Somatic POLE exonuclease domain mutations in endometrial cancer : Insights into the biology of POLE-mutant tumors

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Chapter 2

A panoply of errors: polymerase proofreading domain mutations in cancer

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ABSTRACT

Although it has long been recognized that the exonucleolytic proofreading activity intrinsic to the replicative DNA polymerases Pol δ and Pol ε is essential for faithful replication of DNA, evidence that defective DNA polymerase proofreading contributes to human malignancy has been limited. However, recent studies have shown that germline mutations in the proofreading domains of Pol δ and Pol ε predispose to cancer, and that somatic Pol ε proofreading domain mutations occur in multiple sporadic tumors, where they underlie a phenotype of 'ultramutation' and favorable prognosis. In this review, we summarize the current understanding of the mechanisms and consequences of polymerase proofreading domain mutations in human malignancies, and highlight the potential utility of these variants as novel cancer biomarkers and therapeutic targets.

N.b. Italicized words or phrases are defined in the Glossary following the main text and references.

A PANOPLY OF ERRORS: POLYMERASE PROOFREADING DOMAIN MUTATIONS IN CANCER

Accurate replication of DNA before cell division is a prerequisite for the suppression of mutagenesis and tumor development. The remarkable fidelity of eukaryotic DNA replication – estimated at one incorrect base for every 10^9 to 10^{10} nucleotides replicated[1] – results from a combination of highly accurate base incorporation and exonuclease proofreading by the replicative DNA polymerases Pol δ and Pol ε, and post-replication surveillance by the DNA *mismatch repair* (MMR) apparatus.[2,3] Defects in either polymerase proofreading or MMR increase the mutation rate in *Saccharomyces cerevisiae* and cause tumors in mice.[4-8] Although the importance of MMR deficiency (MMR-D) in human cancer has been recognized for more than two decades,[9,10] until recently, evidence that defective polymerase proofreading contributes to human malignancy has been scarce.[11] However, over the last three years, studies have shown that germline mutations in the proofreading domains of *POLD1* and *POLE* (which encode the catalytic subunits of Pol δ and Pol ε, respectively, in humans) predispose to colorectal cancer (CRC) and other malignancies.[12] Somatic *POLE* proofreading domain mutations are found in 7-12% of endometrial cancers (ECs), in 1-2% of CRCs, and occasionally in tumors of the breast, stomach, pancreas and brain, where they define a distinct, *ultramutated* tumor subgroup.[13-19] *POLE* proofreading domain mutations predict favorable prognosis in EC,[20-24] and may also do so in glioblastoma,[14] possibly because the exceptional number of mutations in these cancers causes an enrichment of antigenic *neopeptides*, leading to an enhanced antitumor immune response.[25,26]

In this review, we summarize the current understanding of the mechanisms and consequences of replicative DNA polymerase proofreading domain mutations in human malignancies. Although we provide an outline of the organization and function of replicative DNA polymerases as a background, we do not cover this subject in detail as it has been described comprehensively in several excellent reviews.[27-30] Instead, we highlight the distinctive clinicopathological and molecular characteristics of replicative DNA polymerase proofreading domain-mutant tumors, and focus on the potential utility of these variants as novel cancer biomarkers and targets for therapy.

DNA polymerase proofreading

Pol δ and Pol ε are the principal eukaryotic DNA replicases, and together are responsible for the bulk of DNA replication, following priming by Pol α.[31-34] They are *B family polymerases* and, unlike Pol α, have a 3′-5′ exonuclease activity that proofreads the newly synthesized DNA strand.[3,35] Both Pol δ and Pol ε comprise four subunits in humans, the largest of which contains the catalytic and proofreading exonuclease active sites, and

is encoded in humans by *POLD1* and *POLE*, respectively.[36] The other smaller subunits (encoded in humans by *POLD2, POLD3* and *POLD4,* and by *POLE2, POLE3* and *POLE4*) also perform several important or essential roles. In the case of Pol δ, they stabilize the holoenzyme complex and stimulate DNA polymerase activity via interactions with the replication processivity factor proliferating cell nuclear antigen (PCNA).[37-39] The essential second subunit of Pol ε mediates the interaction with *GINS* and may help to target the Pol ε holoenzyme to the leading strand during the initiation of DNA replication,[40-42] whereas the non-essential third and fourth subunits are critical for binding double-stranded DNA and for processive DNA synthesis and processive 3′-5′ exonuclease degradation.[43]

Studies of mutant Pol ε and Pol δ polymerases with particular error signatures in *S. cerevisiae* and *Schizosaccharomyces pombe* have suggested a model of DNA replication in which Pol δ replicates the lagging strand following priming by Pol1 (the functional equivalent of the catalytic subunit of human Pol α), whereas Pol ε replicates the leading strand.[44-46] This division of labor has been corroborated by analyses of yeast mutants engineered to misincorporate ribonucleoside triphosphates (rNTPs; reviewed in [47]) and by biochemical reconstitution experiments,[48,49] and is broadly accepted. Indeed, further support for this model – albeit indirect – was provided by the crystal structure of *S. cerevisiae* Pol ε catalytic subunit (Pol2), which revealed the existence of a domain absent in the corresponding Pol δ subunit (Pol3) that could explain its enhanced processivity.[50] However, these roles have been questioned by a very recent publication, which suggests that previous results may have been confused by differential MMR and proposes that Pol δ replicates both the leading and lagging strands, whereas the functions of Pol ε are limited to repair synthesis and proofreading of the leading strand.[51] Although the precise contribution of Pol ε to leading strand replication awaits definitive clarification, current data are concordant in indicating that its exonuclease domain preferentially proofreads the leading strand.[27,51] In addition to their roles in DNA replication, in both yeast and humans both Pol δ and Pol ε also function in base excision repair (BER),[52,53] nucleotide excision repair (NER),[54,55] MMR[56-58] and double-strand break repair.[59,60] Pol ε has also been implicated in cell cycle checkpoint regulation and propagation of chromatin modification states (reviewed in [61]), thus mutations affecting this protein could potentially affect a wider range of cellular activities than just replication fidelity.

The proofreading function of both Pol δ and Pol ε requires several highly conserved exo motifs in their exonuclease domains, within which lie the catalytic site residues that are essential for exonuclease activity (D316 and E318 in Pol δ, and D275 and E277 in Pol ε, in humans; Figure 1).[27,28] Misincorporation of a base into the primer strand results in pausing of the polymerase (due to reduced efficiency in extending a mispaired primer terminus) and a switch from the catalytic to the exonuclease domain.[62] The incorrect

base is then excised and the correct base inserted before DNA synthesis continues.[62] Multiple studies in model organisms have confirmed the essential role of DNA polymerase proofreading in the maintenance of genomic stability. In *S. cerevisiae*, mutants of Pol δ and Pol ε containing alanine substitutions of the exonuclease active site residues have no exonuclease activity, and cells expressing these variants show a ~100-fold increase in mutation rate.[4,63,64] Mice harboring the equivalent substitutions show an

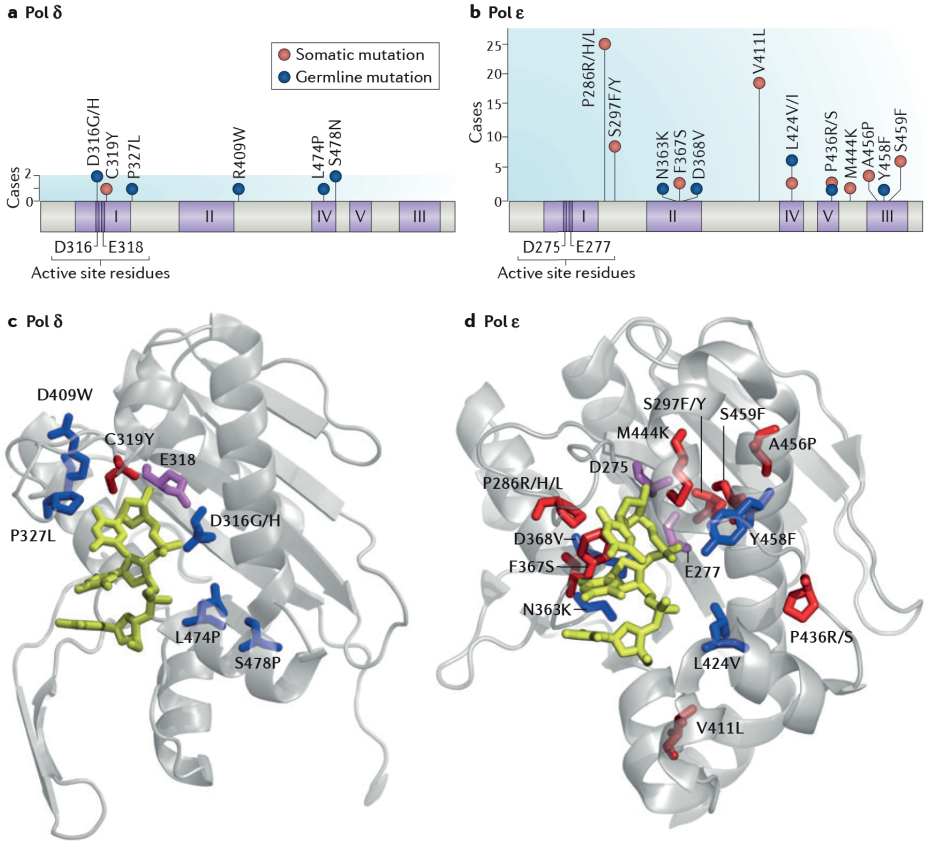

Figure 1. Frequency and location of germline and somatic Pol δ and Pol ε proofreading exonuclease domain mutations in cancers.

A schematic representation of the exonuclease domains of Pol δ (A) and Pol ε (B) showing conserved exo motifs (I-V), exo I active site residues, and the position and frequency of germline and somatic mutations. In the lower panels, the positions of germline (blue) and somatic (red) proofreading domain mutations are mapped to the *Saccharomyces cerevisiae* Pol δ (C, Protein Data Bank identifier (PDB ID) 3IAY[116]) and Pol ε (D, PBD ID 4M8O[50]) exonuclease domain structures. Single-stranded DNA from the aligned bacteriophage T4 polymerase complex (PDB ID 1NOY[118]) is shown in yellow. The exo I motif active site residues are highlighted in magenta (with exception of mutated Pol δ active site residue D316, which is also the site of germline mutations). Note that Pol ε residues L424V and P436R/S are the site of both germline and somatic mutations and are colored according to which alteration is more frequent.

increased mutation rate and develop tumors.[6-8] Notably, cancers only develop in animals homozygous for proofreading-null *Pold1* or *Pole* alleles (the mouse orthologues of *POLD1* and *POLE)*,[6-8] and the tumor spectrum that they develop differs between the two: *Pold1*-mutant mice develop lymphomas and carcinomas of the skin and lung,[7] whereas *Pole* mutants are prone to intestinal tumors and histiocytic sarcomas.[6] A simple explanation for these phenotypes is that defective proofreading leads to an increased mutation rate, as some misincorporated nucleotides escape subsequent correction by MMR, but the reality may be more complicated. For instance, studies in *S. cerevisiae* indicate that elevation of deoxyribonucleoside triphosphate (dNTP) levels by cell cycle checkpoint activation is responsible for the *mutator phenotype* of proofreading-defective DNA polymerases.[65,66]

Proofreading domain mutations in cancer

In 2012, exome sequencing of 224 sporadic CRCs by The Cancer Genome Atlas (TCGA), and a similar analysis of 72 CRC exomes, revealed a subset of ultramutated, yet *microsatellite-stable* tumors with recurrent somatic mutations within the *POLE* exonuclease domain.[13,16] The most common of these involved the replacement of proline by either arginine or histidine at codon 286 (*POLE* P286R/H), and recurring substitutions were also found at codons 411 (*POLE* V411L) and 459 (*POLE* S459F; Table 1).[13,16] Subsequently, two studies detected heterozygous somatic *POLE* proofreading domain mutations, including the P286R and V411L substitutions, in ~7% of sporadic ECs, where they were also associated with ultramutation and microsatellite stability (Table 1).[15,17] These mutations localized to highly conserved or invariant residues within, or close to, the exo motifs that are essential for proofreading activity, and they were predicted to perturb DNA binding by structural mapping (Figure 1B,D).[15] In parallel with these reports, an independent study used linkage analysis and whole genome sequencing to show that families with multiple colorectal tumors but without known predisposition mutations carried heterozygous germline mutations that caused substitutions in the proofreading domains of *POLD1 (POLD1* S478N) and *POLE (POLE* L424V; Table 2).[12] Interestingly, the *POLD1* mutation was also associated with EC. Similar to the somatic *POLE* variants, these mutations affected highly conserved residues in or adjacent to the exo motifs at the DNA-binding interface (Figure 1A,C).[12] Furthermore, the germline variant alleles have been shown to reduce proofreading activity and cause a mutator phenotype in yeast.[12,64]

Current data suggest that germline *POLE* and *POLD1* mutations are present in 0.5-2% of patients in *intestinal polyposis* and CRC cohorts enriched for familial disease.[12,67-69] The *POLE* L424V mutation seems to be the most common deleterious germline variant, with 21 independent carriers identified to date.[68,70,71] Although predominantly associated with CRC, this variant also predisposes to EC, and it may confer moderately increased

Table 1. Pathogenic somatic Pol δ and Pol ε proofreading exonuclease domain mutations that occur in sporadic cancers.

	Evidence in support of pathogenicity				Molecular characteristics*			
Protein change (nucleotide substitution)	**Structural mapping**	**PhyloP score‡**	**Yeast fluctuation assay**	**Biochemical evidence for functional effect§**	**Median number of mutations per exome (range)‖**	**Percentage MSS**	**Percentage of tumors with ≥20% C>A substitutions**	**Tumor type(s)**
Pol δ								
C319Y (956G→A)	Within exo I motif	2.38	Yes[64]	NR	7,349 (44-14,654)	100	NA	GBM[77] and MM[78]
Pol ε								
P286R/H/L (857C→G/A/T)	Flanking exo I motif	2.57	Yes[82]	Yes[18]	5,147 (738-16,248)	100	93	CRC,[13,16,82] EC,[15,17,20,21,24] GBM,[14] EOC,[118] and BrC[119]
S297F/Y (890C→T/A)	Flanking exo I motif	2.67	NR	NR	5,429 (4,918–15,545)	50	66	EC,[15,17,20,21,24] GBM,[14,77] EOC,[75] and CC[119]
F367S (1100T→C)	Exo II active site	2.19	NR	Yes[18,120]	2,934	100	100	CRC[11,13]
V411L (1231G→C/T)	Flanking exo IV motif	2.66	NR	Yes[18]	6,294 (955–14,074)	100	88	CRC,[13] EC,[15,17,20,21,24] GBM,[14] EOC,[118] and GC[79]
L424V/I (1270C→G/A)	Exo IV active site	2.66	Yes[64]	Yes[18,120]	163 (85–6,724)	50	100	EC[17] and BrC[119]
P436R (1307C→G)	Exo V motif	3.53	NR	Yes[120]	6,131	100	100	CRC[13] and EC[21]
M444K (1331T→A)	Flanking exo V motif	2.15	NR	NR	1,204	100	100	EC[17]
A456P (1366G→C)	Within exo III motif	2.61	NR	NR	5,968	100	100	CRC[18,121] and EC[15,17,21]
S459F (1376C→T)	Within exo III motif	2.52	NR	Yes[18,120]	4,780 (1,868–9,907)	100	75	CRC,[13,18,121] GBM,[14] and AA[77]

*Data from exome sequencing studies.

‡PhyloP (phylogenetic conservation) scores were calculated per nucleotide using the alignment of 46 vertebrates dbNSFPv23. For P436R the variant mapped to the third position of a codon so the average PhyloP score for the codon is displayed.

§Data from studies of B family polymerases.

‖Number of exomes sequenced: *POLD1* C319Y, 2; *POLE* P286R/H/L, 16; *POLE* S297F/Y, 5; *POLE* F367S, 1; *POLE* V411L, 10; *POLE* L424V/I, 3; *POLE* P436R, 1; *POLE* M444K, 1; *POLE* A456P, 1; *POLE* S459F, 4.

Abbreviations: AA, anaplastic astrocytoma; BrC, breast cancer; CC, squamous cell cervical carcinoma; CRC, colorectal cancer; EC, endometrial cancer; EOC, endometrioid ovarian carcinoma; GBM, glioblastoma; GC, gastric cancer; MM, multiple myeloma; MSS, microsatellite stable; NA, not applicable; NR, not reported.

Table 2. Pathogenic germline Pol δ and Pol ε proofreading exonuclease domain mutations.

	Evidence to support pathogenicity of variant							Percentage of variant carriers with indicated phenotype									
Protein change (nucleotide substitution)	**Structural mapping**	**PhyloP score***	**Yeast fluctuation assays**	**Biochemical evidence†**	**Segregates with affection status**	**Number of carriers and unrelated carriers (total)§**	**Mean age at diagnosis (range)‖**	**CRC**	**EC**	**BrC**	**DuC**	**OC**	**GBM**	**Duodenal A or P**	**Colonic A or P**	**Other cancers reporter in carriers**	**Refs**
Pol δ																	
D316G (947A→G)	Exo I motif active site	1.93	Yes[64]	Yes[120]	Yes	2 and 1 (3)	51 (44–57)	50	100	50	0	0	0	0	50	0	67
D316H (946G→C)	Exo I motif active site	1.16	Yes[64]	Yes[120]	Yes	2 and 1 (3)	61 (58–64)	50	0	50	0	0	0	0	100	Mesothelioma	67
P327L (981C→G)	Flanking exo I motif	2.16	Yes[82¶]	Yes[18¶]	NR	1 and 1 (2)	70	0	0	0	0	0	0	0	100	0	12
R409W (1225C→T)	Flanking exo II motif	2.26	NR	NR	NR	1 and 1 (2)	32	100	0	0	0	0	0	0	100	0	67
L474P (1421T→C)	Exo IV motif	1.92	Yes[64]	Yes[18¶]	Yes	6 and 2 (8)	40 (23–52)	67	33	0	0	0	0	0	17	0	67, 70
S478N (1433G→A)	Exo IV motif	1.19	Yes[12]	NR	Yes	11 and 3 (14)	35 (26–52)	45	36	0	0	0	0	0	91	Astrocytoma	12
Pol ε																	
W347C (1041G→T)	Outside exo motifs	2.71	Yes[74]	NR	No#	11 and 1 (12)	49 (14–70)	0	0	0	0	0	0	NR	NR	Prostate, cutaneous and uveal melanoma	74
N363K (1089C→A)	Exo II motif active site	4.9	NR	NR	Yes	12 and 1 (13)	41 (28–56)	75	17	0	0	25	0	NR	83	Pancreatic	72
D368V (1103A→T)	Exo II motif active site	5.13	NR	Yes[120]	NR	1 and 1 (2)	47	100	0	0	0	0	0	0	0	0	69

Table 2. (continued) Pathogenic germline Pol δ and Pol ε proofreading exonuclease domain mutations.

	Evidence to support pathogenicity of variant							Percentage of variant carriers with indicated phenotype									
Protein change (nucleotide substitution)	**Structural mapping**	**PhyloP score***	**Yeast fluctuation assays**	**Biochemical evidence‡**	**Segregates with affection status**	**Number of carriers and unrelated carriers (total)§**	**Mean age at diagnosis (range)‖**	**CRC**	**EC**	**BrC**	**DuC**	**OC**	**GBM**	**Duodenal A or P**	**Colonic A or P**	**Other cancers reporter in carriers**	**Refs**
L424V (1270C→G)	Exo IV motif active site	2.66	Yes[64]	Yes[18]	Yes. 2 *de novo* carriers	48 and 21 (69)	39 (16–64)	61	2	2	2	2	4	19	92	ODG and neuro-endocrine carcinoma	12, 68-71
P436S (1306C→T)	Within exo V motif	3.53	NR	NR	*De novo*	1 and 1 (2)	31	100	0	0	0	0	0	100	100	0	68
Y458F (1373A→T)	Exo III motif active site	4.97	NR	Yes[120]	Yes	13 and 2 (15)	48 (38–63)	62	0	0	15	8	0	NR	62	Pancreatic	73

*PhyloP (phylogenetic conservation) scores were calculated per nucleotide using alignment of 46 vertebrates dbNSFPv23. If a variant mapped to the third position of a codon the average PhyloP score for the codon is displayed.

‡Data from functional studies of B family polymerases.

§Reported as of August 2015.

‖Mean age at diagnosis in years refers to cancer or adenoma diagnosis, whichever was earliest.

¶Functional studies of the corresponding residue in Pol ε.

#Six mutation carriers were unaffected.

Abbreviations: A or P, adenomas or polyps; BrC, breast cancer; CRC, colorectal cancer; DuC, duodenal carcinoma; EC, endometrial cancer; GBM, glioblastoma; NR, not reported; OC, ovarian cancer; ODG, oligodendroglioma.

risk of many other tumor types (including carcinomas of the breast, stomach and ovary, brain tumors, and duodenal adenomas and carcinomas; Table 2). The demonstration of *de novo POLE* L424V mutations[70,71] and the absence of a detectable haplotype shared among apparently unrelated families[12] suggests that this is a *mutational hot spot*. Other recently reported germline *POLE* mutations include N363K, which maps to the exo II motif active site and is associated with CRC and pancreatic cancer;[72] Y458F, which affects the exo III motif active site and predisposes to multiple tumor types;[73] and W347C, which lies outside the exo motifs and has been associated with melanoma (Figure 1B,D; Table 2).[74] *POLE* N363K and *POLE* Y458F are highly penetrant, being associated with malignancy in 11 of 12 and 9 of 13 carriers, respectively (Table 2).[72,73] The importance of the W347C variant is uncertain, as it seems to display lower penetrance, with six unaffected carriers in the family, and it does not seem to confer a strong risk of CRC (<10% cases), despite evidence of pathogenicity in *S. pombe*.[74]

Although no additional *POLD1* S478N carriers have been reported since 2012, five other pathogenic germline *POLD1* proofreading domain mutations have been identified (Figure 1A,C; Table 2). One of these, a recurrent mutation at codon 474 (*POLD1* L474P),[67,70] affects the equivalent residue to *POLE* L424V, and another, *POLD1* D316G, affects the exo I motif active site.[67] The cancer risk in carriers of germline *POLD1* proofreading domain mutations seems to be limited to CRC and EC, with no evidence of a predisposition to duodenal or ovarian malignancies.[67,70] However, caution is required, as the modest number of germline *POLD1* variant carriers makes phenotypic characterization less certain than it is for *POLE* variant carriers. Interestingly, similar to the *POLE* active site variants, the germline *POLD1* D316G mutation appears to be highly penetrant (malignancy in 4 of 4 carriers).[67]

Somatic *POLE* proofreading domain mutations occur in ~1-2% of CRCs[13,16] and 7-12% of ECs,[15,17,20-23] and they have also been detected in ultramutated tumors of the brain, pancreas, ovary, breast and stomach,[14,18,75] as well as in uterine carcinosarcomas.[76] The most common *POLE* variants are the P286R and V411L substitutions (Figure 1B,D; Table 1); other recurrent substitutions include S297F, A456P and S459F. Most of these occur within, or close to, the exo motifs; however, unlike the germline mutations, the catalytic sites themselves are seldom affected.[13,15] In contrast to *POLE*, to date only two cases of possibly pathogenic somatic *POLD1* proofreading domain mutations have been identified. The first is a C319Y variant detected in an ultramutated pediatric glioblastoma from a patient with biallelic congenital MMR-D (CMMR-D) due to a compound heterozygous mutation in mutS homolog 6 (*MSH6:* c.3984_3987dupGTCA;c.3959_3962delCAAG).[77] The *POLD1* C319Y mutation has also been detected in a myeloma with a normal mutation burden,[78] and its functional effect is uncertain at present. The second is a D316N

substitution in a highly mutated sporadic gastric cancer.[79] This tumor displays a high proportion of insertions and deletions (indels), and as the *POLD1* variant allele fraction is only 0.13, it is unclear to what extent this substitution has contributed to the mutation burden in this tumor.

The difference in the frequency of somatic proofreading domain mutations between *POLD1* and *POLE* is both notable and unexplained. It seems unlikely to be due to bias in the tumor types analyzed to date: the exomes of more than 400 lung adenocarcinomas – a common tumor type in *Pold1*-proofreading-null mice – have been sequenced at the time of writing.[80,81] Given the current uncertainty regarding the roles of Pol δ and Pol ε at the replication fork,[51] it will be intriguing to see whether future studies reveal to what extent, if any, this discrepancy reflects differential contributions of the two polymerases to DNA replication and repair, or other processes.

Functional insights into pathogenicity

The deleterious consequences of cancer-associated polymerase proofreading domain mutations on exonuclease activity and mutation rate have been confirmed by functional studies. In cell-free assays, the somatically occurring *POLE* P286R/H and *POLE* S459F mutations reduce exonuclease activity to levels similar to those of a proofreading-null *POLE* construct with substitution of both exo I active site residues, whereas the somatic *POLE* V411L and *POLE* F367S variants and the *POLE* L424V variant (which can be either somatic or germline) substantially reduce, but do not eliminate, exonuclease function.[18] Interestingly, the substitution equivalent to human *POLE* P286R was recently shown to confer an exceptionally strong mutator phenotype in *S. cerevisiae*.[82] This far exceeded that of the proofreading-null allele, suggesting that *POLE* P286R may exert effects beyond proofreading alone.[82] Importantly, in this study the mutation rate was also substantially increased in diploid strains heterozygous for the *POLE* P286R analogue.[82] These results provide a possible explanation for why mice with proofreading-null *Pole* alleles only develop tumors in the homozygous state,[6] yet somatic *POLE* proofreading domain mutations in human cancers seem to be mostly, if not all, heterozygous changes.[15,18] This may also explain the paucity of exonuclease active site mutations in sporadic cancers. Given these exciting data, the results of similar analyses of other cancer-associated mutations are eagerly awaited.

Clinicopathological characteristics

Intestinal polyposis seems to be more severe in carriers of germline *POLE* proofreading domain mutations than in those with *POLD1* proofreading domain variants (10-50 compared with <20 colonic polyps per patient, respectively); however, in both cases the polyps themselves are typically of unremarkable histology.[12] Similarly, with the ex-

ception of young age at onset (typically <50 years), CRCs and other malignancies in these patients do not seem to display any distinguishing clinicopathological features, although the number of cases studied to date is limited.[12] Tumors with somatic *POLE* proofreading domain mutations also show a strong tendency to occur in young patients and, when examined, typically exhibit several other notable characteristics, including an association with high tumor grade, a prominent lymphocytic infiltrate and the presence of multiple bizarre *multinucleated giant cells*.[14,25,83]

An association of somatic *POLE* proofreading domain mutations with good prognosis was first suggested by the EC study from TCGA.[17] This has since been confirmed in EC by several groups[20-24] and was also proposed for glioblastomas by a recent report.[14] As many patients in these series received adjuvant radiotherapy, either alone or in combination with chemotherapy, it is currently not possible to draw definitive conclusions on whether *POLE* proofreading domain mutation status is a prognostic or predictive biomarker, or indeed both. However, the lack of recurrences of high-grade *POLE* proofreading domain-mutant ECs in two large clinical trials in which chemotherapy was not used, and in a subset of patients who did not receive radiotherapy in one of these studies ,[20] suggests that *POLE* proofreading domain mutations may portend a good prognosis in the absence of postoperative treatment. While this is reminiscent of the favorable clinical outcome of hypermutated early-stage, MMR-D CRCs,[84,85] it should be noted that the impact of MMR-D on EC prognosis is unclear,[22,86,87] and the prognostic import of *POLE* proofreading domain mutations in CRC awaits confirmation.

Molecular characteristics

Although the number of tumors from germline *POLD1* and *POLE* proofreading domain mutations analyzed to date is relatively modest, most have been found to be microsatellite stable;[12] however, *microsatellite instability* (MSI) has been noted in some cases.[71] Analysis of the mutation spectrum has been limited to the tumors from *POLE* L424V and *POLD1* S478N carriers, which revealed a phenotype of base substitutions and missense substitutions with relatively few frameshift mutations.[12] As noted above, the most striking feature of tumors with somatic *POLE* proofreading domain mutations is their ultramutation.[13,16,18,19] Similar to the tumors from germline *POLE* variant carriers, these are predominantly base substitution mutations,[13,15,16,18,19] and they occur within a unique mutation signature with a 100-fold increase in C→A transversions in the context TCT and a 30-fold increase in C→T transitions in the context of TCG.[15,18,19] This results in a strong bias for particular amino acid changes, with an overrepresentation of serine to tyrosine or leucine, and arginine to isoleucine or glutamine substitutions, and a substantial increase in glutamic acid to stop codon mutations.[18] These manifest as a distinctive pattern of missense and truncation mutations in oncoproteins and tumor suppressors, including:

PI3K catalytic subunit-α (*PIK3CA*) R88Q; *PTEN* R130Q; adenomatous polyposis coli (*APC*) R1114X and *APC* Q1338X; *MSH6* E946X and *MSH6* E1322X; F-box and WD repeat domain-containing 7 (*FBXW7*) E369X; and *TP53* R213X.[12,13,15,16,18,88]

Sporadic *POLE* proofreading domain-mutant cancers display few copy number alterations (CNAs) and, similar to tumors in germline variant carriers, a strong tendency to microsatellite stability, despite frequent mutations in MMR genes.[13,15-17] Accumulating evidence suggests that the interaction between defective DNA polymerase proofreading and MMR is complex and may depend on the extent to which the function of each activity is compromised. For example, whereas the combination of mutator DNA polymerases and partial MMR function causes attenuated growth in *S. cerevisiae,*[89] the combination with complete MMR loss is *synthetically lethal* in *S. cerevisiae* and mice.[6,89,90] Interestingly, a recent study of patients with CMMR-D who developed glioblastomas showed that these tumors occurred following the acquisition of DNA polymerase proofreading domain mutations and were ultramutated, yet predominantly microsatellite stable.[77] Most of the germline MMR mutations in these cases involved either PMS1 homolog 2 (*PMS2*) or *MSH6*, whereas sporadic DNA polymerase proofreading domain-mutant tumors tend to acquire *MSH6* mutations.[13,17,77] Loss of MSH6 or PMS2 function generally causes a milder mutator phenotype, sometimes lacking MSI, than loss of other MMR components such as mutS homolog 2 (MSH2) and mutL homolog 1 (MLH1).[89,91] Although it is tempting to speculate that the combination of defective proofreading and profound MMR deficiency may result in a mutation rate that exceeds the optimum for tumor fitness,[92,93] empirical verification of this is currently lacking, and it must be noted that although they are rare, tumors with DNA polymerase proofreading domain mutations and MSI have been reported.[21,71,77] It will be of particular interest to determine the timing and clonality of both events in these cancers, and to examine whether these tumors harbor secondary *antimutator mutations* that permit continued viability, as has been demonstrated in yeast.[89,94]

Challenges in determining pathogenicity

Although the number of confirmed germline *POLD1* and *POLE* proofreading domain mutations has grown since their initial report in 2012 (Table 2; also curated in the Leiden Open Variation Database (LOVD)), differentiation of pathogenic from non-pathogenic variants in a patient with clinical features that suggest the possibility of DNA polymerase proofreading domain mutations and family history remains challenging. The Exome Aggregation Consortium (ExAC) database currently lists 56 missense or loss-of-function variants mapping to the exonuclease domains of *POLD1* and 75 such variants mapping to the *POLE* exonuclease domain, all of which have a population frequency of <1%. Most of these are likely to have no impact on the proofreading function of these enzymes. In-

deed, of the germline variants reviewed here, only *POLD1* D316H is present in the ExAC database, at a frequency of 0.001% (being present in only 1 of 98,012 alleles genotyped). Therefore, filtering variants according to their presence in ExAC and similar databases will be of limited utility, as it will only exclude variants with relatively high frequencies, such as those with a frequency of >0.1%. Instead, we suggest that the following criteria should ideally be satisfied to prove pathogenicity of a variant: first, segregation with affection status in pedigrees; second, conservation of the affected residue between human DNA polymerases and those from other species; third, evidence of functional effect from at least one of the following: analysis of corresponding residue in model organisms, cell-free exonuclease assays or other polymerase assays;[18,95] and fourth, demonstration of elevated base substitution frequency by sequencing of tumor DNA.

Confirming the pathogenicity of somatic DNA polymerase proofreading domain mutations may also be difficult. Both *POLD1* and *POLE* are large genes, and they are likely to acquire somatic mutations secondary to other causes of increased mutation burden, such as MMR-D. Given the association of somatic *POLE* proofreading domain mutations with prognosis,[14,17,20-24] it is important to differentiate these pathogenic variants from passenger mutations that are of no functional consequence, particularly given examples in the recent literature where a functional role has been inferred for *POLD1* and *POLE* variants of uncertain pathogenicity.[96]

On the basis of their analysis of TCGA data, Shinbrot and colleagues[18] have proposed criteria to identify bona fide pathogenic somatic *POLE* proofreading domain mutations. These include: ultramutation (often exceeding 100 mutations per megabase); an increased proportion of C→A transversions, exceeding 20% of all substitutions (although it should be noted that the most common substitutions are typically C→T transitions); and *POLE* mutation at a residue that is recurrently mutated in cancer (Figure 1A; Table 1). While most *POLE* proofreading domain-mutant cancers display these characteristics, we advocate a degree of flexibility in their application, as the predominant mutations caused by proofreading-deficient human Pol ε *in vitro* are T→A transversions,[97] and it is possible that novel pathogenic *POLE* proofreading domain mutations could cause a different mutational signature. We also suggest that consideration should be given to other features that may indicate a pathogenic variant including: a preponderance of missense mutations (that is, a relative absence of indels); the presence of characteristic flanking nucleotide bias;[15,18,19] and a relative lack of CNA.[15,17] Although the coexistence of MSI does not preclude the presence of a deleterious DNA polymerase proofreading variant, it should prompt careful evaluation of its pathogenicity, ideally using bioinformatic predictors (for example, MutationTaster,[98] SIFT[99] and Polyphen[100]) and structural mapping using the conserved yeast Pol ε structure.[50] Although some tumors carrying

bona fide pathogenic *POLE* proofreading domain mutations will fall outside this classification,[21] we believe that it represents a reasonable starting point that can be refined and improved as further data are accumulated in the future.

A role in early tumorigenesis?

The association of germline variants with malignancy[12] suggests that DNA polymerase proofreading domain mutations are able to initiate cancer, and current data, although limited, are consistent with somatic *POLE* proofreading domain mutations occurring as an early event in sporadic tumors. Analysis of the variant allele fraction in glioblastomas suggests that *POLE* proofreading domain mutations are present in the earliest persisting clone,[14,77] and genes in which driver mutations are known to occur early in CRC and EC frequently display evidence of the *POLE* proofreading domain mutation signature.[13-18] Interestingly, analysis of tumors from patients with CMMR-D suggests that the acquisition of somatic pathogenic DNA polymerase proofreading domain mutations is associated with rapid tumor growth,[77] consistent with the prediction of mathematical models that a strong mutator phenotype confers a preferential advantage in early tumorigenesis.[1,101] Further insights into the timing and consequences of polymerase proofreading domain mutations may be provided by analysis of pre-cancers and multiregion sequencing of tumors carrying these variants.

Explaining associations with prognosis

As noted previously, in addition to ultramutation and good prognosis, *POLE* proofreading domain-mutant tumors frequently display a prominent lymphocytic infiltrate,[14,25,83] similar to that observed in MMR-D CRCs.[102] Recent characterization of this in EC has shown that this infiltrate comprises a population of $CD8^+$ cytotoxic lymphocytes and is accompanied by increased expression of cytotoxic lymphocyte differentiation markers (such as T-box 21 (*TBX21*) and eomesodermin (*EOMES*)), and effectors (such as interferon-γ (*IFNG*), perforin 1 (*PRF1*) and granzyme B (*GZMB*)) compared with other ECs .[25] Bioinformatics analysis revealed that the number of 'antigenic mutations' – that is, mutations in expressed genes that encode neopeptides predicted to bind major histocompatibility complex (MHC) molecules – is substantially greater in *POLE* proofreading domain-mutant tumors than in other ECs, providing a potential explanation for these results.[25,26] Interestingly, *POLE* proofreading domain-mutant tumors displayed increased expression of genes encoding multiple immunosuppressive checkpoints — including programmed cell death protein 1 (*PDCD1*), PD-1 ligand 1 (*PDL1*), cytotoxic T lymphocyte-associated antigen 4 (*CTLA4*), lymphocyte activation gene 3 protein (*LAG3*), T cell immunoglobulin mucin receptor 3 (*TIM3*; also known as *HAVCR3*) and T cell immunoreceptor with immunoglobulin and ITIM domains (*TIGIT*)[25] — suggesting that upregulation of these molecules may be required for tumor growth. It will be important to

investigate whether these findings are observed in *POLE* proofreading domain-mutant tumors of other tissues and to investigate whether tumors from carriers of germline DNA polymerase proofreading variants display evidence of enhanced immunogenicity that may affect their prognosis.

Another possible contributor to the favorable outcome of DNA polymerase proofreading domain-mutant tumors relates directly to their mutator phenotype. Studies of mutator Pol ε and Pol δ variants in yeast have demonstrated the existence of an 'error threshold' that, if exceeded, results in reduced viability.[89,90,94] The combination of defective DNA polymerase proofreading and complete loss of MMR is lethal as it exceeds this threshold.[89,94] Interestingly, sequential biopsy of cancers in patients with CMMR-D demonstrated that although the acquisition of somatic DNA polymerase proofreading domain mutations was associated with the dramatic accumulation of mutations and rapid tumor growth, the total mutation burden subsequently seemed to plateau.[77] The upper limit of 10,000–20,000 exonic mutations suggested by this study is highly concordant with the number of mutations observed in sporadic *POLE* proofreading domain-mutant adult cancers.[19] Tumor mutation burden is not simply a reflection of mutation rate (it also reflects the number of cell divisions during tumor development), and the relationship between the two in DNA polymerase-mutant tumors in uncertain. Nevertheless, it may be speculated that while the proofreading-deficient mutator phenotype confers a growth advantage in early tumorigenesis, it compromises fitness in later-stage cancers. This may be explained by an intriguing recent study that used mathematical modelling to predict the effect of passenger mutations on the fitness of (non-hypermutated) tumors.[103] This showed that whereas strongly deleterious passenger mutations are subject to negative selection, those with individually moderate negative effects are able to fixate (become fixed into the genome), and collectively are predicted to exert a negative impact on tumor growth.[103] As this model was based on tumors with only relatively small numbers of protein-coding mutations (an average of 10-366 per tumor type), it will be interesting to examine the effect of passenger mutations on the fitness of DNA polymerase proofreading domain-mutant tumors.

Clinical management

Diagnosis

The *polymerase proofreading-associated polyposis* (PPAP) phenotype overlaps with that of *Lynch syndrome* and *MUTYH-associated polyposis*, and screening and management algorithms are broadly similar. Valle and colleagues[70] have proposed that screening of the *POLD1* and *POLE* exonuclease domains is indicated in patients with polyposis (10-100 adenomas) and/or those with early-onset CRC (diagnosis before the age of 50), who lack germline mutations in MMR genes, *APC* or *MUTYH*. The concomitant presence of

extracolonic tumors (Table 2), particularly EC and stomach or duodenal tumors, should increase clinical suspicion of germline *POLD1* or *POLE* proofreading domain mutations. However, at present there is insufficient evidence to recommend screening of patients who lack colonic phenotypes. Similar to patients with Lynch syndrome,[104] carriers of germline *POLD1* and *POLE* proofreading domain mutations should be offered colonoscopies at one- to two-year intervals from the age of 25 and upper gastrointestinal endoscopy to check for duodenal tumors (particularly in carriers of *POLE* proofreading domain variants). Although EC screening is not of proven benefit for this cohort, women with germline *POLD1* and *POLE* proofreading domain mutations might pragmatically be offered this from the age of 40, and clinicians should be aware of the potential increased risk of ovarian, brain and breast cancers in these patients, with consideration given to preventive measures if available.

The distinctive characteristics of tumors with somatic *POLE* proofreading domain mutations also have potential implications for patient management. Most notably, the association with good prognosis in EC[17,20-24] suggests that *POLE* proofreading domain mutations might identify a group of patients who are less likely to benefit from adjuvant treatment following surgery. This is clinically relevant, as *POLE* mutations are more common in tumors defined as 'high risk' by conventional criteria,[20,22] for which postoperative radiotherapy and chemotherapy are often recommended. However, as noted earlier, the possibility that the favorable outcome of these tumors reflects increased sensitivity to treatment cannot be excluded at present, and further preclinical and clinical studies will be required before *POLE* proofreading domain mutations can be used to guide management in EC. Similar studies will also be needed to confirm the utility of *POLE* mutation status as a biomarker in other cancer types.

Therapeutic targeting

The remarkable mutation burden of *POLE* proofreading domain-mutant tumors also raises the possibility that they may be particularly sensitive to specific therapeutic strategies. Perhaps the most obvious of these is the use of immune checkpoint inhibitors that target immunosuppressive molecules such as PD-1 and PD-L1. These agents have recently demonstrated striking activity against highly mutated MMR-D CRCs, melanomas and non-small cell lung cancers (NSCLCs),[105-108] in which response seems to correlate with increased tumor antigenic mutation burden[96,109] and the density of pretreatment intratumoral infiltration by $CD8^+$, PD-1^+ and PD-$L1^+$ lymphocytes.[110] These are all prominent features of *POLE* proofreading domain-mutant tumors,[25,26] suggesting that these cancers may be excellent candidates for drugs targeting PD-1-PD-L1 signaling. Furthermore, the recent demonstration that immune checkpoint inhibition may be potentiated by radiotherapy[111] suggests that investigation of these combinations against

DNA polymerase proofreading domain-mutant cancers in preclinical models and clinical trials may be worthwhile.

Another potential therapeutic strategy against DNA polymerase proofreading domain-mutant cancers relates to the concept of the error threshold discussed earlier. In theory, agents such as mutagenic nucleosides or inhibitors of DNA repair[112] could be used to increase the mutation rate in these tumors to a level that exceeds this threshold, resulting in lethal mutagenesis and loss of viability.[93] Clearly, in the first instance such a strategy would be appropriate only in patients with incurable disease who lack other treatment options, although in selected cases this may be worthy of exploration.[93] Preclinical studies suggest that a similar effect may result from modification of the dNTP pool;[67,113] however, differences in nucleotide synthesis and the DNA damage response between yeast and humans mean that further work is required before the possible utility of this approach in humans can be predicted.

Given the modest frequency of DNA polymerase proofreading domain mutations overall, any therapeutic study is likely to have to recruit patients with multiple tumor types (a design frequently referred to as a *basket trial*[114]) or to combine proofreading-deficient tumors with other hypermutated cancers, such as those with defective MMR.

Conclusions and future directions

Evidence has only recently emerged to support the longstanding postulate that defective DNA polymerase proofreading may contribute to human cancer.[115] Nevertheless, it is now clear that germline mutations in the exonuclease domains of *POLD1* and *POLE* predispose to polyposis, CRC and other malignancies,[12,67-73] and that somatic *POLE* proofreading domain mutations cause ultramutation in sporadic ECs, CRCs and several other cancers.[13-19] The exceptional mutation load in somatic *POLE* proofreading domain-mutant ECs is associated with an enhanced immune response[25,26] and an excellent prognosis.[17,20-24] The possibility that mutation rate in these tumors approaches the maximum compatible with continued viability is an intriguing one, the investigation of which may provide novel insights into the consequences of a mutator phenotype in cancer. It will also be of interest to determine whether the ultramutator phenotype in DNA polymerase proofreading domain-mutant tumors represents an Achilles' heel that can be exploited for therapy, as has recently been suggested.[77]

From a clinical perspective, PPAP should be considered and tested for in patients with unexplained polyposis and/or early-onset CRC, particularly if family members have EC or other extracolonic cancers suggestive of germline *POLD1* or *POLE* proofreading domain mutations.[70] Meanwhile, somatic DNA polymerase proofreading domain mutations

exemplify the challenge of implementing precision medicine. For example, the modest frequency of *POLE* proofreading domain mutations in EC (7-12%) has limited the power of some studies to evaluate their impact on prognosis,[21] a problem that is likely to prove even greater in other tumors in which these mutations are less common. As most novel cancer variants occur at a similarly low frequency (<10%), evaluation of these as prognostic and predictive biomarkers will require large-scale collaborations and coordinated analysis. At a more basic level, the results of studies performed to date pose several fundamental questions. For example, why are somatic mutations largely restricted to *POLE*, whereas germline variants of both *POLE* and *POLD1* cause cancer? Why are substitutions of the exo motif catalytic sites proportionally more common among germline than among somatic proofreading domain mutations? Why does the mutator phenotype associated with *POLE* P286R exceed that associated with the exonuclease-null allele? Determining the answers to these questions will be a priority for future studies.

During the past three years, defects in DNA polymerase proofreading have been recognized to drive tumorigenesis in a small but important fraction of common cancers. Given the rapid progress in the field, we are optimistic that the next three years will see similar advances in our understanding of this novel tumor subgroup, with consequent benefits for patients.

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GLOSSARY

Antimutator mutations – mutations that result in an increase in the fidelity of DNA replication; examples have been reported in both *Escherichia coli* and *Saccharomyces cerevisiae*, although their occurrence in human cancer is currently not proved.

B family polymerases – replicative and repair DNA polymerases that are grouped on the basis of sequence and structural similarities, and include the prokaryotic DNA polymerase Pol II, and the eukaryotic DNA polymerases Pol ζ, Pol α, Pol δ and Pol ε.

Basket trial – also known as a bucket trial. A study in which patients with a specific molecular marker are recruited for treatment with a corresponding targeted therapy, irrespective of tumor histology or tissue of origin.

GINS – a multiprotein complex essential for eukaryotic DNA replication. The name is derived from the Japanese go, ichi, ni and san (meaning five, one, two and three), and refers to the constituent components in budding yeast: Sld5, Psf1, Psf2 and Psf3.

Intestinal polyposis – a phenotype of multiple colonic polyps and increased risk of colorectal cancer that may be due to a germline mutation in a known predisposition gene (for example, adenomatous polyposis coli (APC) or MUTYH) or may lack an obvious genetic cause.

Lynch syndrome – a tumor predisposition syndrome caused by germline mutation of mismatch repair genes, which manifests as increased risk of carcinomas of the colon and rectum, endometrium, ovary, stomach and several other organs.

Microsatellite instability (MSI) –the presence of small insertions and deletions (indels) at repetitive DNA microsatellites that result from defective DNA mismatch repair.

Microsatellite-stable – absence of small insertions and deletions (indels) at repetitive DNA microsatellites as a result of proficient DNA mismatch repair.

Mismatch repair (MMR) – a mechanism of post-replicative DNA repair that removes mismatched bases and small insertions and deletions (indels) from the newly synthesized DNA strand.

Multinucleated giant cells – abnormally large cells with multiple nuclei and typically bizarre morphology that are observed in association with chronic inflammation and some

types of malignancy.

Mutational hot spot – a genomic region that is the site of mutations in cancers at a frequency greater than that expected by chance: for example, codon 600 in the serine/threonine kinase BRAF.

Mutator phenotype – elevation of the mutation rate above that expected for normally dividing cells. It has been argued that the acquisition of a mutator phenotype is a characteristic of many cancers.

MUTYH-associated polyposis – a syndrome characterized by multiple intestinal adenomas and an increased risk of colorectal carcinoma. It is caused by germline mutations in the base excision repair gene *MUTYH*.

Neoepitope – novel epitopes, created by somatic mutations in tumors, that are capable of eliciting an antitumor immune response.

Polymerase proofreading-associated polyposis (PPAP) – a high-penetrance, dominantly inherited condition characterized by multiple colorectal adenomas and carcinomas, caused by germline mutations in the genes encoding the catalytic subunits of the DNA polymerases Pol δ and Pol ε (*POLD1* and *POLE*, respectively).

Synthetically lethal – a type of genetic interaction in which the combination of mutations in two genes results in cell death, whereas cells harboring only one of the mutations are viable.

Ultramutated – describes tumors with an exceptional frequency of mutations, often exceeding 100 mutations per megabase and often caused by mutations in the gene encoding the catalytic subunit of DNA polymerase ε (*POLE)*.