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Mutant POLQ and *POLZ/REV3L* DNA polymerases may contribute to favorable survival of tumors with POLE mutations outside the exonuclease domain

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ABSTRACT

Purpose: Tumors with mutations in the exonuclease domain of POLE are associated with ultra-high mutation rates. POLE mutant tumors are best characterized in intestinal and uterine cancers and are associated with a prominent immune infiltrate and favorable prognosis. To determine whether mutations in other DNA polymerases cooperate with POLE mutations to generate the ultramutator phenotype, we analyzed exome sequence data from 15 cancer types with POLE mutations in The Cancer Genome Atlas (TCGA).

Results: 36% of POLE mutant tumors, predominantly colorectal, stomach and endometrial cancers carried mutations in POLQ (E/Q) and/or POLZ/REV3L (E/Z). Mutation burden, microsatellite instability (MSI) status, tumor stage, disease free survival and immune scores were evaluated in these tumors. Compared to the POLE-only mutant tumors, tumors with E/Q, E/Z, and E/Q/Z mutations possessed significantly higher overall mutation frequencies ($p < 0.001$) and increased frequencies of mutations within the POLE exonuclease domain ($p = 0.013$). E/Q, E/Z, and E/Q/Z mutant colorectal, stomach and endometrial tumors within the TCGA cohort demonstrated 100% disease-free survival, even if mutations occurred outside the POLE exonuclease domain ($p = 0.003$). However, immune scores were related to microsatellite instability (MSI) and not POLE mutation status, suggesting that mechanisms in addition to host immune response may contribute to the prolonged disease-free survival.

Conclusion: Our results demonstrate that POLE mutant tumors can be further substratified for outcomes prediction based on additional mutations in POLQ and ERV3L.

KEY WORDS

POLE, POLQ, REV3L, mutation rates

INTRODUCTION

The analysis of thousands of cancers by The Cancer Genome Atlas (TCGA) consortium and academic institutions revealed a small group of cancers with mutations in *POLE* and an ultramutator phenotype (Cancer Genome Atlas 2012; Kandoth et al. 2013). The *POLE* gene encodes the catalytic subunit of DNA polymerase epsilon, which catalyzes the leading strand synthesis during DNA replication. POLE possesses high-fidelity DNA polymerization, proofreading and 3'-5' exonuclease activities, which promote accurate DNA synthesis (Briggs and Tomlinson 2013). First identified and reported in 2 – 6% of colorectal carcinomas (Cancer Genome Atlas 2012; Church 2014; Jesinghaus et al. 2016), *POLE* mutations were also noted at frequencies of 6 – 9% amongst uterine corpus endometrial cancers (Church et al. 2013; Kandoth et al. 2013) and in gastric adenocarcinoma (Comprehensive molecular characterization of gastric adenocarcinoma 2014). Mutations can be found across the entire *POLE* gene, but those in the *POLE* exonuclease domain are most prevalent in cancers with ultra-high mutation rates (>100 mut/Mb). These cancers exhibit higher mutation rates than microsatellite instable (MSI) tumors associated with mismatch repair abnormalities. In addition, *POLE* ultra-mutant cancers also possess a high frequency of C-to-A transversions (Muller et al. 2016; Nebot-Bral et al. 2017). Multiple studies have found significantly improved survival in patients with *POLE* mutated endometrial cancers (Billingsley et al. 2015; Church et al. 2015; Imboden et al. 2019; Kandoth et al. 2013; Meng et al. 2014), while the survival benefit was not as profound in *POLE* mutated colorectal carcinoma (Rayner et al. 2016). A tumor associated inflammatory response, similar to that in MSI tumors, has been reported to occur early on during development of *POLE* mutated endometrial and colorectal cancers (Temko et al. 2018). It is thought to be caused by neo-antigens

that are generated as a result of the high mutation burden (Le et al. 2015; Schwitalle et al. 2008) and renders *POLE* mutant cancers responsive to immunotherapy (Mehnert et al. 2016).

Polymerase theta (POLQ) is a low-fidelity DNA polymerase lacking a 3' to 5' exonuclease function (Hogg et al. 2011). The enzyme is involved in the alternative non-homologous end-joining pathway (alt-NHEJ) (Kent et al. 2015), which is a backup mechanism of double stranded DNA break repair. This pathway predominates in cancer cells when other DNA repair pathways are missing or when telomere ends are deprotected (Ceccaldi et al. 2015; Mateos-Gomez et al. 2015). The loss of POLQ sensitizes cells to ionizing radiation and Polq-deficient mice exhibit increased DNA instability and genomic rearrangements, suggesting a role for POLQ as a guardian of the genome (Yousefzadeh and Wood 2013). Both overexpression and loss of POLQ increase mutation frequencies (Higgins et al. 2010; Killock 2015; Mateos-Gomez et al. 2015). Multiple structural motives in POLQ can interact with DNA, RAD51 and BRCA1 (Brandalize et al. 2014). In addition, POLQ forms a complex with PARP-1 in a pathway of synthetic lethality with BRCA1 and is thus considered a therapeutic target (Ceccaldi et al. 2015; Mateos-Gomez et al. 2015). However, which domains in POLQ should be targeted remains to be determined (Killock 2015).

REV3L (REV3 like, DNA directed polymerase zeta (POLZ) catalytic subunit) is involved in DNA synthesis that reads through damaged DNA (translesion DNA synthesis, TLS). The high efficiency of POLZ bypassing a broad spectrum of DNA lesions led to its recognition as a master TLS polymerase (Lange et al. 2011). POLZ/REV3L has been linked to carcinogenesis in breast, lung, gliomas, and gastric cancers, and modulates cisplatin sensitivity (Makarova and Burgers 2015; Varadi et al. 2011; Wang et al. 2015; Zhang et al. 2013).

Because studies describe overlapping functions and synergy among the over 14 DNA polymerases (Dubarry et al. 2015; Lange et al. 2011; Loeb and Monnat 2008), we undertook an unbiased approach to identify mutations in polymerases within the TCGA tumor compendium. This analysis revealed a greater frequency of mutations in POLQ and POLZ/REV3L compared to other polymerases of similar gene length. Thus, we propose that POLQ and POLZ/REV3L may cooperate with POLE in generating ultrahigh mutation rates.

RESULTS

Of the 33 cancer types in the TCGA database (Colaprico et al. 2016), we identified 15 cancer types (PANCAN) with 2 or more cases that possessed mutations in the *POLE* protein coding region (**Figure 1A and Supplementary Table 1**). These 15 cancer types contained 138 cases with *POLE* mutations anywhere in the exome. 53% of these POLE mutant tumors carried mutations in one or more of the 14 other DNA polymerases. The most common additionally mutated DNA polymerases were *POLQ* and *POLZ/REV3L,* found mutated in 36% of *POLE* mutated tumors. These two polymerases were even more commonly mutated than POLE in our PANCAN cohort (**Supplementary Figure 1**). Altogether, 14 cases with *POLE* and *POLQ* mutations (*E/Q*), 16 cases with *POLE* and *POLZ/REV3L* mutations (*E/Z*) and 20 cases with *POLE, POLQ* and *POLZ/REV3L* (*E/Q/Z*) mutations were identified in the PANCAN cohort (**Figure 1B)**. Mutations in the exonuclease domain of *POLE* are responsible for causing the ultramutator phenotype in colorectal and uterine corpus cancers (Cancer Genome Atlas 2012; Church et al. 2013; Kandoth et al. 2013; Palles et al. 2013). In order to determine the contribution of POLQ and REV3L to the ultramutator phenotype, we compared the mutation frequencies of tumors with mutations in only *POLE to E/Q, E/Z and E/Q/Z* mutant tumors. Mutation frequencies in the cellular genome increased in the following order: no *POLE* mutations < *POLE*-only mutations (anywhere with the POLE exome) < *E/Q*, *E/Z*, *E/Q/Z* mutations (**Figure 1C**). The median mutation count of *E/Q/Z* tumors was more than 10-fold higher ($p < 0.001$) than that of tumors with only *POLE* mutations. E+Q and E+Z mutant tumors also displayed significantly higher mutation counts compared to E-only mutant tumors, suggesting a contribution of Q or Z mutations to the overall cancer mutation rates. Next, we determined the number of mutations in the exonuclease and polymerase domains of *POLE* in the 15 cancer types within our PANCAN compendium (**Figure 1D**). The percentage of *POLE*

mutations in the exonuclease domain was greater in *E/Q*, *E/Z* and *E/Q/Z* mutant tumors compared to *POLE-only* mutant tumors ($p = 0.013$). In contrast, the percentage of mutations in the DNA polymerase domain was similar. Thus, our data confirm the notion that mutations in the exonuclease domain of POLE are responsible for ultra-high mutation rates.

To further investigate these mutant DNA polymerases, we focused on colorectal (CORE), endometrial (UCEC) and stomach (STAD) cancers. These cancer types contain the highest numbers of tumors with *E/Q*, *E/Z* and *E/Q/Z* amongst the 15 cancer types included in PANCAN (**Figure 1A, Supplementary Table 1**). Among these 3 cancer types, we identified 6 cases with *E/Q*, 12 cases with *E/Z* and 16 cases with *E/Q/Z* mutations (**Figure 2A**). In these cancers, the mutation burden in *POLE*, *E/Q*, *E/Z* and *E/Q/Z* mutant tumors paralleled the mutation burden in the whole PANCAN cohort (compare **Figure 2B and Figure 1C, Supplementary Figure 2A and B**). *E/Q/Z* mutant tumors demonstrated, on average, an 8-fold increase in mutation frequencies compared to tumors with only *POLE* mutations. Amongst *POLE* mutant tumors, 26 tumors carried mutations outside the POLE exonuclease domain, while 31 tumors carried mutations within the exonuclease domain. The frequency of POLE exonuclease mutations (15/16 cases in **Figure 2C**) provides a valid explanation for the difference in mutation rates and potential association of POLE exonuclease domain mutations with mutations in POLQ and POLZ/REV3L.

Overall, exonuclease domain mutations were identified in 6/23 cases of *POLE* only mutant tumors, 4/6 *E/Q* cases, 6/12 *E/Z* cases and 15/16 of *E/Q/Z* cases (**Figure 2C**). Stratified by cancer types, *POLE* exonuclease domain mutations occurred in 7/15 colorectal, 18/26 endometrial and 6/16 stomach tumors, demonstrating cancer type specific frequencies (**Supplementary Figure 3A**). In contrast to the *POLE* gene that demonstrates two mutational hotspots in the exonuclease domain, mutational hotspots in the *POLQ* gene are not associated with a functional protein domain

(**Supplementary Figure 3B, C & D**). While *REV3L* does not reveal mutational hotspots, approximately 50% of mutations lead to truncated protein expression (**Supplementary Figure 3E & F**). Another characteristic of *POLE* mutant tumors are C to A and G to T transitions (Cancer Genome Atlas 2012). We observed the greatest increase of nucleotide transitions in cancers with *E/Q/Z* mutations (**Figure 2D**), consistent with the loss of *POLE* exonuclease activity in these tumors.

Since mutations in *POLE* confer increased disease free survival (DFS) in patients with uterine cancer, even in those patients with high-grade tumors (Church et al. 2015; McConechy et al. 2016), we investigated the prognostic role of *POLQ* and *REV3L* mutations in *POLE* mutant tumors. Kaplan-Meier curves were constructed for colorectal, endometrial and stomach cancer cases with follow-up data (**Figure 3A**). Using the TCGA annotations of DFS in individual patients, no cancer recurrences were observed in the E/Q, *E/Z* and *E/Q/Z* mutant groups. POLE exonuclease domain mutations were observed in 29 cases in the good survival group and 1 case in the poor survival group, consistent with the expected long DFS periods of patients with POLE exonuclease domain positive tumors. In addition, 19 cases with mutations in POLE outside the exonuclease domain were in the good survival group. Of those 7 (37%) had concurrent mutations in POLQ or REV3L or in both polymerases **(Figure 3B)**. Furthermore, a Kaplan-Meier analysis in the PANCAN cohort revealed improved DFS associated with these polymerase mutations preferentially in colorectal, endometrial, and stomach cancers and not in diffuse B-cell lymphoma (data not shown). These data demonstrate favorable survival outcomes in tumors with *POLE* mutations outside the exonuclease domain if concurrent mutations in *POLQ*, *REV3L* or both polymerases are present.

Compared to microsatellite stable tumors (MSS), microsatellite instability (MSI) in colorectal cancer confers a better prognosis (Popat et al. 2005). To determine whether the favorable outcome

of *E/Q*, *E/Z* and *E/Q/Z* mutant cancers can be explained by MSI or TMN stage, we examined the relationship between MSI status, tumor stage and polymerase mutations in colorectal, endometrial and stomach cancers (**Figure 3C and Supplementary Figure 4 and Supplementary Table 2**). Despite improved DFS rates, the full range of tumor stages was observed amongst *E/Q*, *E/Z* and *E/Q/Z* tumors ($p = 0.42$) **(Supplementary Table 2A)**. Comparing the POLE-only and E/Q/Z mutant cancers did not reveal a significant difference in tumor stage, but differed in the frequency of MSI cases ($p < 0.001$). In addition, the frequency of MSI cases in *POLE* mutant tumors differed between the 3 cancer types ($p < 0.001$) (**Supplementary Table 2B & C**). Although MSI is enriched in samples with high mutation levels (**Supplementary Table 2D**), as expected, 10-fold higher mutation counts ($P < 0.001$) were observed in cancers with *E/Q/Z* mutations compared to MSI without E/Q/Z mutations (**Supplementary Figure 5**). These results suggest that mutations in *E/Q*, *E/Z* and *E/Q/Z* confer a better prognosis independent of MSI status and TMN stage in colorectal, endometrial and stomach adenocarcinomas.

We next examined the amount of the cancer-associated immune infiltrate. The immune score obtained through ESTIMATE(Yoshihara et al. 2013) corresponded to the categorical score of the immune infiltrate derived from digital H&E images (**Supplementary Figure 6**). Therefore, we used the ESTIMATE immune scores for further analysis of colorectal, endometrial and stomach cancers. As shown in **Figure 4A**, a significant difference was observed in the median immune scores between groups with low, intermediate and high mutation burden, grouped based on mutation burden and not on E, Q, Z mutant status (see Methods) and, as expected, the median immune scores increased with total mutation levels. Surprisingly, the immune scores in *E/Q/Z* mutant tumors did not differ significantly from tumors with a low level of mutations (**Figure 4B**). As expected, MSI tumors possessed higher immune scores than MSS tumors ($p < 0.001$) (Garg

and Soslow 2009) (**Figure 4C**). Finally, immune scores of MSI and *E/Q/Z* mutation tumors were similar to those in the MSI group and higher than MSS and *E/Q/Z* mutation tumors (**Figure 4D**). Within the group of tumors with *POLE* exonuclease domain mutations, MSS tumors possessed insignificantly lower immune scores than MSI tumors ($p = 0.29$) (**Supplementary Figure 7**). Together, results in this TCGA cohort demonstrate the immune response is driven by MSI, rather than POLE exonuclease domain mutations.

DISCUSSION

An analysis of 14 DNA polymerases in tumors with mutations in the *POLE* exome revealed additional mutations most commonly in *POLQ* and *POLZ/REV3L* exomes. Among the 15 cancer types, colorectal, uterine and stomach cancer were most frequently afflicted by these mutations. Cancers with mutations in *POLE* and *POLQ* (*E/Q*), *POLE* and *POLZ/REV3L (E/Z)* and in all 3 polymerases (*E/Q/Z*) were associated with the highest mutation burden and an excellent prognosis independent of MSI status and tumor stage. Mutations in the exonuclease domain were observed in 94% (15/16) of E/Q/Z mutant tumors, but only in 26% of *POLE*-only mutant tumors or in 55% of E/Q + E/Z tumors. However, despite harboring 10-fold more mutations than MSI tumors and 8-fold more mutations than the mutation frequencies associated with *POLE*-only mutant tumors, *E/Q/Z* mutant tumors did not display significantly more inflammation.

The main result from that analysis is that patients with colorectal, stomach and endometrial cancers bearing *E/Q*, *E/Z* and *E/Q/Z* mutations have 100% disease free survival (DFS) at a median follow up time of 33 months. In contrast, patients with tumors bearing mutations in *POLE* only, most of which outside the POLE exonuclease domain, had a DFS of 76% at follow up of 18.4 months. The favorable DFS in *E/Q*, *E/Z* and *E/Q/Z* mutated tumors occurred even in tumors with a non-mutant POLE exonuclease domain. Ths finding thus expands the spectrum of *POLE* mutant tumors with an excellent prognosis. The favorable prognosis included patients with high tumor stage, which echoes prior studies demonstrating a favorable outcome of uterine tumors with *POLE* exonuclease mutations despite adverse standard clinicopathologic indicators including high grade, high stage, and lymphovascular invasion (Billingsley et al. 2016; Church et al. 2015). While the high mutation frequencies may cause an early growth advantage (Fox et al. 2013), as tumors evolve they may succumb to high mutation burden as new mutations can no longer be tolerated and cause

tumor cell death (Bellone et al. 2015; Shlien et al. 2015) or increased sensitivity to therapeutic agents.

The prevailing hypothesis for the favorable prognosis of cancers displaying the hypermutator phenotype is the increased attack by the immune system. Evidence in support of this theory is the observation that tumor infiltrating (TIL) and peritumoral lymphocytes are increased and that cytotoxic activities in CD8+ and CD4+ lymphocyte populations are heightened in *POLE* mutated endometrial cancers (Bakhsh et al. 2016; Howitt et al. 2015; Hussein et al. 2015; van Gool et al. 2015), similar to hypermutated MSI tumors (Nosho et al. 2010). This observation has led to the hypothesis that immune checkpoint inhibitors may be efficacious in *POLE* ultramutated tumors (Howitt et al. 2015). Our results question a direct relationship between mutation burden, tumor immune response and PD-L1 expression, also raised in a larger study across 5722 cases from 21 cancer types in TCGA (Budczies et al. 2018). While we observed a concordance between the computational and histological assessments of the immune infiltrate, the immune score in tumors with *E/Q/Z* mutations depended on MSI status. This result suggests that the immune infiltrate attributable to mutations in *E/Q/Z* mutant tumors may be less, or that its composition may involve immune cells other than lymphocytes. Lesser CD8+ and gamma-interferon gene expression signatures have also been observed in gastrointestinal tumors with a large single nucleotide variant (SNV) burden that was attributed largely to POLE exonuclease mutations (Liu et al. 2018). Perhaps E/Q/Z mutations occur at a later point in tumor evolution (Smid et al. 2016) when immunosuppressive factors already dominate. We also cannot rule out the possibility of increased numbers of cytotoxic lymphocytes intermixed with E/Q/Z mutant tumor cells, because computational methods and inspection of H&E images are not sensitive enough to detect small differences in tumor infiltrating lymphocytes (TILs) that may have large anti-tumoral effects.

Although the relatively small number of tumors cautions the generalization of results, the data provide novel insights on the hypermutator phenotype. First, previous studies attributed hypermutator phenotype to specific mutations primarily within the *POLE* exonuclease domain. Our study reveals that (1) *POLE* exonuclease domain mutations are more common in both double (*E/Z* and *E/Q*) and triple (*E/Q/Z*) mutant tumors (25 of 34 cases) than *POLE* single mutant tumors (6 of 23 cases), and (2) double and triple mutant tumors have higher mutation counts than *POLE* single mutant tumors. Mechanistically, POLQ and POLZ are thought to function in different repair processes: POLQ in alternative (microhomology-mediated) non-homologous DNA repair pathway and POLZ in translesion DNA synthesis. How these DNA repair processes cooperate with replicative DNA polymerase (POLE) to prevent genome instability remains unknown. This will be an important subject for further understanding of the hypermutator phenotype.

Our findings have important clinical implications. They build upon and expand the previously well documented good prognostic impact of *POLE* exonuclease mutations in uterine cancer, that have generated intense interest in part due to the paradox of a favorable prognosis in tumors with pathologic indicators of poor prognosis. While in this study, prolonged DFS is observed in colorectal, endometrial and stomach cancers with *E/Q/Z* mutations, this is not the case in other non-carcinoma cancer types within TCGA. Thus, the positive outcome prediction is cancer type specific. Altogether, results from this study provide a rationale for including POLQ and/or POLZ/REV3L mutations in clinical outcome studies of tumors with POLE mutations.

MATERIALS AND METHODS

Data acquisition

The data used in this study are based upon the whole exome sequence data sets generated by the TCGA Research Network: http://cancergenome.nih.gov/. The locations and frequencies of somatic mutations, MSI status and clinical stage and follow up information in TCGA Provisional datasets were obtained from cBioportal (http://www.cbioportal.org) (Cerami et al. 2012; Gao et al. 2013) up to 06/22/2016 **(Supplementary Table 1).** Functional domains of the proteins were provided by Pfam database (Finn et al. 2016). Data visualization for mutations was performed with MutationMapper in cBioportal.

Case Selection Criteria

We searched all cancer types in TCGA for those that possess 2 or more cases with *POLE* mutations. This yielded 15 tumor types that we named the PANCAN data set in this study. We then determined the frequency of mutations within additional polymerase within PANCAN. We selected the three adenocarcinomas (uterine corpus endometrial adenocarcinoma, colorectal adenocarcinoma, and stomach adenocarcinoma) with the highest frequency of double or triple mutated DNA polymerase status for more detailed analysis

Studies performed

Kaplan Meier survival plots were generated using clinical follow-up data available within the TCGA database. To determine the global mutational spectrum, we classified 6 types of nucleotide transitions or transversions. The frequency of each mutation type was calculated.

Digital images of all *E/Q* mutant tumors and representative cases of tumors with neither *POLE* nor *POLQ* mutations, and of MSI tumors were assessed by one author (JR) for the amount of tumor immune infiltrate. The combination of tumor infiltrating lymphocytes and the peritumoral lymphoid infiltrate was graded on a scale between 0 and 3. Tumor associated lymphocytes were graded as none (0), minimal (rare) to 1 per high powered field (HPF) (1), 2 to 5 per HPF (2) and >5 per HPF (3). Peritumoral lymphocytes were assessed at the deepest advancing tumor front, graded at low power: none (0), minimal (1), mild (2), moderate (3) and marked (4).These visual semi-quantitative scores were compared with the immune scores evaluated using ESTIMATE (Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data) (Yoshihara et al. 2013). We separated the colorectal, stomach and endometrial cancer cases into 3 quartiles defined by the highest 25%, intermediate 50%, and lowest 25% of mutational counts, and calculated the immune score for each group, as well as by MSI status.

Statistical data analysis

Statistical analysis was performed in R Program (R Core Team 2015) and data visualization methods as described previously (Huang et al. 2016). The horizontal lines in the boxplots represent the 1st, 2nd and 3rd quartiles and whiskers outside the box show the 1.5 interquartile range. The significance of the differences of data illustrated in the boxplots was calculated using the Wilcoxon rank-sum tests. The Chi-square test was performed to test the significance of differences in frequencies of all tables. The significance in the Kaplan–Meier survival plot was calculated using the log rank test. Statistical significance was accepted at $p<0.05$.

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STATEMENT OF AUTHOR CONTRIBUTIONS

FH, JKR, BSK designed study; FH collected and analyzed the data; All authors were involved in writing the paper and had final approval of the submitted versions.

ADDITIONAL INFORMATION

Competing financial interests: the authors involved in this study do not declare a conflict of interest.

FIGURES LEGENDS

Figure 1. ***Cancer types (PANCAN) with POLE/Q/Z mutations in TCGA.*** **A)** Number of cases with *POLE- only*, *E/Q*, *E/Z* and *E/Q/Z* mutations in 15 cancer types (cohort referred to as PANCAN) within TCGA. The x-axis shows the actual number of cases with *POLE* (green), *E/Q* (orange), *E/Z* (pink) and *E/Q/Z* (blue) mutations. The y-axis displays the 15 cancer types: Uterine corpus endometrial carcinoma (UCEC), Stomach adenocarcinoma (STAD), Colon and rectum adenocarcinoma (CORE), Skin cutaneous melanoma (SKCM), Lymphoid Neoplasm Diffuse Large B-cell Lymphoma (DLBC), Lung adenocarcinoma (LUAD), Breast invasive carcinoma (BRCA), Sarcoma (SARC), Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), Pancreatic adenocarcinoma (PAAD), Lung squamous cell carcinoma (LUSC), Head and neck squamous cell carcinoma (HNSC), Bladder urothelial carcinoma (BLCA), Kidney renal clear cell carcinoma (KIRC), and Liver hepatocellular carcinoma (LIHC). **B)** Venn diagram displaying the number of cases in PANCAN with mutations in 1, 2 or 3 POL genes. **C)** Mutations per Mb (y-axis) of PANCAN cases without *POLE* mutations (other) or with *POLE*, *E/Q*, *E/Z* and *E/Q/Z* (x-axis) mutations. Number of cases in each group are listed in parenthesis. **D)** Mutation frequencies in *POLE* exonuclease and polymerase domains as a percentage of total number of mutations in the POLE exome. The cases are grouped by their polymerase mutation status on the y-axis, and the number in parenthesis represents the total number of POLE mutations within each group.

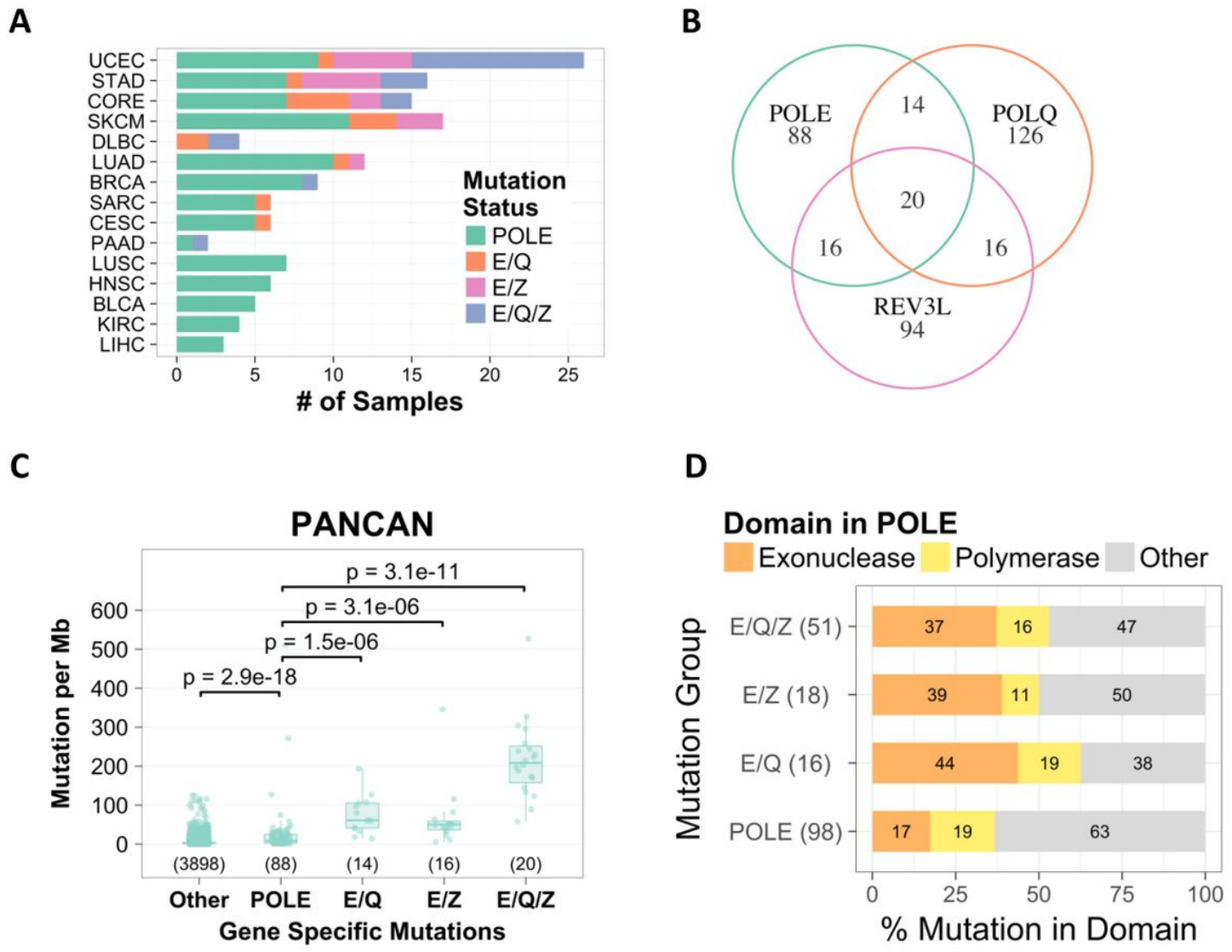
Figure 2. ***POLE/Q/Z*** **mutations** ***in colorectal (CORE), endometrial (UCEC) and stomach (STAD) cancers.*** **A)** Venn diagram displaying the number of cases with mutations in 1, 2 or 3 POL genes. **B)** Mutation groups of cases without polymerase mutations (other), or with mutations in *POLE* only, *E/Q*, *E/Z* and *E/Q/Z*. The number of cases in each group is listed in parenthesis. **C)** Number of cases with mutations in *POLE* exonuclease domain in various mutation groups. **D)**

Percentages transitions (Ti) and transversions (Tv) are shown on the Y-axis for CORE, STAD and UCEC. The x-axis shows the mutation groups.

Figure 3. ***Survival and clinical characteristics of patients with polymerase mutations colorectal (CORE), endometrial (UCEC) and stomach (STAD) cancers.*** **A)** Kaplan-Meier curves of Disease-Free Survival (DFS) for 3 groups of patients: POLE only (n=21, median follow-up =18.4 months), green line; E/Q (n=6, median follow-up = 19.0 months), orange line; E/Z (n=9, median follow-up = 34.3 months), pink line, and E/Q/Z (n=16, median follow-up = 37.5 months), blue line; tumors without mutations in *POLE*, *POLQ* or *REV3L* exomes, grey line. **B)** Polymerase mutation analysis of cases in the good survival group in panel A. The red bar indicates cases with POLE exonuclease mutations. **C)** Cancer type-specific illustration of mutation count, *POLE*, *POLQ* and *REV3L* mutations, microsatellite instability (MSI) and tumor stage.

Figure 4. ***ESTIMATE immune scores by mutation frequency quartiles, E/Q/Z mutation groups, and MSI in colorectal (CORE), endometrial (UCEC) and stomach (STAD) cancers.*** **A)** ESTIMATE immune scores in cancers within high, intermediate and low overall mutation quartiles. **B)** Immune scores of samples with *E/Q*, *E/Z* and *E/Q/Z* mutations compared to the low mutation quartile from panel A. **C)** Immune scores in groups of cancers separated by MSI status. **D)** Immune scores in MSI and MSS *E/Q/Z* cases compared to all other MSI cases. For each panel the number of cases within each group is included in parentheses on the x-axis.

Figures



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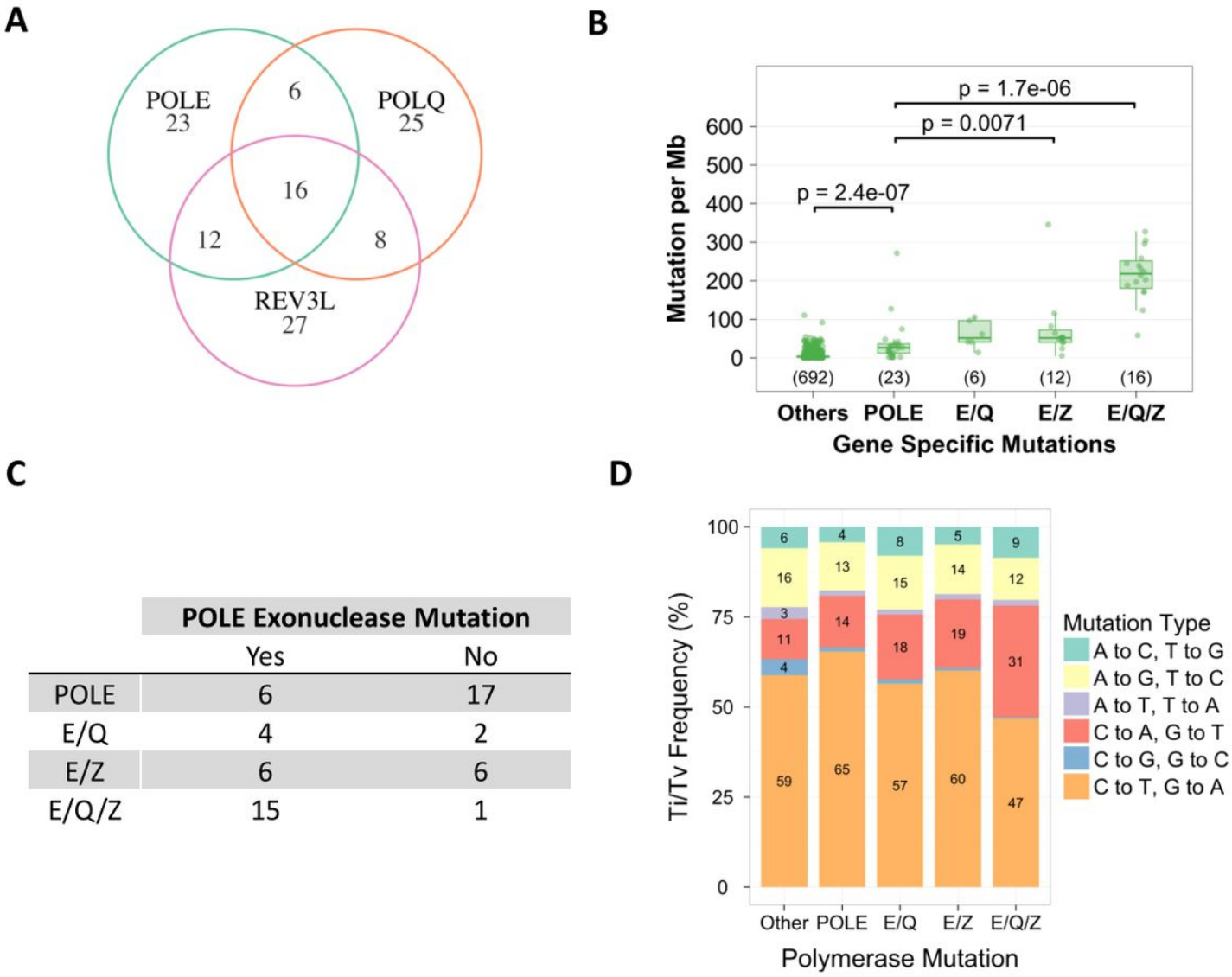
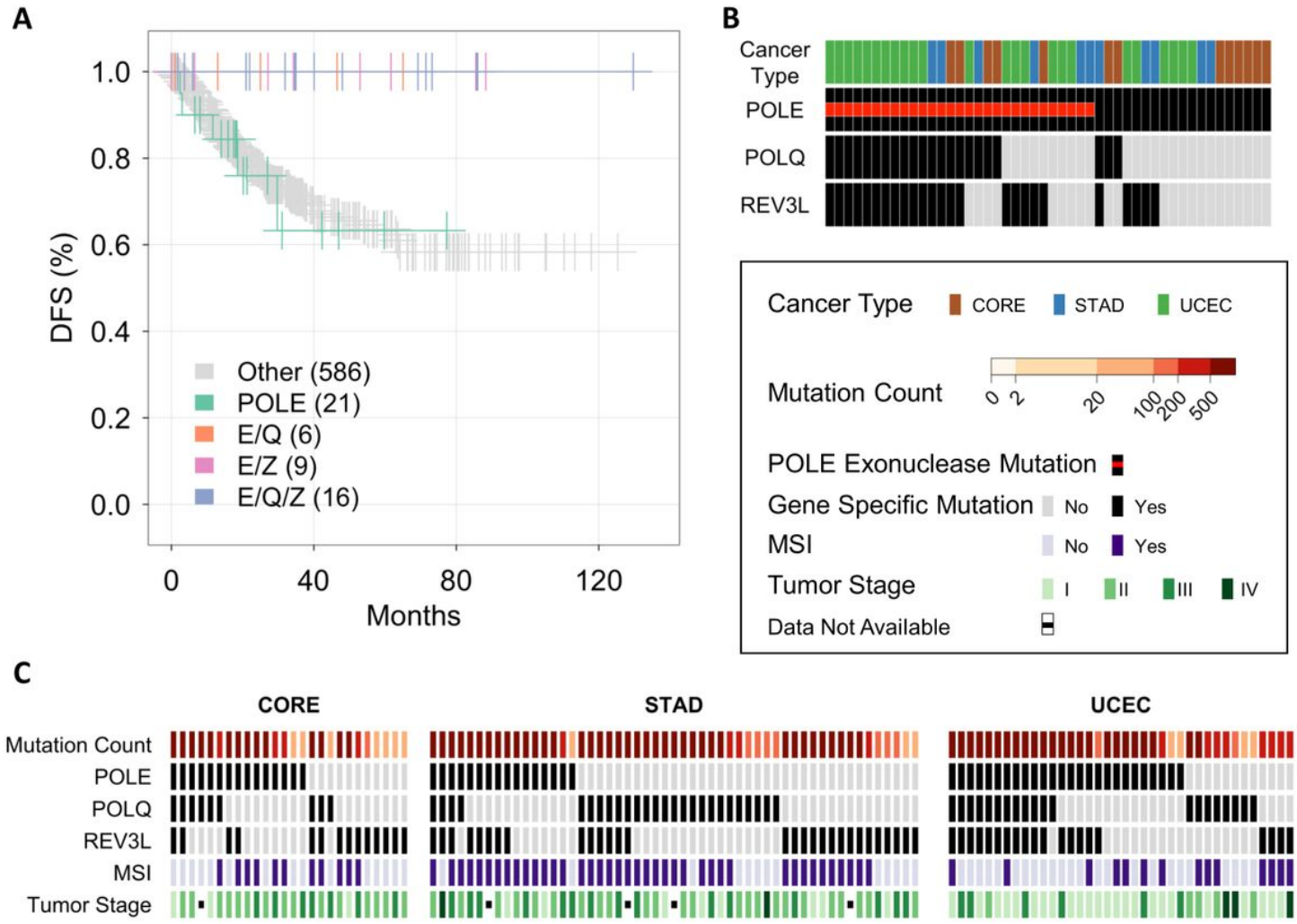


	POLE Exonuclease Mutation	
	Yes	No
POLE	6	17
E/Q	4	2
E/Z	6	6
E/Q/Z	15	1

Figure 2

POLE/Q/Z mutations in colorectal (CORE), endometrial (UCEC) and stomach (STAD) cancers. A) Venn diagram displaying the number of cases with mutations in 1, 2 or 3 POL genes. B) Mutation groups of cases without polymerase mutations (other), or with mutations in POLE only, E/Q, E/Z and E/Q/Z. The number of cases in each group is listed in parenthesis. C) Number of cases with mutations in POLE exonuclease domain in various mutation groups. D) 25 Percentages transitions (Ti) and transversions (Tv) are shown on the Y-axis for CORE, STAD and UCEC. The x-axis shows the mutation groups.



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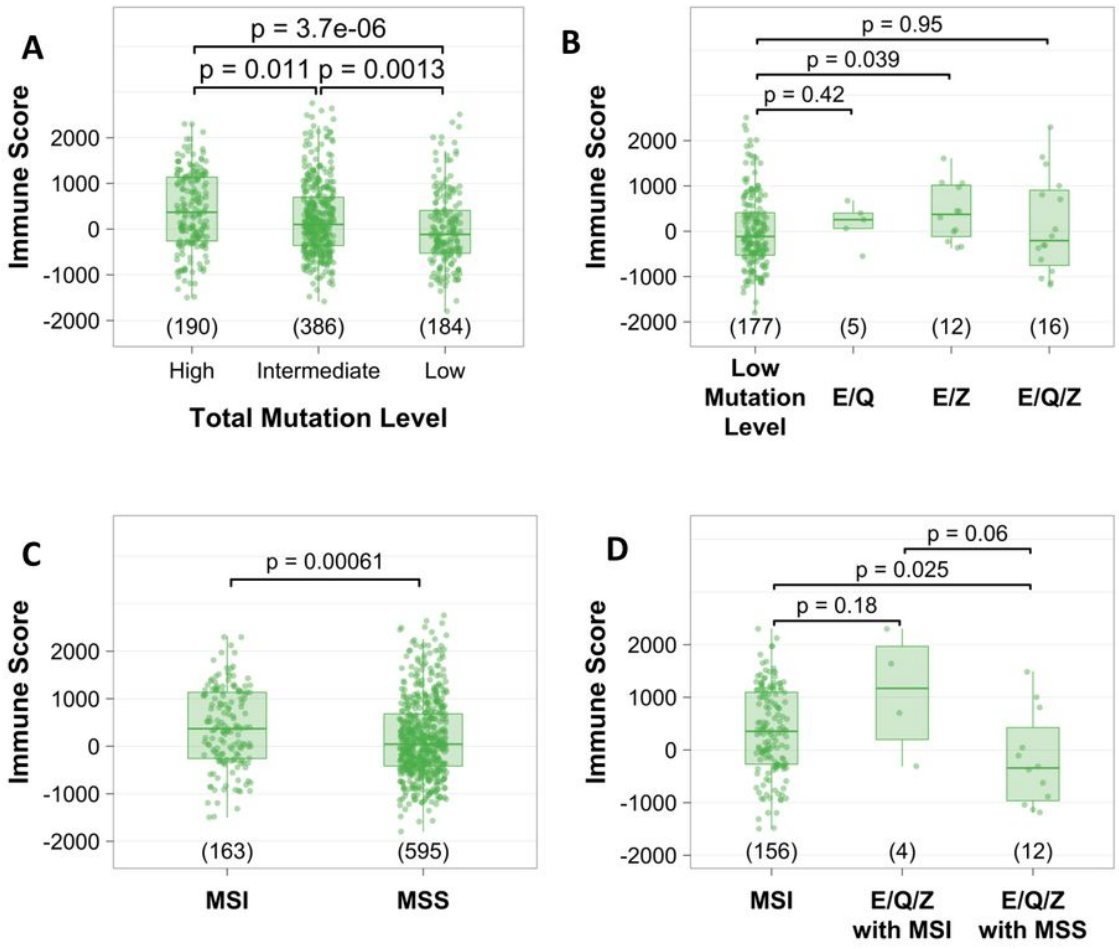

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