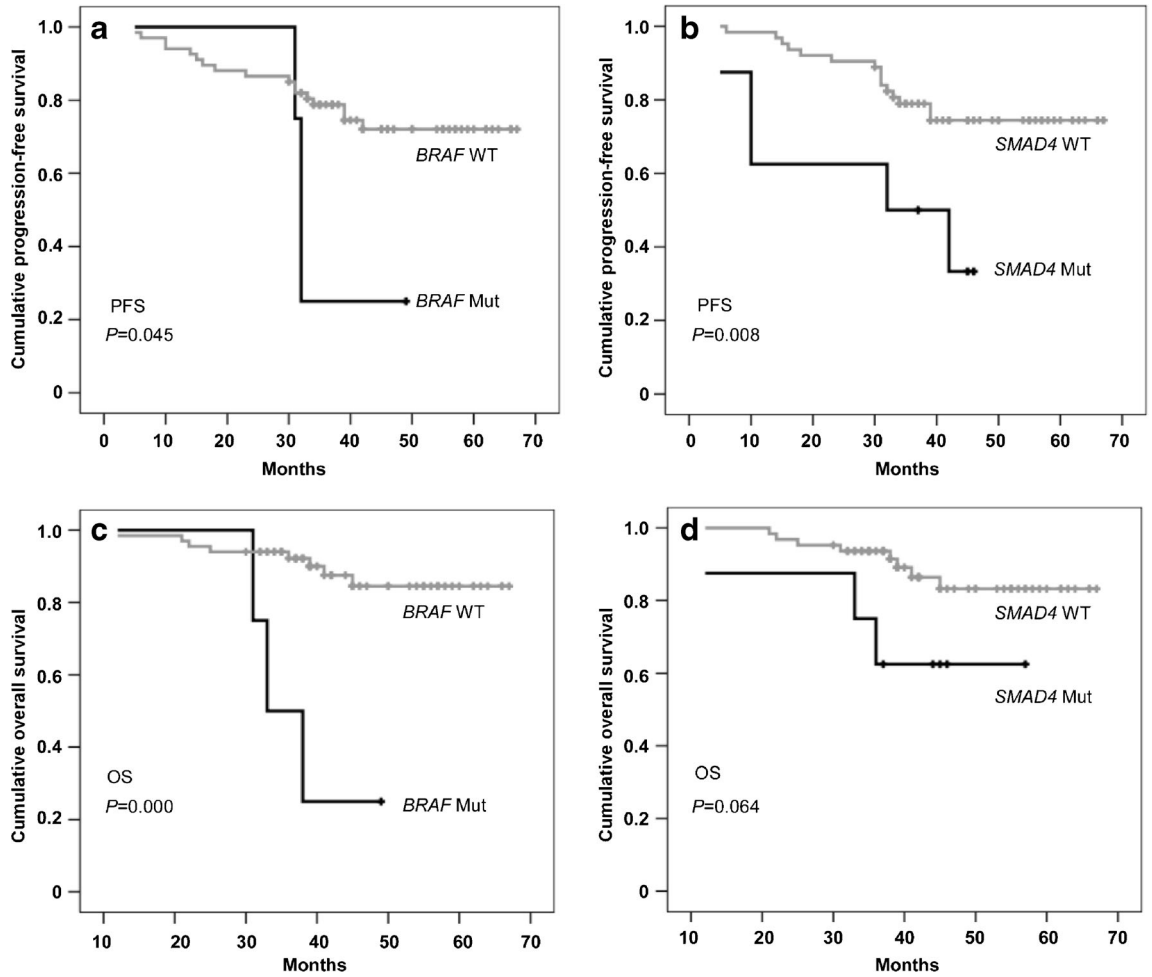

Fig. 3 Progression-free survival and overall survival by *BRAF* status and *SMAD4* status in this cohort of patients. (**a**) and (**b**) show the progression free survival (PFS). (**c**) and (**d**) show the overall survival (OS). Mut, mutation; WT, wild-type

furthermore, poor prognosis. Larger cohort of patients is needed for analysis in the future.

KRAS is a broadly studied gene in CRC. The patient who has *KRAS* mutation would not benefit from anti-*EGFR* antibodies cetuximab and panitumumab [7]. For the function as a biomarker for tumor response in rectal cancer treated with nCRT, different previous studies obtained different results. Some studies found *KRAS* mutations were more common in non-pCR tumors [3], or *KRAS* codon 13 mutations were associated with poor tumor regression response [6, 8]. However, some studies demonstrated *KRAS* mutation was not a predictive factor for either pathologic response or downstaging [4, 5, 9, 10]. In our study, *KRAS* mutation status was not a predictive marker for non-pCR patients or for T category downstage. Our data confirmed the finding proposed from a previous study that there was no tumor with pCR harboring codon 13 mutation [8]. Furthermore, we found mutations in some extend codons only occurring in TRG 2–3 tumors.

Another interesting finding was a case (case number 64) carrying *KRAS* (p. Q22K) and *BRAF* (p. D594G) at the same time. This result contradicts the previous idea that *KRAS* and *BRAF* mutations are mutually exclusive [18, 19]. However, the *KRAS* gene mutation was only found in the post-treatment tissue, not in the pre-treatment biopsy tissue, which might be explained by heterogeneity of the tumor or evolution of the tumor after nCRT. Whether these two mutations co-exist in single tumor cells or exist in different tumor populations, still needed further study.

*PIK3*CA and *PTEN* are key components of *PI3K*/*PTEN*/*AKT* signaling pathway which participate in multiple biological cellular processes. Even the prognostic role and predictive role to anti-*EGFR* antibody therapy of *PIK3*CA and *PTEN* gene status are not well established at present in CRC [20, 21]. The patients with CRC tumors which have *PIK3CA* mutation would have improved survival by using aspirin after diagnosis [22]. In this study, *PIK3*CA had higher mutational frequencies in TRG 3 tumors (25%) than in TRG 0–2 tumors (6.5%), and there was only one TRG 3 tumor that carried *PTEN* mutation. However, more cases are needed for further analysis to clarify the correlations between these genetic mutations and tumor response to nCRT because no significant result was obtained in this study.

TP53, known as molecular switch which can control the fate of cells, is one of the most famous tumor repressors, which is broadly studied in many malignancies. *TP53* is not a prognostic marker, while *TP53* wild-type might be a predictor for benefit from anti-*EGFR* antibody therapy in CRC [23]. Even though *TP53* mutation could not be a predictor to nCRT in some earlier studies [3, 10], it could predict stable T stage after short-term radiotherapy [24]. Indeed, our results demonstrated that *TP53* was not a predictor because the mutational frequencies of *TP53* were similar among different TRG tumors. Moreover, *TP53* mutation status was not associated with T stage reduction in our study, which yielded a different result from the previous study. More cases are needed to be analyzed in order to identify the relationship between *TP53* mutation and nCRT and between *TP53* status and T category downstaging.

There were some cases that had mismatching mutational results in pre- and post-nCRT tissues. More genetic mutations in pre-treatment biopsies might be interpreted as due to some surgical samples not being fixed well during the specimen processing, which may interfere with the test results. Another reason for this mismatch might be due to some cancer cells harboring these genetic mutations which were eliminated during nCRT. The extra *PIK3CA* (p. E726G) and *KRAS* (p. Q22K) mutation that was found in the post-treatment surgical sample may be caused by additional gene changes after the treatment or the evolvement of the tumor pathogenesis. Further researches will be performed to confirm all these explanations.

MMR deficiency or a high level of microsatellite instability (MSI-H) status is validated as a good prognosis marker for stage II CRC, and the patients would not likely to respond to 5-fluoroucil [11]. Rarely was a previous study focused on the function of MMR/MSI in nCRT. A report alleged MSI status could not be altered after neoadjuvant therapy [25]. In our study, MMR/MSI status might not influence tumor response to nCRT in LARCs.

In summary, we found that *BRAF* and *SMAD4* gene mutation might be important molecular markers to predict poor response to nCRT, by taking NGS sequencing from a group of LARCs. Furthermore, the patients whose tumors harbored *BRAF* mutation showed shorter PFS and OS. The patients with *SMAD4*-mutated tumors had shorter PFS. *KRAS* gene mutations of some extend codons were only found in TRG 2–3 tumors. However, *KRAS*, *PIK3CA*, *TP53*, and MMR could not be used as predictors for nCRT. Our study provides an insight to help identify the patients who would poorly respond to nCRT and the patients who might have poor prognosis. However, larger series of cases are needed to confirm these findings in the near future.

Authors' contributions DJ and XW designed the study; YW, MX, JZ, and LS conducted experiments; DJ, XW, WM, and DH analyzed and interpreted the data; DJ, DP, and KL wrote the manuscript. All authors have read and approved the manuscript.

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Compliance with ethical standards The authors declare that all the cases used in this study had informed consent from the patients of using their tissues.

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

Ethical responsibility of authors' section All individuals listed as co-authors of the manuscript qualify for every one of the four criteria listed in the ICMJE recommendation for qualification of authorship.

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