Original Article

Assessment of *POLE* and *POLD1* mutations as prognosis and immunotherapy biomarkers for stomach adenocarcinoma

Mingyu Zhu[1,2#^], Haiyan Cui[1,2#], Lu Zhang[1,2#^], Kuo Zhao[3], Xiaochen Jia[4], Hao Jin[1,2]

[1]Tianjin Medical University Cancer Institute & Hospital, Tianjin, China; [2]Cancer Precise Diagnosis Center, Tianjin Cancer Hospital Airport Hospital, Tianjin, China; [3]Department of Medical Oncology, Tianjin Cancer Hospital Airport Hospital, Tianjin, China; [4]Department of Breast Oncology, Tianjin Cancer Hospital Airport Hospital, Tianjin, China

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[#]These authors contributed equally to this work.

Correspondence to: Lu Zhang. Tianjin Cancer Hospital Airport Hospital, No. 99, East 5th Road, Tianjin Airport Economic Zone, Tianjin, China. Email: 13920026618@163.com.

Background: Cancer patients with *POLE* or *POLD1* mutations may be excellent candidates for immune checkpoint inhibitors (ICIs) therapy and have favorable prognosis, but their potential in stomach adenocarcinoma (STAD) remains unknown. Therefore, the clinical significance of *POLE* and *POLD1* mutations in STAD was evaluated.

Methods: A summary of *POLE/POLD1* mutations and clinical characteristics was performed on all 613 STAD samples, from which 360 samples were screened for analysis of the potential clinical relevance of *POLE/POLD1* mutations to prognosis and immunotherapy.

Results: The total frequency of both *POLE* and *POLD1* mutations was 7.99% in STAD patients, correlating with an older age of onset and more frequently in the antrum anatomic subdivisions. Several genes that related to prognosis and immunotherapy also had high mutation frequencies in *POLE/POLD1*-mutant STADs. Furthermore, the STAD subgroup with *POLE/POLD1* mutations had longer progression free survival (PFS) and overall survival (OS) in the subpopulation under 80. More importantly, STAD patients with *POLE/POLD1* mutations exhibited adaptive immune resistance tumor microenvironment (TME) and deficient mismatch repair (dMMR) status, and possessed significantly higher PD-L1 expression level, higher tumor mutational load (TMB), higher microsatellite instability (MSI) percentage, and lower aneuploidy score, all of which may have potential implications for better ICIs treatment outcomes.

Conclusions: *POLE* and *POLD1* mutations are promising useful biomarkers to improve the clinical efficiency of practicing precision medicine in STAD patients, including as positive prognostic markers and predictive biomarkers of immunotherapy outcomes for STAD patients.

Keywords: *POLE*; *POLD1*; prognosis, immune checkpoint inhibitor (ICI); stomach adenocarcinoma (STAD)

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^ORCID: Mingyu Zhu, 0000-0002-8553-8058; Lu Zhang, 0000-0002-2074-8687.

Introduction

DNA polymerases ε and δ, the catalytic subunits of which are encoded by the *POLE* and *POLD1* genes respectively (1), are essential for proofreading and fidelity in DNA replication of the leading and lagging strands respectively (2-5). The fidelity of replication relies on three error avoidance mechanisms acting in series: nucleotide selectivity of replicative DNA polymerases, 3'-5' exonucleolytic proofreading, and post-replicative DNA mismatch repair (MMR) (6,7). The proper functioning of *POLE* and *POLD1* genes is essential to suppress gene mutations and tumorigenesis (8,9). Mutations in *POLE* and *POLD1* genes have been associated with high tumor mutational load (TMB) (10), prognosis (11-13), and clinical benefits of immunotherapy (14-17) in many cancer types, including endometrial cancer (EC), colorectal cancer (CRC), non-small cell lung cancer (NSCLC) and glioblastoma. However, the status of *POLE/POLD1* gene mutations and its clinical significance in stomach adenocarcinoma (STAD) remain unclear.

Immunotherapies based on immune checkpoint inhibitors (ICIs) have achieved remarkable clinical success and gained regulatory approval in many advanced cancers, including STAD (18-20). Gastric cancer is one of the most commonly diagnosed cancers, ranking fifth for incidence and fourth for mortality globally, and the prevalence is markedly higher in East Asia region (21). In China, it is the second most common malignancy and the third leading cause of cancer-related death, with an estimated of 403,000 new gastric cancer cases and 291,000 cancer deaths occurring in 2015 (22). STAD is the most common subtype of gastric cancer, accounting for more than 90% (23). Nowadays, PD-L1 expression, microsatellite instability (MSI)-H/deficient mismatch repair (dMMR) and TMB have been approved by the US Food and Drug Administration (FDA) as biomarkers for ICIs in the treatment of certain cancers (24-26). In addition, aneuploidy (27), Epstein-Barr virus (EBV) (28), T-cell inflamed gene profiling and interferon-γ gene signature (29,30), and circulating tumor DNA (ctDNA) (31,32) have also been reported as possible biomarkers to predict clinical outcomes of ICIs immunotherapy. Nevertheless, the assessment of these biomarkers has not reached a unified standard, and the multiplicity of these biomarkers makes us face selection difficulties in clinic. Moreover, responses to ICIs also do not fully correlate with any of these existing biomarkers (19,20), and only limited success in a minority of STAD patients has been reported in several clinical trials, with objective response rates (ORRs) of only 10–20% (20,30,33). To improve the efficacy of ICIs for STAD patients, the identification of novel accurate predictive biomarkers is becoming increasingly urgent and challenging.

In this study, we comprehensively analyzed the variations of *POLE* and *POLD1* genes in STAD patients, examined the impacts of these mutations on the prognosis of STAD patients, and we further explored the relationship between *POLE* and *POLD1* gene mutations and current immunotherapy biomarkers, including PD-L1 expression, TMB, aneuploidy, MSI, dMMR, and the tumor microenvironment (TME). Our research demonstrated the possibility of *POLE/POLD1* mutations as novel precise biomarkers for the overall prognosis and patient selection of ICIs immunotherapy in STAD patients. We present the following article in accordance with the REMARK reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-1601/rc).

Methods

Patient selection

The study population consisted of 613 STAD patients selected and retrieved from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov), Guo *et al.* (34) and Kakiuchi *et al.* (35) (Figure S1) for comprehensive integrated analysis. The cohort was obtained after removing duplicates and non-mutation information samples from cBioPortal database (https://www.cbioportal.org) (36,37). All 613 samples were summarized for *POLE/POLD1* mutations and clinical characteristics, from which a total of 360 samples with both somatic mutation, mRNA expression profiling, and copy number alteration data were screened for prognostic and immunotherapy biomarker analyses. *POLE/POLD1* mutations were defined as all mutations in coding regions except synonymous and intron mutations, including missense mutations, nonsense mutations, indels and splice mutations. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was deemed exempt from institutional board approval and patient informed consent was waived, due to the retrospective nature and publicly available data source of the study.

Demographic and clinicopathological variables

Baseline demographic and clinicopathological variables were treated as either categorical (e.g., grade, stage, sex)

or continuous (age) as appropriate. By comparing the relationship between *POLE/POLD1* mutations and these variables, the preference of these mutations was discussed.

TME analysis

We analyzed the mRNA expression levels of some cytotoxic T-cell markers (IFNG, GZMA, GZMB and GNLY) and effector cytokines (CXCL9, CXCL10 and STAT1) (12,38) through RNA-seq data (in FPKM format) to evaluate the immune activation status in STAD patients with *POLE/POLD1* mutations. In addition, according to the mRNA expression level of PD-L1 and the degree of lymphocytes infiltration in the tumor, we classified the tumor immune microenvironment of *POLE/POLD1*-mutant STADs (39,40). The mRNA expression level of CD8A was used to indicate the presence or absence of tumor-infiltrating lymphocytes (TILs), and positive PD-L1 and CD8A were defined as above-median expression.

Immunotherapy biomarkers analysis

Currently, the biomarkers used to predict the efficacy of ICIs therapy mainly include PD-L1 expression, TMB, MSI and MMR status. Moreover, tumor aneuploidy is also considered to be negatively related to patient response to immunotherapy (27). Therefore, we analyzed the relationship between these biomarkers and *POLE/POLD1* mutations to explore the possibility of using *POLE/POLD1* mutations to predict the efficacy of STAD immunotherapy.

TMB was calculated with the total number of nonsynonymous somatic mutations in coding sequence (CDS). All single nucleotide variants (SNVs) and indels were included. The expression of PD-L1 and MMR-related genes were evaluated through RNA-seq data (in FPKM format). Aneuploidy scores were obtained by calculating the sum of the number of chromosome arms that were amplified or deleted. MSI status had previously been determined in the cohort by standard techniques. The effects of *POLE/POLD1* mutations on these biomarkers were then comprehensively assessed.

Statistical analysis

We used the nonparametric Mann-Whitney test and Kruskal-Wallis test for all comparisons of continuous data, and analyzed the correlation between variables by Pearson rho. Categorical variables were compared using the Fisher exact test. For the analysis of the association of *POLE/POLD1* mutations with clinical outcomes, survival curves were plotted using the Kaplan-Meier method and compared by the log-rank test. The OS and PFS data evaluated by Response Evaluation Criteria in Solid Tumors, version 1.1 (RECISTv1.1) were available for 341 and 343 patients, respectively. PFS was defined as the time from treatment to the date of disease progression or death from any cause. OS was defined as the time from treatment to the date of death from any cause. All statistical tests were two-sided, and p value under 0.05 was considered statistically significant. Except where indicated, statistical tests were unadjusted. Statistical analyses were performed using SPSS 25.0 and Prism 8.0 (GraphPad, USA).

Results

POLE/POLD1 mutations and STAD patient characteristics

The status of *POLE/POLD1* mutations in STAD patients is summarized in Table S1. Across all 613 STAD patients, the mutation frequencies of *POLE* and *POLD1* genes were 6.04% (37 of 613) and 2.77% (17 of 613), respectively. There were 5 patients (0.82%) had both *POLE* and *POLD1* mutations. Overall, *POLE/POLD1* mutations were detected in 49 STAD patients (7.99%), irrespective of mutation site. Among the 360 samples selected, 31 cases had *POLE/POLD1* mutations, of which 24 cases had *POLE* mutations, 10 cases had *POLD1* mutations, and 3 cases had both *POLE* and *POLD1* mutations (Figure S2). All mutations, including P286 (41,42) and V411 (43), occurred only once except for c.347delC (mutated in 2 patients), suggesting that no hotspot mutations exist in both *POLE* and *POLD1* genes in STAD patients. All mutations of *POLE/POLD1* were distributed throughout the coding regions of the genes, including the splice regions, exonuclease domains, polymerase domains and other regions. The software, Sorts Intolerant From Tolerant (SIFT) (44) and Polymorphism Phenotyping v2 (PolyPen-2) (45) were used to predict possible impact of gene mutations on the structure and function of a protein. The lower the SIFT score, the higher the pathogenicity of the mutation, while PolyPen-2 is completely the opposite. Mutations with scores ranging from 0 to 0.05 and 0.957 to 1 are considered to be damaging, respectively. The variation was judged as a deleterious mutation when both predictions of these two software were consistent. Due to most frameshift mutations are considered damaging and may affect normal protein function, we found that most

POLE/POLD1 mutations were deleterious mutations and they could occur in any regions of the entire genes, not just the exonuclease domains.

We separately analyzed those top 10 genes with high mutation frequency and statistically significant differences from wild-type samples in *POLE* or *POLD1*-mutant STAD patients (Figure S3A,B, all P-values were less than 0.001). The *TTN*, *ARID1A*, *MUC16* and *KMT2D* genes were present in both groups. What's more, some of these genes have been confirmed in previous studies to be related to prognosis and immunotherapy, such as LRP1B (46,47), ARID1A (48,49) and MUC16 (50,51). Most of the STADs with *POLE/POLD1* mutations in the cohort also had LRP1B, ARID1A or MUC16 mutations, while most wild-type STADs did not have mutations in these three genes (Figure S4). These further prove that *POLE/POLD1* mutations may be associated with prognosis and immunotherapy.

According to the status of *POLE/POLD1* gene mutations, Table S2 shows the demographic and clinicopathological characteristics of this STAD cohort. Compared with wild-type STADs, patients with *POLE/POLD1* mutations were correlated with older cancer onset (median age 70.5 *vs.* 67 years, P=0.042), and the tumors were more commonly located in the antrum anatomic subdivisions at diagnosis (48.98% *vs.* 31.38%, P=0.047). Besides, there were no significant differences between the *POLE/POLD1*-mutant group and WT group in terms of sex, stage, grade, treatment, and residual tumor.

Clinical outcomes by POLE/POLD1 mutations in STAD

We examined the association of *POLE/POLD1* mutations with clinical outcomes in our entire STAD cohort. PFS of patients with *POLE/POLD1*-mutant STADs was not statistically significantly greater than that of other patients (*Figure 1A*, HR =0.47, 95% CI: 0.26–0.86, P=0.067). The same result was also observed in terms of OS (*Figure 1B*, HR =0.61, 95% CI: 0.35–1.05 P=0.14). Although not statistically significant, STAD patients with *POLE/POLD1* mutations showed potential for favorable prognosis.

Due to the strong association of *POLE/POLD1* mutations with age, especially older age onset, we speculated that their apparent prognostic effect would be most evident in related subgroup. According to research, the age of cancer onset peaks at 80 years old (22), and most of the mutant samples come from individuals under 80 (90.32%). Of 336 STAD patients under 80 years old, there were 322 and 320 patients with available PFS and OS data, respectively. In this cohort, we found a statistically significantly improved PFS (*Figure 1C*, not reached *vs.* 34.5 months, HR =0.37, 95% CI: 0.21–0.67, P=0.039) and OS (*Figure 1D*, not reached *vs.* 27.4 months, HR =0.46, 95% CI: 0.26–0.79, P=0.037) in *POLE/POLD1*-mutant group. As a result, *POLE/POLD1* mutations can be used as prognostic markers for patients with STAD, especially in those aged 80 and younger.

POLE/POLD1 mutations increase PD-L1 expression and facilitate an adaptive immune resistance TME

Immune checkpoints PD-L1 (CD274) expression is one of the earliest biomarkers developed to enrich the population that are sensitive to PD-1/PD-L1 targeted immunotherapy in cancer patients (18,30,52), and it has been reported that it may be insufficient to be used as a stand-alone biomarker to predict the benefit of ICIs (20). To explore the potential use of *POLE/POLD1* in clinical benefit of immunotherapy in STAD, we evaluated the relationship between *POLE/POLD1* mutations and the mRNA expression level of PD-L1 in these patients. As illustrated in *Figure 2A*, *POLE/POLD1*-mutant STADs display significant upregulation of the expression level of PD-L1 mRNA than wild-type group (*Figure 2A*, P=0.0072), implying that *POLE/POLD1* mutations are correlated with higher PD-L1 mRNA expression.

The previous study has shown that *POLE* mutated ECs harbor higher neoantigen loads and stimulate more potent cytotoxicity (53). To find out if *POLE/POLD1* variations lead to similar response in STAD patients, we further checked the TME in *POLE/POLD1*-mutant STAD patients. Compared with *POLE/POLD1* wild-type group, the expression of some cytotoxic T-cell markers (IFNG, GZMA, GZMB and GNLY) and effector cytokines (CXCL9, CXCL10 and STAT1) (12,38) were slightly up-regulated in STAD patients with *POLE/POLD1* mutations (*Figure 2B*). Collectively, these data indicate the presence of a potential preexisting TILs and antitumor immunity in STAD patients with *POLE/POLD1* mutations.

Based on the mRNA expression level of PD-L1 and TILs (the presence or absence of TILs), four different types of TMEs have been proposed: TME immune type (TMIT) I (high PD-L1/high CD8A), II (low PD-L1/low CD8A), III (high PD-L1/low CD8A), and IV (low PD-L1/high CD8A) (39,40). In this study, we found that the *POLE/POLD1*-mutant group displayed a higher proportion of dual positive PD-L1 and CD8A (PD-L1$^+$/CD8A$^+$) than the

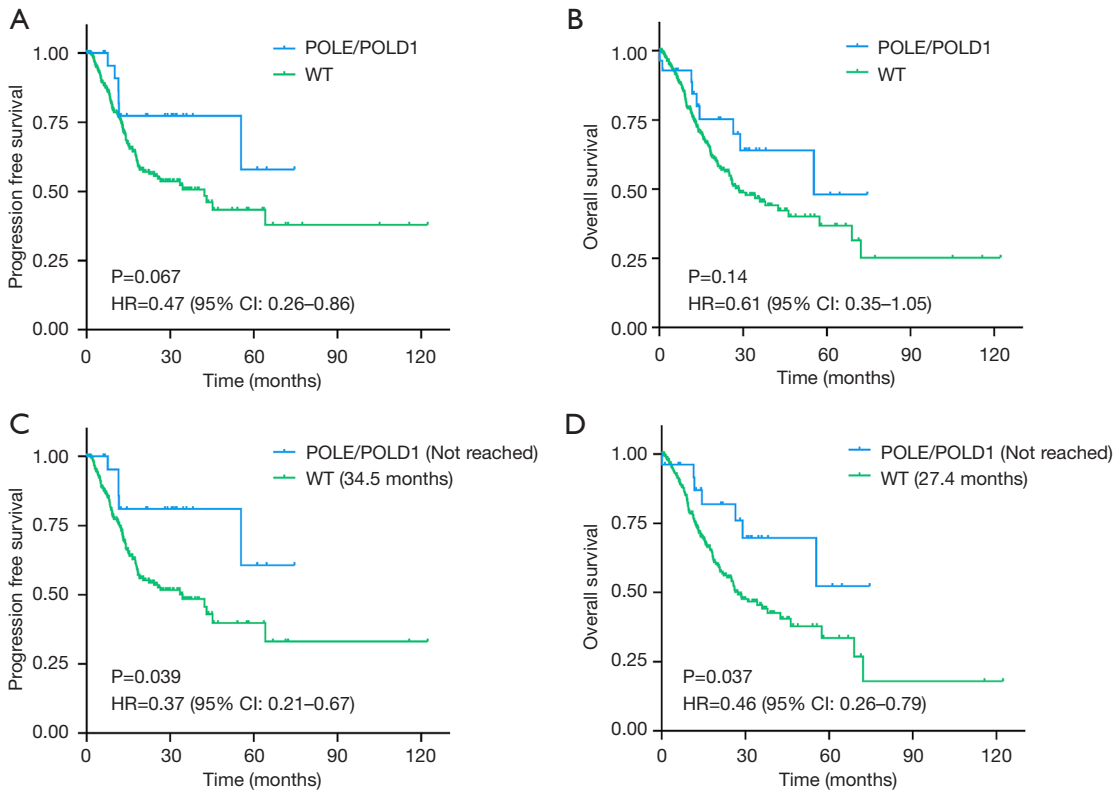

Figure 1 Clinical outcomes of STADs according to tumor *POLE/POLD1* mutation status. (A,B) Progression free survival and overall survival of *POLE/POLD1*-mutant and wild-type STADs in the cohort. (C,D) Progression free survival and overall survival of POLE/*POLD1*-mutant and wild-type in STADs aged 80 and younger. WT = *POLE* and *POLD1* wild-type. P values indicated comparisons between *POLE/POLD1*-mutant and wild-type STADs by unadjusted log-rank test. STAD, stomach adenocarcinoma.

wild-type group (*Figure 2C*, 45.16% *vs.* 33.43%, P=0.004). This suggests that *POLE/POLD1*-mutant STAD patients exhibit TMIT I, a type of adaptive immune resistant TME defined as high PD-L1 expression and the presence of $CD8A^+$ cytotoxic T lymphocytes (39), which is likely a good predictive factor for the response to anti-PD-1/PD-L1 therapy.

Higher TMB in STAD Patients with POLE/POLD1 Mutations

TMB is measured by the total number of mutations in the coding region of the tumor cell genome and has been used as an effective indicator for response prediction in immunotherapy of many cancer types (54,55). STAD patients with *POLE/POLD1* mutations in the cohort exhibited a much higher number of nonsynonymous somatic mutations than wild-type patients (*Figure 3A*, 1,262 *vs.* 105, P<0.001). Consistent with the previous reports in other cancers (56,57), the TMB of MSI-H samples in STAD was significantly higher than that of MSI-L and MSS samples (*Figure 3B*, P<0.001). Although there was no significant difference between hypermutator phenotypes caused by *POLE* and *POLD1* mutations (P=0.6756), the number of nonsynonymous somatic mutations in patients with *POLE* mutations was higher than that in MSI-H patients (MSI-H excludes samples with *POLE/POLD1* mutations here; *Figure 3C*, 1,271 *vs.* 954, P=0.0168), while not the same case in *POLD1*-mutant group (*Figure 3C*, P=0.3586), suggesting MMR inactivation in the context of *POLE* mutations may result in a synergistic increase of mutation rate.

To evaluate the accuracy of using *POLE/POLD1* mutations to predict the level of TMB in STAD patients, 10 mutations/Mb was used as the cut-off value for TMB as reported in most studies (58,59). The positive rate of TMB-H (>10/Mb) among *POLE/POLD1*-mutant patients was 100% (31/31), while that of wild-type patients was 11.85% (39/329), P<0.001 (Fisher exact test). The presence

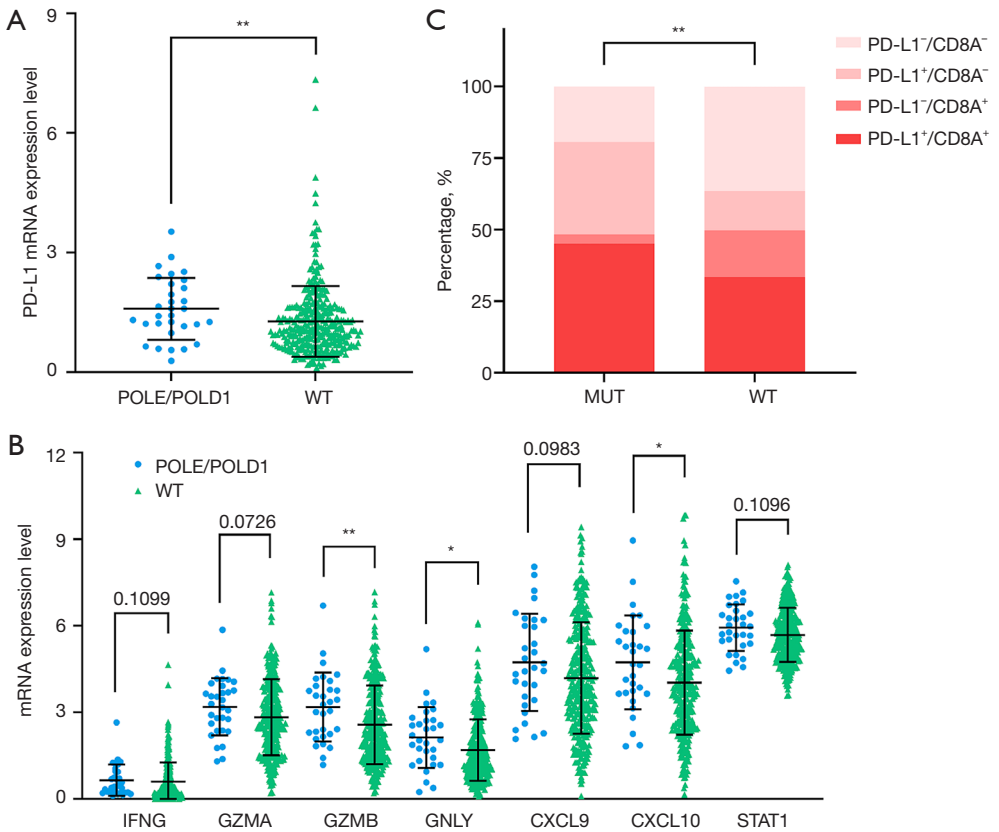

Figure 2 *POLE/POLD1* mutations increase PD-L1 expression and facilitate an adaptive immune resistance tumor microenvironment. (A) Quantitative analysis of PD-L1 mRNA expression based on *POLE* and *POLD1* mutation status. (B) The expression of cytotoxic T-cell markers (IFNG, GZMA, GZMB and GNLY) and effector cytokines (CXCL9, CXCL10 and STAT1) in the *POLE/POLD1*-mutant group were slightly up-regulated. (C) POLE/POLD1-mutant group displayed a higher proportion of dual positive PD-L1 and CD8A (PD-L1$^+$/CD8A$^+$). WT = *POLE* and *POLD1* wild-type. Statistical comparisons between different groups were made by Mann-Whitney test (shown in a and B) and Fisher exact test (shown in C). *, P<0.05, **, P<0.01.

of high TMB in all types of *POLE/POLD1* mutations, including indels, missense and splicing mutations, suggests that high TMB is independent of a particular mutation type, and further supports the idea that clinically significant *POLE/POLD1* mutations are indeed distributed across all coding regions of the full genes.

Furthermore, to determine the sensitivity and specificity of *POLE/POLD1* mutations for predicting TMB, ROC curve analysis was performed using TMB>10/Mb as the standard. The area under the ROC curve (AUC) of *POLE/POLD1* mutations was 0.721, 95% CI: 0.642–0.801, with a sensitivity of 44.3% and a specificity of 100% (*Figure 3D*, P<0.001).

The aneuploidy score is significant lower in POLE/POLD1-mutant population

Aneuploidy, also known as somatic copy number alterations (SCNAs), is widespread in cancer and correlates with markers of immune evasion and reduced response to immunotherapy (27,60). We further explored the status of aneuploidy in *POLE/POLD1*-mutant STADs. As in previous research, we found that in hypermutated tumors caused by *POLE/POLD1* mutations, the aneuploidy score showed a weak negative correlation with the count of mutation (*Figure 4A*, r=–0.29, P=0.117). Further detailed analysis found that the aneuploidy score of the *POLE/POLD1*-mutant group was significantly lower than that of the wild-

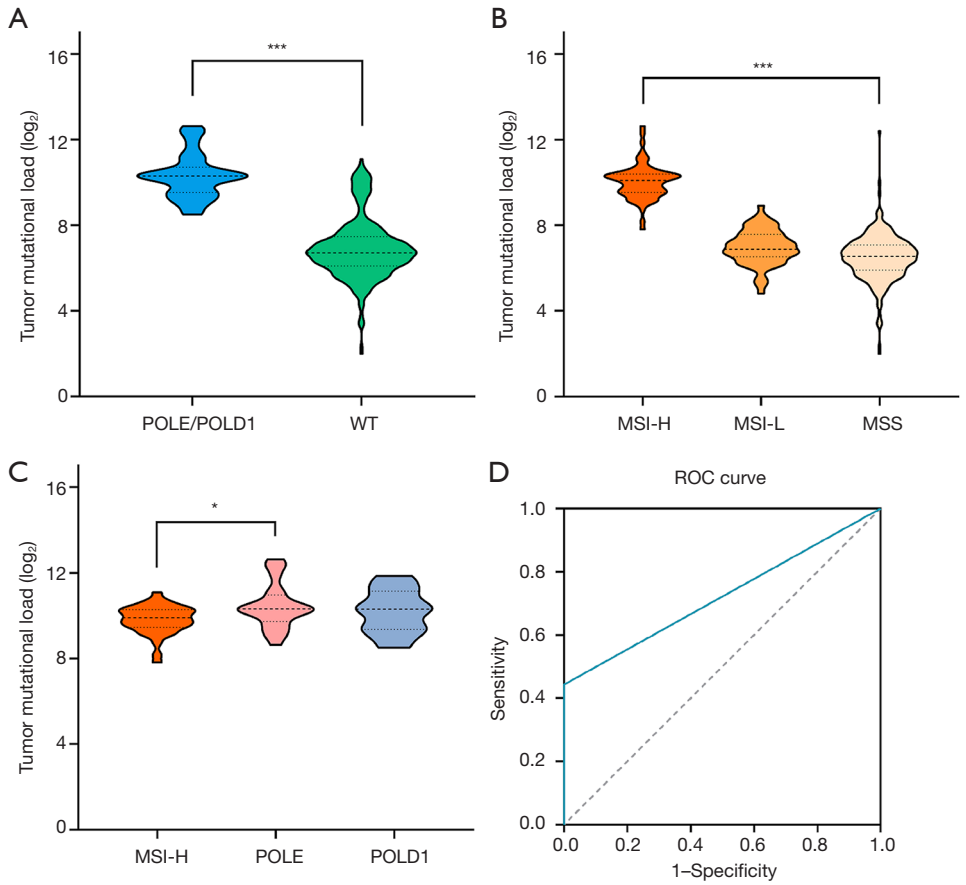

Figure 3 Comparisons of tumor mutational load between different groups in the dataset and high specificity of *POLE/POLD1* mutation status for predicting high tumor mutational load (>10/Mb). (A-C) The correlation between tumor mutational load and different groups classified based on *POLE/POLD1* mutation and MSI status. Tumor mutational load was calculated with the total number of nonsynonymous mutations in coding regions, including all SNVs and indels. WT = *POLE* and *POLD1* wild-type. Statistical comparisons between different groups were made by Kruskal-Wallis test (shown in B) and Mann-Whitney test (shown in a and C). *, P<0.05, ***, P<0.001. (D) ROC curve analysis was used to determine the sensitivity and specificity of *POLE/POLD1* mutations for the differential diagnosis of tumor mutational load>10/Mb. The area under the ROC curve was 0.721, 95% confidence interval was 0.642–0.801, P<0.001.

type group (*Figure 4B*, P<0.001). These data validate the conclusion that TMB is much higher in STAD patients with *POLE/POLD1* mutations, indicating that *POLE/POLD1*-mutant STADs are more likely to benefit from ICIs immunotherapy.

MSI-H is more common in STAD patients with POLE/POLD1 mutations

Multiple studies have previously shown contradictive data on the relationship between *POLE/POLD1* mutations and MSI, including most *POLE*-mutant CRCs and ECs are hypermutant and MSS (14,61), while *POLD1* mutations are correlated with higher MSI in most tumors (62). Recently, another study demonstrated that 74% *POLE/POLD1*-mutant patients were MSS across 47,721 patients with different cancer types (15). MSI-H tumors have been reported to have high response rate to ICIs therapy, with ORR >50% (24,63). We examined the association of *POLE/POLD1* mutations with MSI in STAD patients. Among the 360 samples of STAD patients assessed in this study, 18.06% (65/360) were positive for MSI-H, and 43.08% (28/65) had *POLE/POLD1* mutations. Strikingly, we found the frequency of MSI-H in both *POLE* and *POLD1*-mutant STAD patients were significantly higher than that in the wild-type group, with 91.67% (22/24) *POLE*-mutant

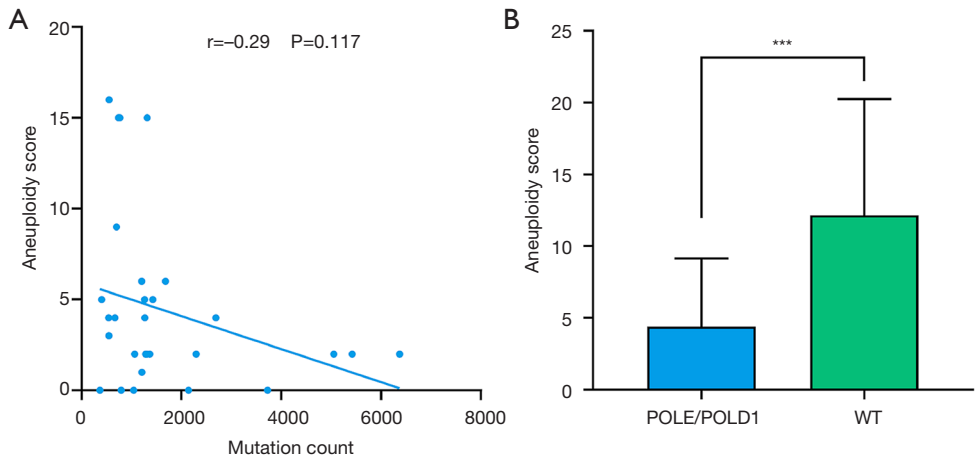

Figure 4 Aneuploidy in *POLE/POLD1*-mutant STADs. (A) The aneuploidy score of the *POLE/POLD1*-mutant group was negatively correlated with the mutation count. The correlation between variables was analyzed by Pearson correlation analysis. r=–0.29. (B) *POLE/POLD1*-mutant group possessed significantly lower aneuploidy score than that of the wild-type group. WT = *POLE* and *POLD1* wild-type. Statistical comparison was made by Mann-Whitney test. ***, P<0.001. STAD, stomach adenocarcinoma.

and 90% (9/10) *POLD1*-mutant STADs were MSI-H, respectively. Overall, there were 90.32% (28/31) *POLE/POLD1*-mutant STAD patients correlated with higher MSI, while only 11.25% (37/329) of wild-type STADs with MSI-H (*Figure 5A*, P<0.001). These imply that *POLE* and *POLD1*-mutant STAD patients exhibit unique characteristics and need to be further studied extensively.

MMR deficiency is the leading cause of MSI-H (63). It has been reported that dMMR occurs in approximately 8% of early gastric cancer (stage I to stage III) and 4% of metastatic gastric cancer (63,64). The inability of the MMR proteins to function normally leads to an accumulation of errors in DNA microsatellite regions, resulting in MSI (63). We further explored the relationship between MMR-related genes (*MLH1/MSH2/MSH6/PMS2/PMS1/MLH3*) and *POLE/POLD1*, and found that the somatic mutation rate of MMR-related genes in *POLE/POLD1*-mutant subset was significantly higher than that of wild-type (*Figure 5B*, P<0.001). Combined with the above finding that most *POLE/POLD1*-mutant STADs are MSI-H, we hold the view that these mutations may not be passenger mutations caused by *POLE/POLD1* mutations. Specifically, the *POLE/POLD1*-mutant group showed significantly decreased MLH1 (P<0.001) and MLH3 (P=0.0185) mRNA expression levels than that of the wild-type group (*Figure 5C*). Taken all these together, *POLE/POLD1* mutations are correlated with higher MSI in STAD patients, and may be accompanied by higher mutation frequency of MMR-related genes and mainly decreased mRNA expression levels of MLH1 and MLH3.

Discussion

In this study, we conducted a comprehensive analysis of *POLE/POLD1* gene variants in 613 STAD patients. We found the mutation frequencies of *POLE* and *POLD1* genes in STAD are 6.04% and 2.77%, respectively. Overall, the incidence of both *POLE* and *POLD1* gene mutations is 7.99% in STAD. And several genes believed to be associated with immunotherapy and prognosis in previous studies also had high mutation frequency in *POLE/POLD1*-mutant STADs. Besides, our data demonstrate that STAD patients with *POLE/POLD1* mutations also exhibit favorable survival, with longer PFS and OS, especially in patients aged under 80. More importantly, the subset of STAD with *POLE/POLD1* mutations displayed substantially higher PD-L1 expression, TMB and MSI, lower aneuploidy score and presented a higher proportion of Type I TME.

The frequencies of *POLE* and *POLD1* gene mutations in STAD patients exhibited a unique pattern. The mutation frequency of *POLE* gene in STAD is lower than that in EC (7–12%) (14) and higher than that in CRC (1–2%) (17). Interestingly, the frequency of *POLD1* mutations in STAD is higher than previously reported that *POLD1* mutations are uncommon (43). Compared with a recent study from Dr. Xu's group, the total frequency of *POLE/POLD1*

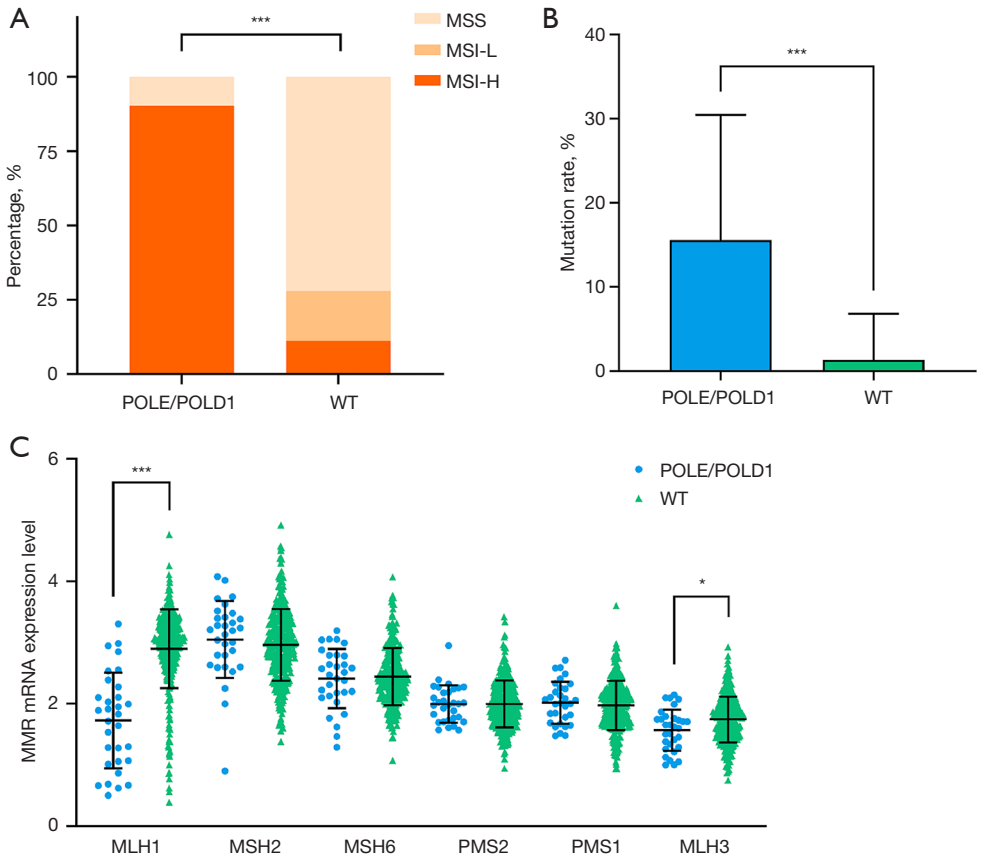

Figure 5 *POLE/POLD1* mutations were accompanied by MMR genes mutations and decreased mRNA expression levels of MMR-related proteins. (A) Significant increase in MSI-H ratio in *POLE/POLD1*-mutant STADs. (B) The somatic mutation rate of the six MMR-related genes (*MLH1/MSH2/MSH6/PMS2/PMS1/MLH3*) in *POLE/POLD1*-mutant subset was significantly higher than that of wild-type group. (C) The mRNA expression levels of MLH1 and MLH3 decreased significantly in *POLE/POLD1*-mutant group. WT = *POLE* and *POLD1* wild-type. Statistical comparisons between different groups were made by Fisher exact test (shown in A) and Mann-Whitney test (shown in B and C). *, P<0.05, ***, P<0.001. STAD, stomach adenocarcinoma.

mutations in STAD is lower than nonmelanoma skin cancer (16.59%), EC (14.85%) and melanoma (14.7%), and a slightly higher than CRC (7.37%) (15). And STAD patients with *POLE* mutations were associated with an older age of onset, which is contradict with the previous reports that *POLE* mutations are associated with an earlier age of onset in endometrial (65) and CRC (12). More notably, STADs with *POLE/POLD1* mutations also had high incidence of *LRP1B*, *ARID1A* and *MUC16* mutations. *LRP1B* is a tumor suppressor gene that belongs to the low-density lipoprotein (LDL) receptor gene family (66), and patients with LRP1B mutation have higher TMB in lung cancer and melanoma (46,47,67). Mutations in *ARID1A* and *MUC16* genes are related to increased immune activity of gastrointestinal tumors (48), and OS is significantly prolonged in STADs with MUC16 mutations (50). The high correlation with these clinically important genes further indicates that *POLE/POLD1* gene mutations may have important significance in multiple cancer types, particularly in STAD.

EC and CRC patients with *POLE* mutations show favorable prognosis (11,12), and our study on the role of *POLE/POLD1* mutations in STAD also obtained the same results, particularly in patients under 80 years old. Combined with what we have observed, despite the upregulation of PD-L1 mRNA and a higher proportion of PD-L1$^+$/CD8A$^+$ TILs in STAD subset with *POLE/POLD1* mutations, the expression levels of some cytotoxic markers and effector cytokines still slightly increased, illustrating that the degree of adaptive immune resistance may be insufficient to fully suppress cytotoxicity in *POLE/POLD1*-

mutant STADs (68), which may also be related to the better prognosis of STAD with *POLE/POLD1* mutations. Besides, *POLE/POLD1* positive STADs that were neither MSI-H nor PD-L1 highly expressed accounted for only a small portion (6.45%, 2 of 31), which proves that *POLE/POLD1* genetic testing is helpful for treatment options of STAD patients. Moreover, STAD patients with *POLE/POLD1* mutations exhibit biologically distinct characteristics. The TMB in the *POLE*-mutant population was even higher than that of MSI-H, suggesting STADs with *POLE* mutations may harbor more neoantigens and be more immunogenic. Although the analysis showed that the TMB of STAD with *POLE/POLD1* mutations was not significantly different from that of MSI-H samples, this may be due to the relatively small impact of *POLD1* mutations on the overall number of mutations. As dramatic ICIs responses occur only in a minority of STAD patients, and most biomarkers that have been developed and used to predict ICIs outcomes are not sufficient, therefore, the prominence of all features among *POLE/POLD1*-mutant STADs, including upregulation of immune checkpoint PD-L1, increased mutational load, lower aneuploidy score, and higher MSI-H percentage, implying that *POLE/POLD1* mutations can be independent risk factors to predict beneficial outcome from ICIs therapy in STAD.

However, our study has some limitations. Firstly, given the retrospective nature and a relatively small population of this study, the conclusions obtained need to be verified by a large-scale prospective analysis. Secondly, the rarity and small number of *POLE/POLD1*-mutant STADs among the cohort warrant our findings to be interpreted with caution. Thirdly, most mutations occur in coding regions outside the polymerase and exonuclease domains, making it impossible for us to assess whether the effects of different regions within *POLE/POLD1* genes are different. Fourthly, RNA-seq data rather than immunohistochemical data are used to evaluate the expression levels, and the effect of translation level on protein expression cannot be predicted. Fifthly, we did not conduct a specific analysis of the subgroup of metastatic or inoperable STADs, which is more likely to receive immunotherapy in clinical practice. Finally, the clinical outcomes of immunotherapy in these STAD patients have not been conducted in this subset, and the clinical significance of *POLE/POLD1* mutations needs further study.

Considering the lack of hotspot mutations in *POLE* and *POLD1* genes in STAD, the whole exon or even the whole gene level mutations need to be comprehensively analyzed, which makes high-throughput genetic sequencing (NGS) the ideal method to perform in the current clinical setting. All together, we propose that it is beneficial to perform genetic testing on every STAD patient before making a treatment plan, as a large amount of information can be obtained from precious patient sample, including the mutation status of the target genes (including *POLE/POLD1*), TMB and MSI status. In addition, germline mutations of *POLE/POLD1* genes are also available through genetic testing, which can help determine whether the patient has a related genetic syndrome (69). All these data are integrated and will provide more useful and accurate information for refining risk stratification and predicting the outcomes of immunotherapy treatment in STAD.

To the best of our knowledge, this is the first study to comprehensively analyze *POLE/POLD1* mutations and their correlation with current biomarkers of immunotherapy in STAD patients. Collectively, these data reveal *POLE/POLD1* mutations may be very promising novel biomarkers for the risk stratification and the screening of ICIs therapy candidates in STAD. We recommend that *POLE/POLD1* mutations, either alone or combined with other biomarkers, should be tested in all clinical research and practice of STAD patients. However, further studies are needed to better understand the clinical significance of *POLE/POLD1* mutations as prognostic markers and predictive biomarkers for ICIs immunotherapy in STAD, which will lay a solid foundation for subsequent clinical trials and translational medicine research.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-1601/rc

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-1601/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was deemed exempt from institutional board approval and patient informed consent was waived, due to the retrospective nature and publicly available data source of the study.

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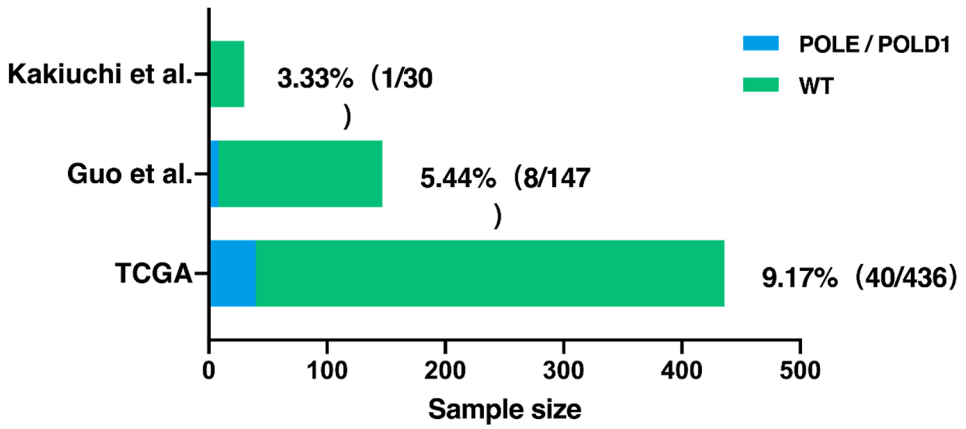

Figure S1 Sample composition and size of the study cohort. The percentages represented the mutation frequency of *POLE/POLD1* in these three groups.

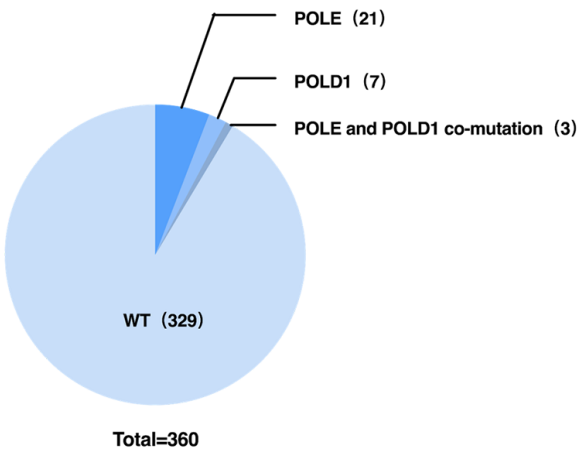

Figure S2 The distribution of *POLE/POLD1* mutations in 360 samples selected. Among the 360 samples, 31 cases had *POLE/POLD1* mutations, of which 24 had *POLE* mutations, 10 had *POLD1* mutations, and 3 had both *POLE* and *POLD1* mutations. WT = *POLE* and *POLD1* wild-type.

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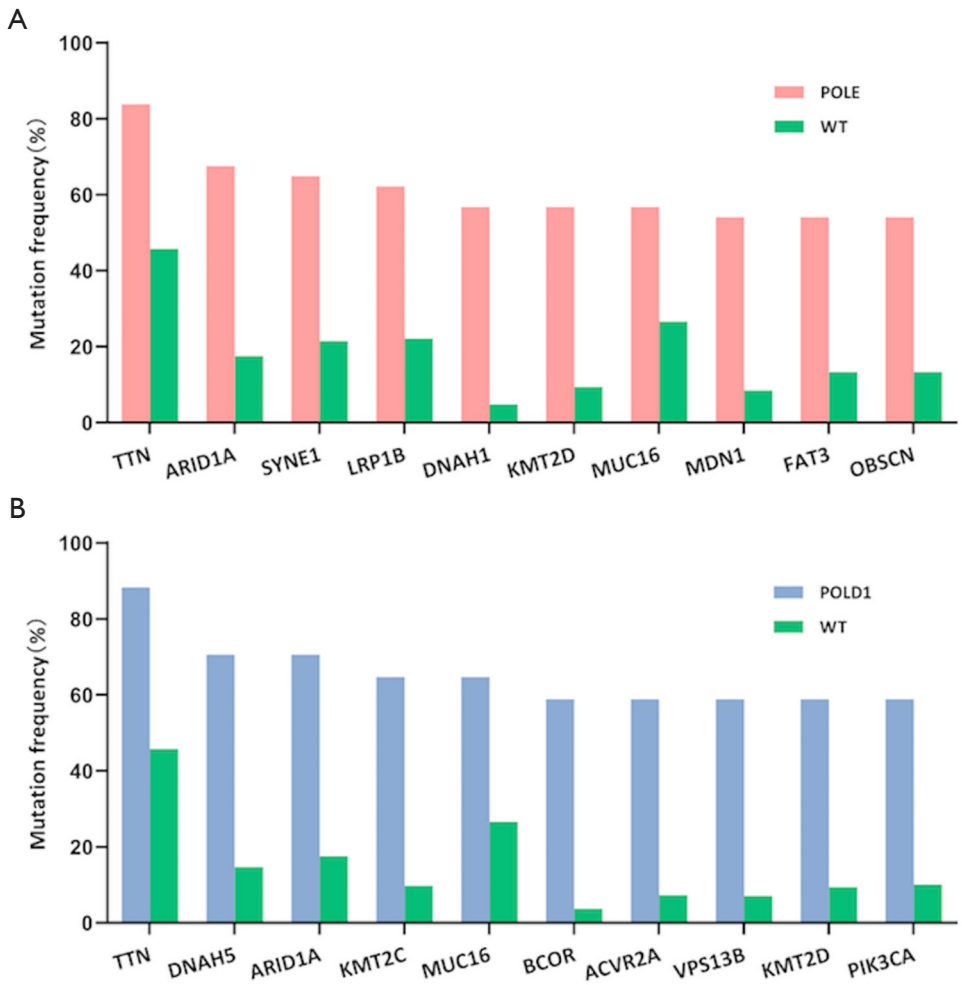

Figure S3 Related genes with high mutation frequency in mutant groups. Top ten genes with high mutation frequency in the *POLE* (A) and *POLD1* (B) mutation groups. WT = *POLE* and *POLD1* wild-type. Fisher's exact test was used to statistically compare the *POLE* or *POLD1* mutants with the wild-type group.

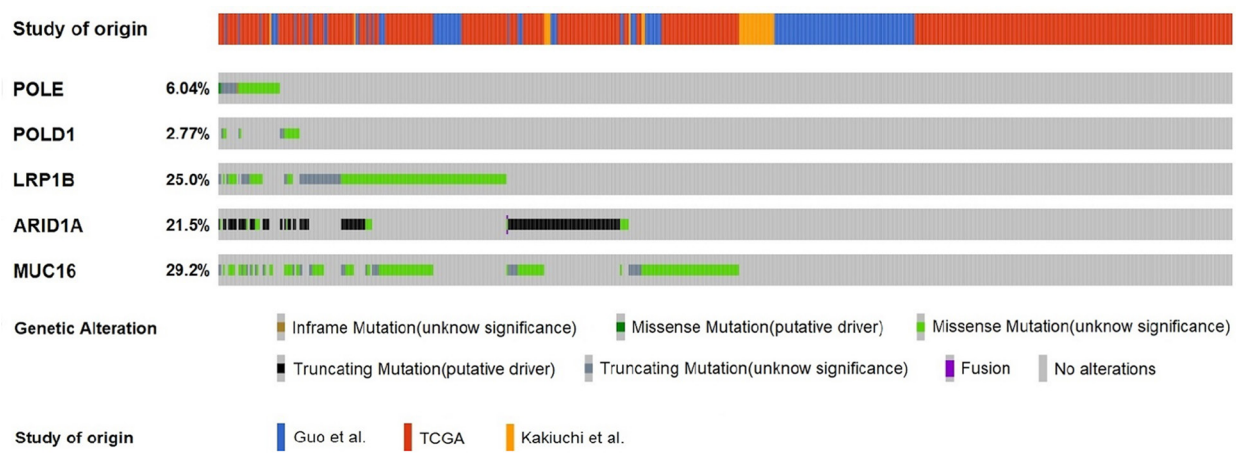

Figure S4 The oncoprint showing gene mutation status and distribution of *POLE*, *POLD1*, *LRP1B*, *ARID1A* and *MUC16* in the cohort. The percentages represented the proportion of mutations in each gene in the entire population. Most samples with *POLE/POLD1* mutations also had *LRP1B*, *ARID1A* or *MUC16* mutations.

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Table S1 Sites and predicted consequences of POLE and POLD1 mutations

Nucleotide change	Amino acid change	Mutation type	Site	SIFT score	Polyphen-2 score
POLE					
c.70G>C	p.G24R	Missense Mutation	Other	0.01	0.813
c.207C>T	p.T69=	Splice Region	Splice Region	-	-
c.208G>A	p.E70K	Missense Mutation	Other	0.04	0.98
c.331-2A>G	p.X111_splice	Splice Site	Splice Site	-	-
c.335G>T	p.C112F	Missense Mutation	Other	0.7	0.002
c.557C>T	p.A186V	Missense Mutation	Other	0.51	0.03
c.630G>C	p.K210N	Missense Mutation	Other	0.14	0.003
c.673G>A	p.D225N	Missense Mutation	Other	0	1
c.727dupA	p.W243Lfs*20	Frameshift Ins	Other	-	-
c.857C>T	p.P286L	Missense Mutation	Exo.	0	1
c.1231G>T	p.V411L	Missense Mutation	Exo.	0	1
c.1346C>T	p.T449M	Missense Mutation	Exo.	0.21	0.01
c.1420G>A	p.V474I	Missense Mutation	Other	0.06	0.987
c.1516A>G	p.M506V	Missense Mutation	Other	0	0.989
c.1651delC	p.V551Ffs*12	Frameshift Del	Pol.	-	-
c.1741G>C	p.A581P	Missense Mutation	Pol.	0.01	0.921
c.2041A>C	p.S681R	Missense Mutation	Pol.	0.01	0.614
c.1993C>T	p.R665W	Missense Mutation	Pol.	0	1
c.2091dupC	p.F699Vfs*11	Frameshift Ins	Pol.	-	-
c.2134C>T	p.R712C	Missense Mutation	Pol.	0	0.995
c.2377C>T	p.R793C	Missense Mutation	Pol.	0.01	0.992
c.2461C>T	p.R821C	Missense Mutation	Pol.	0	1
c.2485A>G	p.M829V	Missense Mutation	Pol.	0.01	1
c.2539C>T	p.R847W	Missense Mutation	Pol.	0	1
c.2743G>A	p.E915K	Missense Mutation	Pol.	0.06	0.003
c.2865-4_2865-3insC	p.X955_splice	Splice Site	Splice Site	-	-
c.3109C>T	p.R1037C	Missense Mutation	Pol.	0	0.996
c.3332G>A	p.R1111Q	Missense Mutation	Pol.	0.04	0.998
c.3970C>T	p.R1324C	Missense Mutation	Other	0	0.999
c.3989C>T	p.P1330L	Missense Mutation	Other	0.08	0.914
c.4162C>A	p.L1388I	Missense Mutation	Other	0	0.999
c.4193_4194delAT	p.Y1398*	Frameshift Del	Other	-	-
c.4247C>T	p.A1416V	Missense Mutation	Other	0.07	0.032
c.4555C>T	p.R1519C	Missense Mutation	Other	0	1
c.4556G>A	p.R1519H	Missense Mutation	Other	0.01	0.999
c.4647delG	p.K1550Nfs*12	Frameshift Del	Other	-	-
c.5096C>A	p.A1699D	Missense Mutation	Other	0.27	0.015
c.5213C>A	p.T1738N	Missense Mutation	Other	0	0.98
c.5239G>A	p.D1747N	Missense Mutation	Other	0	0.998
c.5333C>A	p.A1778D	Missense Mutation	Other	0.63	0.003
c.5539_5541delCTT	p.K1847del	Inframe Del	Other	-	-
c.5666A>G	p.Y1889C	Missense Mutation	Other	0	0.999
c.5842G>T	p.D1948Y	Missense Mutation	Other	0.01	0
c.5867A>G	p.E1956G	Missense Mutation	Other	0.08	0.001
c.5900C>T	p.A1967V	Missense Mutation	Other	0.27	0.001
c.6008A>G	p.Y2003C	Missense Mutation	Other	0	1
c.6049C>T	p.R2017C	Missense Mutation	Other	0.02	0.024
c.6349A>G	p.N2117D	Missense Mutation	Other	0.43	0.012
c.6446G>A	p.R2149H	Missense Mutation	Other	0.12	0
c.6676G>A	p.G2226R	Missense Mutation	Other	0.4	0.066
c.6748-2A>C	p.X2250_splice	Splice Site	Splice Site	-	-
POLD1				-	-
c.-2G>T	p.X1_splice	Splice Site	Splice Site	-	-
c.347delC	p.P116Hfs*53	Frameshift Del	Other	-	-
c.377G>A	p.R126H	Missense Mutation	Other	0.01	0.993
c.537dupG	p.R180Efs*72	Frameshift Ins	Other	-	-
c.931C>T	p.R311C	Missense Mutation	Exo.	0	0.999
c.971G>T	p.G324V	Missense Mutation	Exo.	0	1
c.997C>A	p.P333T	Missense Mutation	Exo.	0	0.944
c.1504G>A	p.D502N	Missense Mutation	Exo.	0.2	0.081
c.1520G>A	p.R507H	Missense Mutation	Exo.	0	0.996
c.1573C>T	p.R525W	Missense Mutation	Exo.	0	0.994
c.1762G>A	p.E588K	Missense Mutation	Pol	0	0.99
c.1837G>T	p.A613S	Missense Mutation	Pol.	0	0.99
c.2182A>C	p.I728L	Missense Mutation	Pol.	0.07	0.514
c.2251-1G>T	p.X751_splice	Splice Site	Splice Site	-	-
c.2414G>A	p.S805N	Missense Mutation	Pol.	1	0.001
c.2489A>G	p.E830G	Missense Mutation	Pol.	0	1
c.2629G>A	p.D877N	Missense Mutation	Pol.	0	1
c.3315G>T	p.E1105D	Missense Mutation	Other	0.31	0.012

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Table S2 Demographic and clinicopathological characteristics in STAD cohort according to *POLE/POLD1* mutation status

	POLE/POLD1	WT	p value
Number	49	564	
Age	70.5 [44-90]	67 [30-90]	0.042†*
Sex			
Female	21 (42.86%)	177 (31.38%)	0.201
Male	26 (53.06%)	338 (59.93%)	
Unknown	2 (4.08%)	49 (8.69%)	
Stage			
I	8 (16.33%)	60 (10.64%)	
II	8 (16.33%)	135 (23.94%)	0.326
III	19 (38.78%)	209 (37.06%)	
IV	10 (20.41%)	81 (14.36%)	
Unknown	4 (8.16%)	79 (14.01%)	
Grade			
G1	0	12 (2.13%)	
G2	15 (30.61%)	140 (24.82%)	0.802
G3	25 (51.02%)	235 (41.67%)	
Unknown	9 (18.37%)	177 (31.38%)	
pT stage			
T1	3 (6.12%)	31 (5.50%)	
T2	8 (16.33%)	111 (19.68%)	0.079
T3	15 (30.61%)	239 (42.38%)	
T4	20 (40.82%)	133 (23.58%)	
Unknown	3 (6.12%)	50 (8.87%)	
pN stage			
N0	17 (34.69%)	133 (23.58%)	
N1	12 (24.49%)	138 (24.47%)	0.444
N2	8 (16.33%)	126 (22.34%)	
N3	9 (18.37%)	109 (19.33%)	
Unknown	3 (6.12%)	58 (10.28%)	
pM stage			
M0	39 (79.59%)	462 (81.91%)	0.403
M1	5 (10.20%)	41 (7.27%)	
Unknown	5 (10.20%)	61 (10.82%)	
Anatomic subdivision			
Antrum	24 (48.98%)	177 (31.38%)	
Cardia	5 (10.20%)	132 (23.40%)	0.047*
Fundus / body	14 (28.57%)	162 (28.72%)	
Other	1 (2.04%)	16 (2.84%)	
Unknown	5 (10.20%)	77 (13.65%)	
Residual tumor			
R0	33 (67.35%)	313 (55.50%)	
R1	2 (4.08%)	16 (2.84%)	0.897
R2	1 (2.04%)	16 (2.84%)	
Unknown	13 (26.53%)	219 (38.83%)	
Radiation therapy			
Yes	8 (16.33%)	67 (11.88%)	0.343
No	23 (24.49%)	293 (51.95%)	
Unknown	18 (36.73%)	204 (36.17%)	
Targeted therapy			
Yes	13 (26.53%)	161 (28.55%)	0.851
No	18 (36.73%)	195 (34.57%)	
Unknown	18 (36.73%)	208 (36.88%)	

Data are n (%) or median [range]. WT = *POLE* and *POLD1* wild-type. †, determined by Mann-Whitney test. Other statistical comparisons between groups were made by Fisher exact test. *, P<0.05.

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