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A phase II clinical trial of toripalimab in advanced solid tumors with polymerase epsilon/polymerase delta (*POLE/POLE*) mutation

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Patients carrying mutations in polymerase epsilon/polymerase delta have shown positive responses to immune checkpoint inhibitors. Yet, prospective trials exploring the efficacy in those with polymerase epsilon/polymerase delta mutations are still lacking. A phase II clinical trial was initiated to evaluate the efficacy of toripalimab, a humanized IgG4K monoclonal antibody to human PD-1, in patients with advanced solid tumors with unselected polymerase epsilon/polymerase delta mutations but without microsatellite instability-high. A total of 15 patients were enrolled, 14 of whom were assessed for treatment efficacy. There was a 21.4% overall response rate, with a disease control rate of 57.1%. The median overall survival and median progression-free survival were 17.9 (95% CI 13.5-not reach) months and 2.5 (95% CI 1.4-not reach) months, respectively. For patients with exonuclease domain mutations, the objective response rate was 66.7% (2/3), with a disease control rate of 66.7% (2/3). For those with non-exonuclease domain mutations, the rates were 9.1% (1/11) and 54.5% (6/11), respectively. Notably, patients with *PBRM1* gene mutations exhibited a high response rate to toripalimab at 75.0% (3/4). This study showed that neither the exonuclease domain mutations nor non-exonuclease domain mutations could fully predict the efficacy of immunotherapy, urging the need for more investigations to clarify potential immune sensitization differences within polymerase epsilon/polymerase delta mutation variants.

Signal Transduction and Targeted Therapy (2024)9:227

; <https://doi.org/10.1038/s41392-024-01939-5>

INTRODUCTION

Cancer stands as one of the primary causes of disease-related mortality in the world. Recent global cancer statistics show an alarming estimate of 20 million new cancer diagnoses and almost 10 million cancer-related fatalities each year, with these numbers on the increase.¹ While conventional treatments such as surgery, chemotherapy, and radiotherapy have advanced in enhancing the overall survival rates for certain cancers, the overall effectiveness remains inadequate for the majority of cancers. Recently, immune checkpoint inhibitors (ICIs), specifically programmed cell death-1/programmed cell death-ligand 1 (PD-1/PD-L1) antibodies, have demonstrated considerable potential in treating various types of cancer such as melanoma, non-small cell lung cancer, and renal cell cancer. However, the efficacy of ICIs is relatively low in most tumor types, and varies markedly between unselected and selectively treated patients.² Therefore, it is important to identify specific biomarkers to distinguish patients who could truly benefit from ICIs.

Various possible indicators for predicting the effectiveness of ICI treatment have been discovered, such as high levels of

microsatellite instability/mismatch repair deficiency (MSI-H/dMMR) or high tumor mutation burden (TMB-H), as well as increased expression of PD-L1 in certain tumors.^{2,3} In a study conducted by Le et al., 53% of patients with dMMR cancers in various tumor types showed a positive response, with a complete response seen in 21% of patients.⁴ Nevertheless, the occurrence of MSI-H/dMMR is limited in clinical practice. TMB is also a predictive biomarker for the efficacy of ICI treatment in different cancers. In the phase 2 KEYNOTE-158 study, patients with TMB-H status (i.e. TMB ≥ 10 mutations per megabase) demonstrated a greater response rate than those with TMB-low (TMB-L) status (29% vs 6%, respectively).⁵ Despite the observed association between TMB and response to immunotherapy, TMB alone does not discriminate responders clearly. There are some patients with TMB-L status responding to ICIs and vice versa. Prior research has also identified additional possible predictive biomarkers, including tumor-infiltrating lymphocyte density, T-cell receptor clonality, peripheral blood markers, immune gene signatures, transcriptomic and epigenetic signatures, commensal microbiota, etc.^{2,6}

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Received: 14 December 2023 Revised: 24 June 2024 Accepted: 29 July 2024

Published online: 02 September 2024

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Like MSI/MMR and TMB, these biomarkers have their own limitations. Hence, there is a requirement for improved biomarkers to differentiate patients who may benefit from immunotherapy and those who may not.

The Polymerase epsilon (*POLE*) and polymerase delta 1 (*POLD1*) genes are responsible for encoding the main catalytic subunit of the DNA polymerase Pol ϵ and Pol δ ,⁷ which are involved in different proofreading and DNA repair processes such as nucleotide excision, double-strand break repair, base excision, and mismatch repair.⁸ The exonuclease region of Pol ϵ and Pol δ is responsible for identifying and eliminating mismatch bases formed during DNA replication. As a result, mutations in the exonuclease region of *POLE/POLD1* genes (*POL*-EDMs) lead to the loss of proofreading ability, leading to an increase in genetic mutations and the creation of neoantigens in the cell. A growing number of studies have reported that damaging germline or somatic mutations in *POLE/POLD1* genes may lead to genomic instability, heightened mutation rates, and carcinogenesis of multiple malignancies.^{9–13} Therefore, mutations of the *POLE/POLD1* (*POL*-MUTs) may enhance the sensitivity of tumors to immunotherapy and have the potential to act as a biomarker of ICI therapy.

In a previous report, our team indicated that *POL*-MUTs could be used as an independent predictive marker for the effectiveness of ICI treatment in different types of cancer.¹⁴ In this comprehensive analysis of a vast dataset, patients with *POL*-MUTs had a higher TMB compared to those without these mutations. Patients in the ICI treatment group who had *POL*-MUTs experienced markedly improved overall survival compared to those without these mutations (34 months vs 18 months, respectively). It should be noted that *POL*-MUTs outside of the exonuclease domain (*POL*-non-EDMs) also showed a comparable correlation with the overall survival of patients who received ICI therapy. Our further investigation revealed that domain location did not determine the predictive significance of *POL*-MUTs. Within the combined groups of 2862 cancer patients treated with immunotherapy, we discovered that both unselected *POL*-non-EDMs and *POL*-non-EDMs served as biomarkers for identifying patients who were likely to benefit from ICI therapy in addition to *POL*-EDMs. Patients with *POL*-non-EDM achieved a significantly higher response rate than the patients with wild type (58.7% vs 23.8%, respectively). *POL*-non-EDM was also linked to an immune response, even in patients with low TMB status.¹⁵ Despite this, there is still debate over the potential benefits of immunotherapy for patients with *POL*-non-EDMs. Some researchers suggested that tumors would only react to anti-PD-1 monotherapy if they had particular *POLE* pathogenic mutations in the DNA binding or catalytic site of the exonuclease domain.¹⁶ Since the majority of *POL*-MUTs were located outside the exonuclease domain, it is essential to investigate the effectiveness of ICIs in treating this patient subgroup. For this purpose, we carried out a prospective phase II clinical trial with a single arm to examine the effectiveness of toripalimab, a humanized IgG4-blocking monoclonal antibody targeting PD-1, in patients with advanced solid cancer and *POL*-MUTs who are not MSI-H.

RESULTS

Patients and treatments

Between April 2019 and August 2023, a total of 15 patients diagnosed with locally advanced or metastatic colorectal adenocarcinoma, colorectal sarcoma, renal medullary carcinoma, malignant peripheral nerve sheath tumor, cervical cancer, endometrial cancer, and hepatocellular carcinoma were enrolled. All patients had confirmed germline or somatic *POL*-MUTs before enrollment. Their median age was 50 (range, 24 to 72) years. The majority of patients were diagnosed with stage IV disease and had undergone a minimum of two prior treatment regimens. Seven patients had

Table 1. Demographic and Baseline Characteristics of the Patients

Characteristic		No. of patients % (n = 15)
Sex	Male	5 (33.3)
	Female	10 (66.7)
Age, years	Median	50
	Range	24–72
ECOG PS	0	7 (46.7)
	1	8 (53.3)
BMI	Median	21.7
	Range	18.0–27.6
Cancer type	Colorectal adenocarcinoma	9 (60.0)
	Colorectal sarcoma	1 (6.7)
	Renal medullary carcinoma	1 (6.7)
	Malignant peripheral nerve sheath tumor	1 (6.7)
	Endometrial cancer	1 (6.7)
	Cervical cancer	1 (6.7)
	Hepatocellular carcinoma	1 (6.7)
Stage IV cancer		14 (93.3)
No. of metastasis sites	<2	8 (53.3)
	≥2	7 (46.7)
Liver metastases	Yes	6 (40.0)
	No	9 (60.0)
Previous systematic therapies	<2	4 (26.7)
	≥2	11 (73.3)
Previous resection of primary tumor		13 (86.7)
Previous radiation therapy		2 (13.3)
TMB, mut/MB	Median	6.6
	Range	1.1–203.3
Gene Mutation	<i>POLE</i>	10 (66.7)
	<i>POLD1</i>	4 (26.7)
	<i>POLE</i> & <i>POLD1</i>	1 (6.7)

two or more metastatic organs, and two-fifths had liver metastasis. Most of the patients (66.7%) had *POLE* mutation, and one had both *POLE* and *POLD1* mutation (Table 1 and Table 2). As of the latest follow-up, two patients had completed the two-year protocol treatment, while ten patients discontinued treatment due to disease progression, two because of grade three or higher treatment-related adverse events, and one for surgery without a radiographic assessment.

Anti-tumor activity

Response to toripalimab was evaluated in 14 patients who underwent post-baseline assessment. We observed an objective response in three patients (one complete response and two partial responses), and five patients had stable disease (Fig. 1 and Table 2). The overall response rate was 21.4% with a confidence interval of 95% between 4.7% and 50.8%, and the disease control rate was 57.1% with a confidence interval of 95% between 28.9% and 82.3%. Among the three responders, two patients demonstrated both an EDM and a non-EDM *POLE* mutation, with one showing a complete response and the other a partial response. The third patient (a partial response) had a germline non-EDM *POLD1* mutation. The study could advance to the second stage if there were at least 3 out of 18 cases showing objective responses in the first stage, leading to the enrollment of an additional 17

Table 2. Individual patient data

No.	Sex	age	Cancer type	TMB	Response	PFS (months)	OS (months)	POLE/ POLD1 mutation	Mutation alteration	cDNA change	Variant type	POL-EDM	Functional mutation	OncoKB curated alterations
SYSUCC01	F	55 yr	HCC	4.6	SD	4.9	6.1	POLE	p.H1440T	c.4318 C > T	missense	N	VUS	Unknown
SYSUCC02	F	65 yr	EC	1.1	SD	2.6	28.2	POLE	p.A252V (germline)	c.755 C > T	missense	N	VUS	Unknown
SYSUCC03	F	58 yr	CRC	2.8	PD	1.4	53.0	POLE	p.V533M	c.1597 G > A	missense	N	VUS	Unknown
SYSUCC04	F	24 yr	malignant peripheral nerve sheath tumor	25.1	NE	NA	17.9	POLE	p.I2070- K2072delIQR	c.6209- 6217delITTCAGAAAGA	DEL	N	VUS	Unknown
								POLE	p.T2273- E2275delITLE	c.6817- 6825delAACCTGGAG	DEL	N	VUS	Unknown
								POLD1	p.L523- L526delLRL	c.1566-1577del12	DEL	Y	VUS	Unknown
SYSUCC05	M	41 yr	CRC	2.6	SD	2.7	38.7	POLE	p.T1104M	c.3311 C > T	missense	N	VUS	Unknown
SYSUCC06	M	52 yr	CRC	5.3	PD	1.2	13.5	POLD1	p.T238M	c.713 C > T	missense	N	VUS	Unknown
SYSUCC07	F	64 yr	Renal medullary carcinoma	2.6	PR	39.9	40.2	POLD1	p.R682Q (germline)	c.2045 G > A	missense	N	VUS	Unknown
SYSUCC08	F	47 yr	CRC	92.8	PD	1.5	22.5	POLE	p.V411L	c.1231 G > T	missense	Y	Functional Mutation/ Hypermuation	Likely Oncogenic
SYSUCC09	F	35 yr	CRC	130.3	PR	24.8	39.4	POLE	p.S297F	c.890 C > T	missense	Y	Functional Mutation/ Hypermuation	Likely Oncogenic
SYSUCC10	M	45 yr	CRC	203.3	CR	25.0	33.4	POLE	p.S1906Y p.P286R	c.5717 C > A c.857 C > G	missense missense	N Y	VUS Functional Mutation/ Hypermuation	Unknown Likely Oncogenic
SYSUCC11	F	72 yr	Cervical cancer	20.2	PD	1.3	17.5	POLD1	p.F1907L	c.5721 C > A	missense	N	VUS	Unknown
SYSUCC12	F	50 yr	CRC	5.3	SD	2.6	14.4	POLE	p.S1034F	c.3101 C > T	missense	N	VUS	Unknown
SYSUCC13	F	48 yr	CRC	16.3	PD	1.4	5.0	POLE	p.Q520P	c.1559 A > C	missense	N	VUS	Unknown
SYSUCC14	M	56 yr	CRC	6.6	PD	0.4	0.4	POLD1	p.E575K p.R978C	c.1723 G > A c.2933 C > T	missense missense	N N	VUS VUS	Unknown Unknown
SYSUCC15	M	39 yr	CRC	6.7	SD	2.5	8.7	POLE	p.I1739V	c.5215 A > G	missense	N	VUS	Unknown

cDNA complementary DNA, CR complete response, CRC colorectal cancer, EC endometrial cancer, F female, HCC hepatocellular carcinoma, M male, N no, OS overall survival, PFS progression-free survival, POL-EDM POL-EDM exonuclease domain mutation, PR partial response, SD stable disease, TMB-H high tumor mutational burden, TMB-L low tumor mutational burden, VUS variant of unknown significance, Y yes

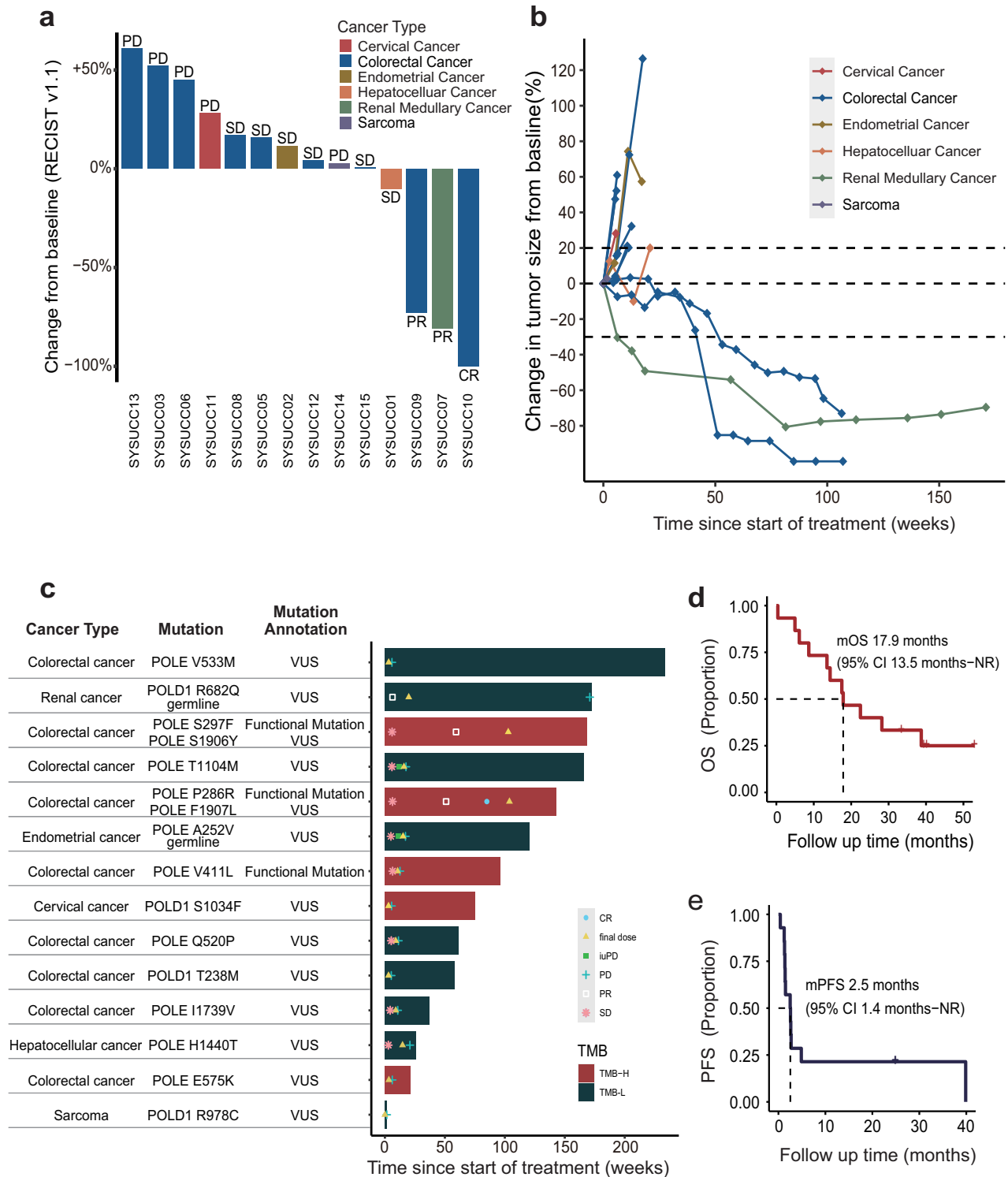


Fig. 1 Tumor response assessment and Kaplan-Meier survival plots. **a** Waterfall plot depicting maximum changes in tumor size from baseline of the 14 evaluated patients. **b** Spider plot depicting longitudinal changes in individual tumor measurements over time for the 14 evaluated patients. **c** Swimmer plot depicting treatment exposure, clinical response and survival of the 14 evaluated patients. **d** Kaplan-Meier plot of overall survival of the 15 investigated patients. **e** Kaplan-Meier plot of the progression-free survival of the 15 patients

patients. Despite the high disease control rate of 54.5% in patients with *POL*-non-EDMs, the overall response rate was only 9.1%. These results indicated that this group of patients needs more intensive treatment. Following conversations with the data monitoring committee and researchers, it was determined that the initial findings of the ongoing study would be disclosed, and a

new clinical trial would be organized to investigate the effectiveness of combining immunotherapy with chemotherapy in patients with *POL*-non-EDMs.

Figure 1a–c present the overall results stratified by TMB, cancer type, and *POL* functional mutation/hypermutation.¹⁷ The median overall survival was 17.9 months, with a 95% confidence interval

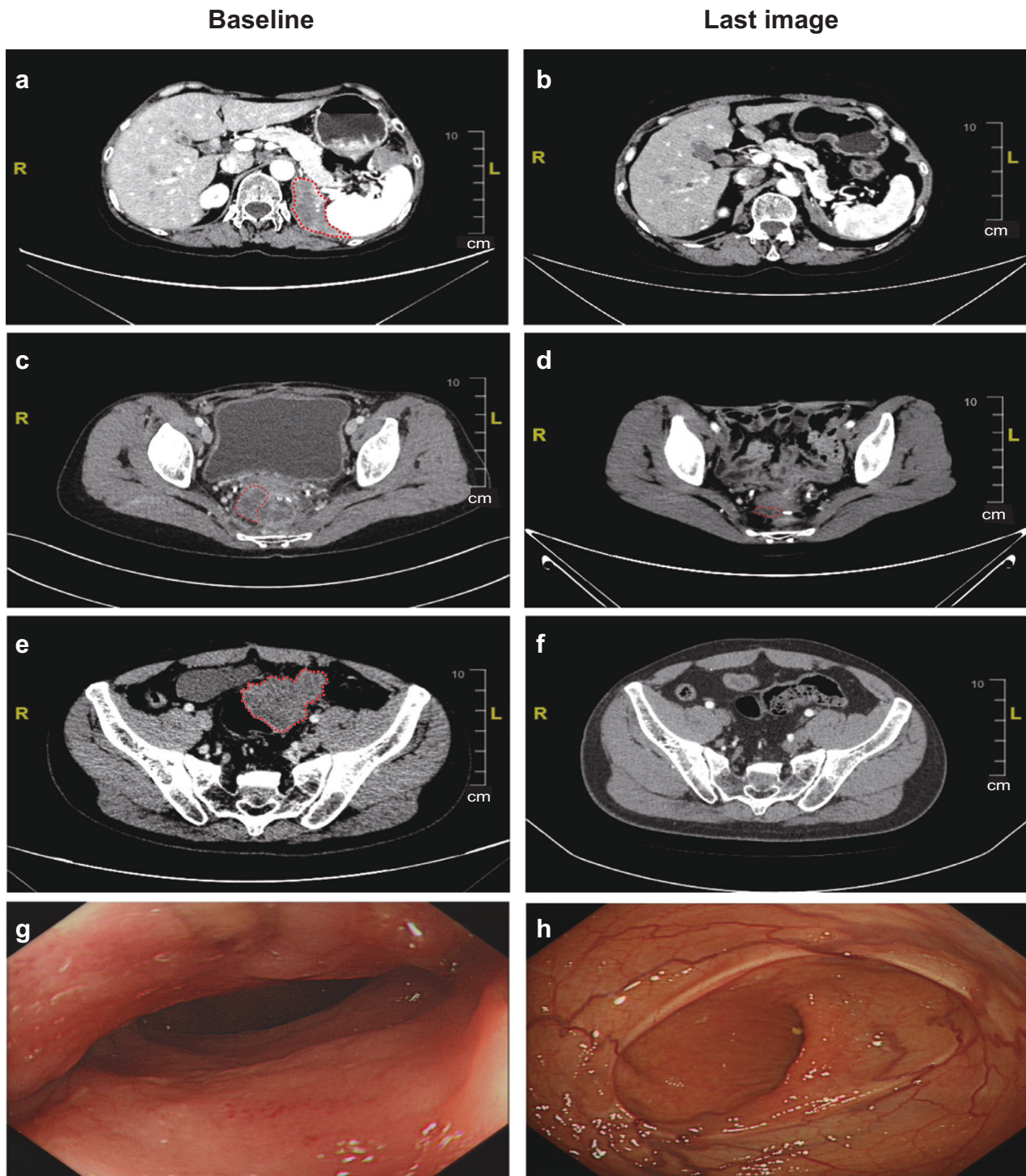


Fig. 2 Objective tumor responses in three patients with metastatic solid tumor. CT scan showing peritoneal metastasis and its evolution between baseline (**a**) and after toripalimab (**b**) of patient SYSUCC07 with metastatic renal medullary carcinoma harboring R682Q germline *POLD1* mutation. **c, d** The regression of the peritoneal metastasis of patient SYSUCC09 diagnosed with colon cancer harboring S297F and S1906Y *POLE* mutation. **e, f** The locally recurrent primary tumor and its evolution between pretreatment and after toripalimab in patient SYSUCC10 diagnosed with colon cancer harboring P286R and F1907L *POLE* mutation, who achieved complete response. **g, h** The colonoscopy image at baseline and after treatment of patient SYSUCC10, respectively

of 13.5 to not reached (NR), as shown in Fig. 1d. The median progression-free survival was 2.5 months, with a 95% confidence interval of 1.4 to NR, as shown in Fig. 1e. Figure 2 illustrates the baseline and post-treatment examinations of three patients with objective tumor responses. Patient SYSUCC07, diagnosed with R682Q *POLD1* germline mutant renal medullary carcinoma,

achieved partial response after two doses of toripalimab. Despite discontinuing treatment after seven cycles due to grade three treatment-related pneumonitis, the patient continued to exhibit a persisting partial response with a progression-free survival of 39.9 months (Fig. 2a, b). Patient SYSUCC09, who had local recurrent colon cancer with S297F and S1906Y *POLE* mutations

and had previously received two lines of palliative systemic treatment, also achieved a partial response after 18 doses of toripalimab. She has completed 2-year treatments with a persisting partial response at the last follow-up (Fig. 2c, d). One patient (SYSUCC10) achieved a complete response after receiving protocol treatment. This 45-year-old male had local recurrent colon cancer and liver metastasis with P286R and F1907L *POLE* mutations. After standard palliative chemotherapy, he was enrolled in this clinical trial. He achieved a partial response after receiving 14 doses of toripalimab and a complete response after 26 cycles of treatment, with both colonoscopy and PET/CT scan confirming no tumor residue. At the last follow-up, he had completed two-year treatments and achieved a confirmed complete response (Fig. 2e–h).

Patients SYSUCC02 and SYSUCC06 were assessed with progressive diseases during the second and first radiological assessments, respectively. However, they were both clinically stable with preserved performance status. After thorough evaluation, investigators considered that the patients may continue to benefit from the study treatment. Both patients, who were subsequently reassessed as having stable disease, received additional doses of toripalimab before disease progression—SYSUCC02 received four more doses, and SYSUCC06 received eight more doses.

Treatment-related toxicities

Among the 15 patients, nine (60.0%) reported at least one treatment-related adverse event: three (20.0%) experienced grade three or higher adverse events, and one (6.7%) experienced grade five adverse events. One patient with colorectal sarcoma and liver metastasis died from hepatic hemorrhage and hepatic failure after one cycle of the protocol therapy, which was believed to be related to the treatment. The most common treatment-related adverse events included elevated transaminase (33.3%), anemia (20.0%), and hypothyroidism (20.0%). Grade three treatment-related adverse events comprised hepatic hemorrhage, hepatic failure, anorexia, diarrhea, and pneumonitis (Table 3).

POLE/POLD1 mutation and treatment response

POLE/POLD1 variants are detailed in Table 2 and Fig. 3. Of all 15 patients, ten had *POLE* mutation (one A252V variant was germline), four had *POLD1* mutation (one R682Q variant was germline), and one had malignant peripheral nerve sheath tumor with both *POLE* and *POLD1* mutations. Most patients had one *POLE* or *POLD1* mutation, while three had more than one mutation: one with

S297F and S1906Y *POLE* mutations, one with P286R and F1907L *POLE* mutations, one with L523-L526del *POLD1* mutation, I2070-K2072del and T2273-E2275del *POLE* mutations. Among three patients with more than one mutation, two had an objective response, and one did not undergo a CT scan after one dose of toripalimab.

Both *POL*-EDMs and *POL*-non-EDMs might obtain clinical benefits from immunotherapy. Patients with *POL*-EDMs had an overall response rate of 66.7% and a disease control rate of 66.7%, compared to 9.1% and 54.5% for *POL*-non-EDMs, respectively. Of all 15 patients, 12 had *POL*-non-EDMs, while four had *POL*-EDMs, including one with P286R *POLE* mutation, one with V411L *POLE* mutation, one with S297F *POLE* mutation, and one with L523-L526del *POLD1* mutation. Three patients with *POLE*-EDMs were likely oncogenic (annotated by OncoKB: <https://www.oncokb.org/>) or functional mutation/hypermethylation, two of them (66.7%) achieved an objective response (SYSUCC09 with PR and SYSUCC10 with CR), while all *POL*-non-EDMs were variants of unknown significance (VUS).

POL-related mutation signatures may reflect *POL* functional mutation status more accurately than *POL* point mutation. Ma X et al. constructed a model based on 4 *POL*-MUT signatures to identify tumors with functional *POL*-MUT sensitive to ICB.¹⁷ The 4 *POL*-MUT signatures include SBS10a, SBS10b, SBS20 and SBS14. To investigate the role of *POL*-MUT signatures in our trial, we compared the attribution of *POL*-MUT signatures between responders and non-responders. We found the mutations of all responders are dominated by *POL*-MUT signatures: *POL*-MUT signatures attributed to 55%, 40%, and 65% mutations in SYSUCC10, SYSUCC07, and SYSUCC09, respectively (Supplementary Fig. 1). As for the non-responders, most of them were dominated by other non-*POLE/POLD1* mutation-related signatures (e.g. SBS30). Only one patient with stable disease was dominated by *POL*-MUT signatures. Surprisingly, in one patient with partial response (SYSUCC09), *POL*-MUT signatures were eliminated after immunotherapy. These results indicated that the *POL*-MUT signatures might be favorable predictive factors for immunotherapy.

Patients with *POLE/POLD1* and *PBRM1* co-mutation respond better to immunotherapy

To delineate the profile of patients who responded to immunotherapy, we focused on genomic differences between responders and non-responders. Notably, four patients harbored *PBRM1* gene mutation, and three of them responded to immunotherapy (Fisher's Exact Test $P = 0.024$) (Fig. 3c). Acknowledging the scarcity of patients with concurrent *POL*-MUTs and *PBRM1* mutations in publicly available immunotherapy cohorts, we explored the role of *POL* and *PBRM1* co-mutation in two large pan-cancer cohorts: the MSKCC metastasis cancer cohort¹⁸ and the TCGA pan-cancer cohort.¹⁹ All patients in these cohorts were classified into four groups: both *POL* and *PBRM1* wild type (WT), only *POL*-MUT (*POL*-o), only *PBRM1* (*PBRM1*-o) and *POL*-*PBRM1* co-mutation (*POL*&*PBRM1*) respectively. In the MSKCC cohort, after removing MSI-H patients and adjusting age, gender, and cancer types, the *POL*&*PBRM1* group had the highest TMB value ($P < 0.001$) (Supplementary Fig. 2a, Supplementary Table 1).

Given the profound influence of the mutation site in *POL* on TMB, patients with *POL*-MUTs were stratified by the mutations in EDM and non-EDM. Supplementary Fig. 2b showed that patients with *PBRM1*&*POL* also harbored much higher TMB than those with *POL*-o regardless of the mutation location (EDM: $P < 0.001$, non-EDM: $P < 0.001$) (Supplementary Table 1). In the three cancer types with the most *POL*&*PBRM1* patients (colorectal cancer, endometrial cancer, and non-small cell lung cancer), the *POL*&*PBRM1* group also had the highest TMB among the four groups (Supplementary Fig. 2c). Higher TMB in these three cancer types with *POL*&*PBRM1* mutation was observed both in the EDM group and non-EDM

Table 3. Summary of treatment-related adverse events

Adverse events		All Grades (%)	Grade 3-5 (%)
Blood or lymphatic system disorders	Anemia	3 (20.0%)	0 (0.0%)
	Leukopenia	1 (6.7%)	0 (0.0%)
Gastrointestinal disorders	Anorexia	1 (6.7%)	1 (6.7%)
	Diarrhea	1 (6.7%)	1 (6.7%)
Hepatobiliary disorders	Elevated transaminase	5 (33.3%)	0 (0.0%)
	Hepatic failure	1 (6.7%)	1 (6.7%)
	Hepatic hemorrhage	1 (6.7%)	1 (6.7%)
Endocrine disorders	Hypothyroidism	3 (20.0%)	0 (0.0%)
Pulmonary disorders	Pneumonitis	1 (6.7%)	1 (6.7%)
Renal and urinary disorders	Proteinuria	2 (13.3%)	0 (0.0%)
Fatigue		2 (13.3%)	0 (0.0%)

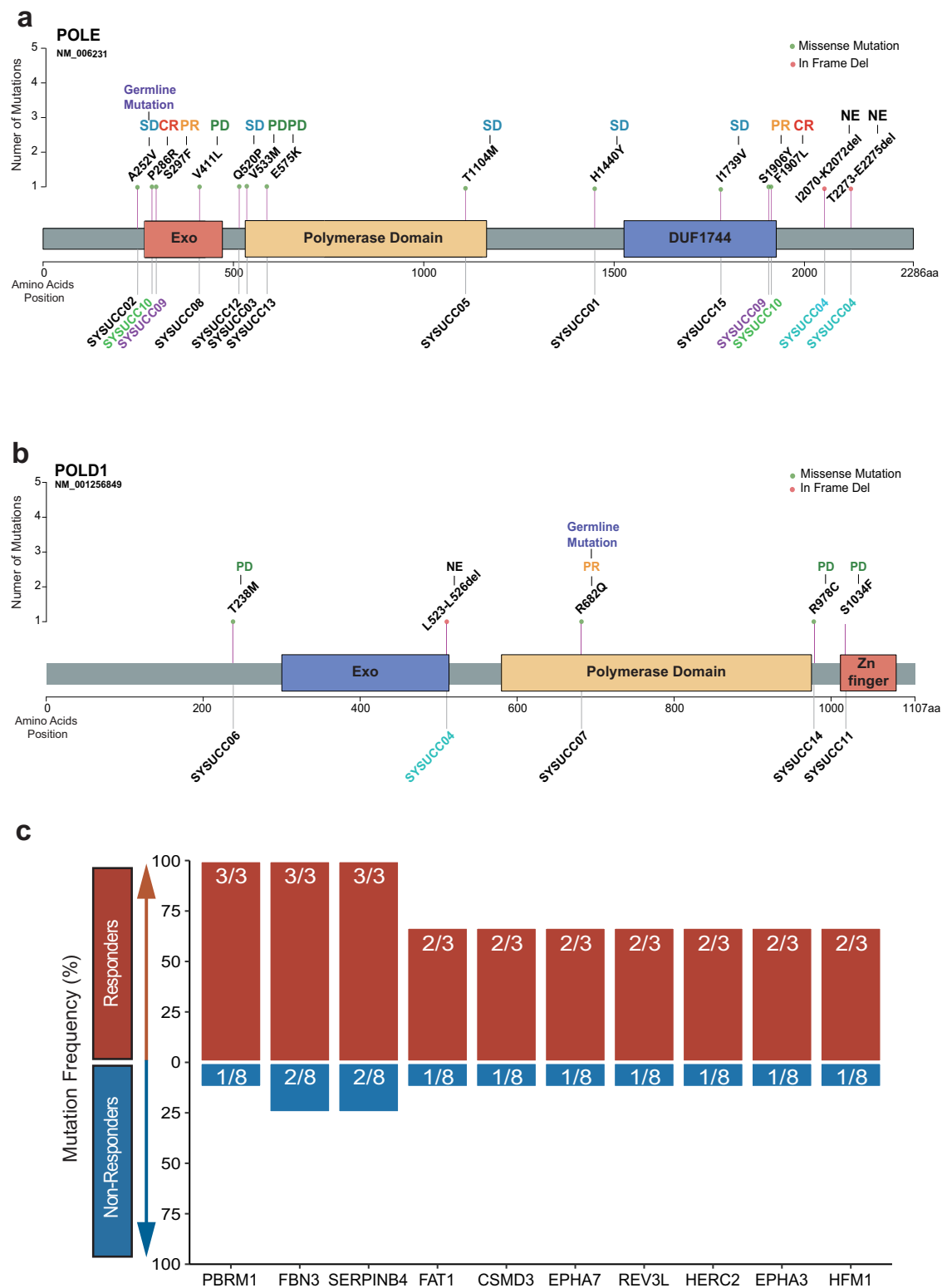


Fig. 3 *POLE/POLD1* Variant and Co-mutation Gene. **a**, **b** Individual responses for each *POLE/POLD1* variant. CR complete response, NE not evaluated, PR partial response, SD stable disease, PD progressive disease. **c** Mutation frequency of top 10 genes with the greatest mutation difference between responders and non-responders. The upper panel displays the mutation frequency among responders, and the lower panel presents the same for non-responders. The fraction in the bar indicates the specific number of patients carrying the mutation among responders or non-responders. Only *PBRM1* enriched in responders (Fisher's Exact Test $P < 0.05$)

group compared to those with POL-o (Supplementary Fig. 2d). Similar results were also observed in the TCGA pan-cancer cohort (Supplementary Fig. 3a–d, Supplementary Table 2). To validate the role of *POL&PBRM1* in immunotherapy further, we evaluated six transcriptional immunotherapy response signatures (T cell-inflamed gene expression profiles (T Cell GEP) signature,²⁰ Tertiary lymphoid structures (TLS) signature,²¹ interferon-gamma (IFNG) signature,²² Tumor Inflammation Signature (TIS),²³ Immunologic Constant of Rejection (ICR) signature²⁴ and cytotoxic T lymphocytes (CTL) signature²²) in TCGA. After adjusting for cancer types and filtering out MSI-High samples, the *POL&PBRM1* group had the highest signature scores in all response signatures (GEP: FDR < 0.001, IFG: FDR < 0.001, TLS: FDR = 0.088, TIS: FDR < 0.001, ICR: FDR < 0.001, CTL: FDR < 0.001) (Supplementary Fig. 4a, Supplementary Table 3). Next, we investigated the pathway and tumor microenvironment differences between *POL&PBRM1* and others. GSEA showed that immune-related-pathways, including the TCR signaling pathway, chemokine receptors bind chemokines, interferon gamma signaling, and antigen processing–cross presentation, were significantly activated in the *POL&PBRM1* group (Supplementary Fig. 4d–f). DNA repair and DNA double-strand break response were suppressed in the *POL&PBRM1* group. Given the significant heterogeneity of tumor microenvironment among various types of cancers, we focused on two cancer types with the most *POL&PBRM1* patients: colorectal cancer and endometrial cancer. In colorectal cancer, the *POL&PBRM1* group had more CD4⁺ memory T cells, and CD8⁺ T cells (CD8⁺ naive T cells, CD8⁺ effector memory T cells, and CD8⁺ central memory T cells) (Supplementary Fig. 4b). Similarly, more CD4⁺ T cells and CD8⁺ T cells were also observed in the *POL&PBRM1* group in endometrial cancer (Supplementary Fig. 4c). In summary, our findings revealed that patients with *POL&PBRM1* co-mutations exhibited significantly higher TMB, higher immunotherapy response signature scores, and more active anti-tumor immunity, suggesting a potentially favorable response to immunotherapy in these patients.

Disease progression accompanied by the increase of POL VAF. Previous studies^{25–27} have established that ctDNA monitoring serves as a potent instrument for treatment management, in which an increase in ctDNA levels during therapy indicates disease progression. Therefore, it is worthwhile to track the ctDNA dynamic change during treatment. In our cohort, four patients underwent ctDNA monitoring at more than three-time points (Supplementary Fig. 5, Supplementary Table 4). A dynamic change in POL maximal variant allele frequency (POL VAF) was consistent with tumor burden and treatment response for the patients with somatic POL-MUTs. In two responders (SYSUCC09 and SYSUCC10), POL VAF was detected at an extremely low level before treatment (0.63% for SYSUCC09 and 0.52% for SYSUCC10), and it remained undetectable throughout the clinical response. For patient SYSUCC06, the POL VAF remained at an almost undetectable level before the second dose of treatment, but then dramatically increased from 4.1% to 25.3% when the volume of the tumor increased, following which the patient was confirmed with progressive disease after ten doses of treatment. The POL VAF of patient SYSUCC07, who achieved partial response, did not change because the tumor harbored a *POLD1* germline mutation. According to the series ctDNA analysis, increasing ctDNA POL VAF levels may provide moderated valuable insights into the progression of the tumor in patients with POL-MUTs.

DISCUSSION

This prospective clinical trial evaluated the anti-tumor effect of toripalimab in non-MSI-H patients with unselective *POLE/POLD1* mutations. Three patients achieved an objective response, meeting the primary endpoint of the first stage with an overall response rate of 21.4%.

The findings indicated that patients with solid tumors plus *POL*-EDMs who received toripalimab exhibited a significant response rate (66.7%), consistent with previous studies.^{16,28} As *POL*-EDMs only account for a small proportion of all *POL*-MUTs, it is important to explore the predictive significance of *POL*-non-EDMs in immunotherapy. The recently reported Acsé Nivolumab trial focused on patients with advanced *POLE*-mutated solid tumors with mismatch repair-proficient phenotype. The study showed that monotherapy with nivolumab achieved a 38% overall response rate and 58% disease control rate at 12 weeks.¹⁶ Researchers suggested that immunotherapy could be advantageous exclusively for patients with variants impacting the proofreading function, DNA binding clusters, or the catalytic site, while no anti-tumor activity was noted in patients possessing *POLE* non-exonuclease domain mutations.¹⁶ However, our previous study discovered that both unselected *POL*-MUTs and *POL*-non-EDMs could act as biomarkers for identifying responders to immunotherapy.¹⁵ Notably, *POL*-non-EDMs were linked to an activated immune response, even in low-TMB tumors. This finding indicates that certain *POL*-MUTs might elicit an immune response through mechanisms not dependent on hypermutant status.¹⁵ In another study at the MD Anderson Cancer Center,²⁸ 82 patients who were diagnosed with *POLE* mutant advanced solid tumors were treated with PD-1/PD-L1 inhibitor with or without a CTLA-4 inhibitor, resulting in a 35% overall response rate and 60% disease control rate. Further analysis of the impact of *POLE* mutation locations revealed that patients with mutations in both the inside and outside of the exonuclease domain could benefit from ICI therapy (overall response rate, 33 and 36%; disease control rate, 78 and 58%, respectively). Dong et al. analyzed the MSK-IMPACT cohort of 1278 patients who were diagnosed with advanced cancers harboring low or intermediate TMB and received ICIs. The study showed that missense *POLE* mutations outside the exonuclease domain were predictive markers of ICI benefit.²⁹ In the current study, anti-PD-1 therapy was not exclusively ineffective among patients with *POL*-non-EDMs, though the response rate was relatively low. One patient with *POL*-non-EDM mutation achieved PR and had a PFS over 3 years, and one patient achieved disease control and had an approximately 5-month PFS, suggesting that some patients could still derive clinical benefits from ICI therapy. In addition, one patient with *POLE*-EDM and high TMB showed no response to PD-1 antibody and progressed quickly. Further investigation is needed to better classify this subgroup and identify patients who could truly benefit from immunotherapy.

This study revealed that *POL*-EDM or non-EDM status alone cannot fully predict the benefit of ICI treatment, highlighting the need for additional biomarkers. We found that patients carrying *PBRM1* mutation were enriched in responders. In pan-cancer cohorts, we discovered that patients carrying *POL* & *PBRM1* mutations may have better responses to ICIs. Previous studies showed patients with truncating or loss-of-function mutations of *PBRM1* benefited from ICI.^{30,31} BAF180, encoded by *PBRM1*, is a submodule of the PBAF, playing a key tumor suppressor role by regulating cellular differentiation, proliferation, and DNA repair.^{32,33} Mutation of BAF180 affects DNA repair in three ways: increasing the distance of centromeres, impairing the DNA post-replication repair pathway, and losing function to silence transcription near the site of damage.^{34,35} In addition to increasing TMB, Pan et al. discovered that *PBRM1* expression in tumor cells negatively correlated with T-cell cytotoxicity markers.³⁶ Loss of PBAF function increased tumor cell sensitivity to interferon- γ and *PBRM1*-deficient tumor triggered T cell-mediated killing more easily by activating interferon- γ than those with intact *PBRM1*.³⁶ If patients carried *PBRM1* and *POL*-MUTs simultaneously, massive numbers of mutations would be produced and could not be cleared in time because of the synergistic effect on the DNA repair system, leading to increasing TMB. At the same time, *PBRM1* mutation would up-regulate immune-related pathways and recruit more effector T cells. Therefore, patients with *PBRM1* and *POL*

co-mutation may respond better to ICIs. Moreover, the synergistic effect of *POL* & *PBRM1* mutation has sparked inspiration for a novel therapeutic strategy for patients with *POL*-MUTs: inhibiting key molecules of the DNA repair system, such as *PBRM1*, will further increase TMB, which holds the potential to enhance the effectiveness of immunotherapy.

Some limitations of this study should be acknowledged. First, the sample size was relatively small due to the low prevalence of *POL*-MUTs. Although the preliminary results showed the potential clinical benefits of ICI, more data from prospective studies are needed to confirm these findings. Second, selection bias might have existed as most of the cancers in this study were colorectal cancers. Third, blood samples were missing from several treatment cycles for some patients, and the treatment duration of some patients was too short due to disease progression, thereby affecting the assessment of ctDNA change during therapy. More ctDNA data is needed to validate the value of ctDNA *POL* VAF in clinical benefit prediction.

In summary, this study demonstrates that anti-PD-1 therapy elicited a favorable objective response in *POLE/POLD1* exonuclease domain mutant tumors and a good disease control rate in *POLE/POLD1* non-exonuclease domain mutant tumors, indicating the need to explore the efficacy of immunotherapy combined with chemotherapy in patients with *POL*-non-EDMs. Further classification of the *POL*-non-EDMs to identify the potential subgroups sensitive to immunotherapy is also a valuable area for investigation.

MATERIALS AND METHODS

Study design and patients

Investigators initiated a phase II clinical trial at Sun Yat-sen University Cancer Center in Guangzhou, China, and involved a single arm with an open-label design. The study criteria included patients between the ages of 18 and 75 with pathologically confirmed metastatic or unresectable solid tumors, microsatellite stability (MSS), microsatellite instability-low (MSI-L), or proficient mismatch repair (pMMR), germline or somatic *POLE/POLD1* mutation (excluding synonymous mutation), refractory or intolerant to systemic chemotherapy or target therapy, adequate organ function, and an Eastern Cooperative Oncology Group (ECOG) performance status of 2 or less. Major exclusion criteria included prior use of PD-1 antibody or other immune checkpoint inhibitors, presence of suspected or confirmed brain metastasis, history of another cancer, autoimmune disease, or long-term immunosuppressant use, as well as HIV infection, active hepatitis, severe infections needing systemic antibiotics, or unexplained fever. Although Shanghai Junshi Biosciences donated the study drug, they did not participate in any aspect of data collection or analysis.

The study protocol was approved by the institutional review board (B2018-153-03) and conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines. All patients provided written informed consent for participation before enrollment. This study has been registered on "<https://clinicaltrials.gov/>" (identifier: NCT03810339).

Procedures

After enrollment, the patients received 240 mg of toripalimab through intravenous infusion every three weeks, with evaluations of their response every 6 weeks. At baseline and every six weeks until disease progression, computed tomography or magnetic resonance imaging was performed. Patients who had received at least one cycle of toripalimab and had undergone at least one post-baseline assessment were assessed for tumor response based on Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1). Patients with disease control were eligible to continue treatment for a maximum of two years unless they experienced intolerable adverse events, confirmed progressive disease or

withdrew consent. For patients assessed as pseudoprogression, treatment would continue until confirmed progressive disease.

Patients had safety monitoring visits at the end of each cycle, with follow-up visits at 30 days and 6 months after the last dose. Dose delays were allowed to manage grade three or four adverse events (National Cancer Institute Common Terminology Criteria for Adverse Events [NCI-CTCAE; version 5.0]). Dose reductions or dose escalations were not permitted.

Tumor tissue and plasma samples were collected for genomics and ctDNA analysis. Tissue samples preserved in formalin and embedded in paraffin were gathered for DNA sequencing of tumors with a 680-gene next-generation sequencing (NGS) panel that is commercially accessible.

Outcomes

The primary endpoint of the research was the overall response rate, which was defined as the percentage of patients who achieved either a complete response or partial response based on RECIST 1.1. Additional outcome measures consisted of disease control rate, overall survival, progression-free survival, and safety assessments. The disease control rate was determined by calculating the percentage of patients who achieved complete response, partial response, or stable disease as their best overall response. Overall survival was defined as the period from initial toripalimab administration until death, while progression-free survival was determined as the period from initial toripalimab administration until the occurrence of progressive disease or death, whichever came first. Adverse events were assessed for severity in patients who underwent at least one treatment cycle using NCI-CTCAE version 5.0.

DNA extraction and library construction

All tissue and blood samples were acquired with informed consent. In total, 56 samples of patients at baseline, post-treatment, and after disease progression were obtained for tissue DNA or circulating tumor DNA (ctDNA) analysis.

Blood samples were centrifuged within two hours of collection to isolate peripheral blood lymphocyte (PBL) debris and plasma. Next, circulating cell-free DNA (cfDNA) was isolated from the plasma with the QIAamp Circulating Nucleic Acid kit (QIAGEN), while genomic DNA (gDNA) was extracted from corresponding PBLs using the RelaxGene Blood DNA System (TianGen Biotech Co., Ltd., China). Tumor DNA was isolated from tumor tissue samples preserved in formalin and embedded in paraffin using the QIAamp DNA FFPE tissue kit from Qiagen. Extracted DNA was measured using Qubit 2.0 from Thermo Fisher Scientific in the USA in accordance with the manufacturer's instructions.

Initially, FFPE tumor tissue DNA and genomic DNA (gDNA) underwent fragmentation via enzymatic digestion using dsDNA Fragmentase from NEB. Subsequently, size selection of the DNA fragments (150–250 bp) was achieved utilizing Ampure XP beads (Beckman Coulter, Inc., Brea, CA, USA). The KAPA Library Preparation kit from Kapa Biosystems, Inc. in Wilmington, MA, USA, was used to create sequence libraries. This involved checking the DNA fragment concentration, repairing the ends, adding A-tails to the 3'-end, and performing PCR amplification following the manufacturer's instructions.

Targeted capture and sequencing

Targeted capture was executed utilizing DNA probes specifically designed to encompass all exons and select intron regions across 680 cancer-related genes, in addition to 117 microsatellite instability sites. The hybridization of the amplified sample libraries and the SeqCap EZ Library was used following the manufacturer's instructions. Following the hybrid selection process, the DNA fragments that were captured underwent amplification with 1× KAPA HiFi Hot Start Ready Mix and Post-LM-PCR Oligos. Afterward, the two reactions were then pooled and purified using Agencourt

AMPure XP beads. Next, the libraries underwent sequencing with 150 bp paired-end reads on the Illumina NovaSeq 6000 platform (Illumina).

Raw data processing and alignment

Raw sequencing data were pre-processed by Fastp (version 0.18.0), which encompassed adaptor trimming, meticulous removal of low-quality and short reads to ensure data integrity.³⁷ The resulting clean reads were then precisely aligned to the human reference genome hg19 genome (GRCh37) using the Burrows-Wheeler Aligner v0.7.15 r1140 with default settings.³⁸ Subsequently, Gencore (version 0.12.0) was employed to eliminate duplicate reads, streamlining the dataset. Finally, Samtools (version 0.1.19) was utilized to generate mpileup files, focusing solely on paired reads with a mapping quality score ≥ 60 , ensuring high confidence in variant calling.^{39,40}

Mutation calling, filtering and annotation

VarScan2 (version 2.3.8) was utilized to identify single nucleotide variants (SNVs) and short insertions/deletions (indels).⁴¹ After deduplication, the average sequencing depth achieved for cfDNA and tumor tissue DNA were $\geq 500\times$ and $\geq 1500\times$, respectively. For cfDNA, stringent filter criteria were applied, including a variant allele frequency (VAF) threshold of 0.1%, requiring at least five unique reads with at least one on each strand for somatic variants (SNVs or indels), and exclusion of variants with a mutant allelic frequency greater than 0.5% in the paired normal sample (PBLs). Furthermore, cfDNA SNVs and indels were subjected to background polishing using healthy subject cfDNA samples to minimize false positives. For tumor tissue DNA, the filter criteria were adjusted with a VAF threshold of 2%, maintaining the requirement for at least five unique reads, with at least one read present on each strand, and $<0.5\%$ mutant allelic frequency in PBLs. An additional layer of quality control involved manual visual inspection using GenomeBrowse to eliminate potential artifacts. Subsequently, ANNOVAR (version 2018-04-16) was employed to annotate all identified SNVs and indels.⁴² Blood tumor mutational burden (bTMB) was estimated from ctDNA by calculating the total number of SNVs and indels with a VAF exceeding 0.5% per million bases. Tumor mutational burden (TMB) was estimated for tumor tissue DNA based on SNVs and indels with a VAF above 5% per million bases. MSIsensor2 (<https://github.com/niu-lab/msisensor2>) was applied to assess the microsatellite instability (MSI) status.

Mutation signature analysis

Additional 14 tumor samples from the 15 patients were collected for NGS, among which 11 were obtained before treatment, and three were obtained at the end of treatment. One sample with less than 10 mutations was filtered.¹⁷ The mutation data were transformed into a trinucleotide context matrix and then normalized, taking into account the relative abundance of each trinucleotide context category within the HaploX gene panel. The normalized trinucleotide context matrices were refitted to 60 COSMIC V3 Single Base Signature (SBS) using the deconstructSigs R package. The SBS signatures with no contribution to all samples were removed, and the contributions of the rest SBS signatures were shown by the heatmap R package.

External cohort collection

Mutation data of MSKCC metastasis cancer cohort¹⁸ were obtained from cBioPortal (<https://www.cbioportal.org/datasets>). TCGA pan-cancer mutation data and RNA-sequencing data were downloaded from Xena (<https://xenabrowser.net/>).¹⁹

TMB, survival, and transcriptome analysis

We utilized a standard generalized linear regression model, incorporating age, gender, and cancer type as covariates, to investigate the relationship between TMB and gene mutation

groups. T-test was applied to compare the TMB value in groups. The R packages “survival” and “survminer” were used to perform survival analysis. The multivariate Cox-proportional hazard modeling was utilized to estimate the hazard ratio among gene mutation groups. Cancer type, age, and gender were set as the covariates in the model. The immune response signature scores were estimated by ssGSEA in the GSVA R package. A generalized linear regression model, similar to TMB, was built to validate the relation between these signature scores and gene mutation groups. Different gene expression analysis was performed by DESeq2. We performed GSEA analysis on the different gene expression results obtained from DESeq2 based on the clusterProfiler R package. Tumor microenvironment was estimated by xCell.

Quantification and statistical analysis

Simon’s two-stage design was chosen to calculate sample size with $P_0 = 0.1$ (as the null hypothesis) and $P_1 = 0.3$ (as the alternative hypothesis), at a significance level of 0.05 and a power of 90%. In the first stage, 18 patients were needed, and the study had to stop if two or fewer patients achieved an objective response. If three or more patients demonstrated an objective response, another 17 patients were to be recruited in the second stage. If more than six of these 35 patients achieved an objective response, the regimen was considered effective.

Overall survival and progression-free survival were analyzed using the Kaplan–Meier method and the stratified Log-rank test to estimate medians and 95% CIs. The overall response rate and disease control rate were calculated using the exact Clopper-Pearson CIs. Statistical analyses were performed using the R 4.0.2 software.

DATA AVAILABILITY

The unprocessed sequence information mentioned in this article has been stored in the Genome Sequence Archive⁴³ at the National Genomics Data Center,⁴⁴ China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA007914), which can be accessed by the public at <https://ngdc.cncb.ac.cn/gsa-human>. The analysis code can be found at https://github.com/runjie-huang/POL_Mut. For further details needed to reassess the data presented in the article, the lead contact can provide upon reasonable request.

ACKNOWLEDGEMENTS

We thank the patients, their families, the study investigators, the coordinators, and the nurses. The study drug (toripalimab) was provided by Shanghai Junshi Biosciences. We thank HaploX Biotechnology for NGS testing and analysis. We thank Sharvesh Raj Seeruttun for providing assistance in editing this manuscript. This work was supported by National Natural Science Foundation of China (82321003, 81930065, 82173128); CAMS Innovation Fund for Medical Sciences (CIFMS) (2019-12M-5-036); Science and Technology Program of Guangzhou (202206080011); Medical Scientific Research Foundation of Guangdong Province (A2022054); Cancer Innovative Research Program of Sun Yat-sen University Cancer Center (CIRP-SYUCC-0004); Sun Yat-sen University Clinical Research 5010 Program (84000-31630002); the Fundamental Research Funds for the Central Universities, Sun Yat-sen University (22ykjl06); Young Talents Program of Sun Yat-sen University Cancer Center (YTP-SYUCC-0018); CSCO foundation (Y-QL202202-0089).

AUTHOR CONTRIBUTIONS

R.X., F.W. and Y.S. conceived, designed and supervised the study. Y.J., W.G., Z.W., Y.S., Y.L., J.X. and X.Z. contributed to patient selection, recruitment, and conducting the trial. Y.J., R.H., W.G., Z.M. and Z.W. contributed to data collection. Y.J., R.H., W.G., Z.M., S.C., M.L., Q.Z., F.W. and R.X. contributed to data analysis. Y.J., R.H., W.G., Z.M., F.W. and R.X. contributed to writing the paper. All authors have read and approved the article.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41392-024-01939-5>.

Competing interests S.C. and M.L. were hired by HaploX Biotechnology. The remaining authors declare no competing interests.

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