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

Gool, I.C. van

Chapter 6

Adjuvant treatment for *POLE* proofreading domain-mutant cancers: sensitivity to radiotherapy, chemotherapy and nucleoside analogs

Inge C. Van Gool, Emily Rayner, Elisabeth M. Osse, Remi A. Nout, Carien L. Creutzberg, Ian P.M. Tomlinson, David N. Church, Vincent T.H.B.M. Smit, Niels de Wind, Tjalling Bosse, Mark Drost

Clinical Cancer Research 2018

ABSTRACT

Objective

Pathogenic *POLE* proofreading domain mutations are found in many malignancies where they are associated with ultramutation and favorable prognosis. The extent to which this prognosis depends on their sensitivity to adjuvant treatment is unknown, as is the optimal therapy for advanced-staged or recurrent *POLE*-mutant cancers.

Methods

We examined the recurrence-free survival of women with *POLE*-mutant and *POLE*-wild-type endometrial cancers in the observation arm of the randomized PORTEC-1 endometrial cancer trial (N=245 patients with stage I endometrial cancer for analysis). Sensitivity to radiotherapy and selected chemotherapeutics was compared between *Pole*-mutant mouse-derived embryonic stem (mES) cells, generated using CRISPR-Cas9 (*Pole* mutations D275A/E275A, and cancer-associated P286R, S297F, V411L) and isogenic wild-type cell lines.

Results

In the observation arm of the PORTEC-1 trial (N=245), women with *POLE*-mutant endometrial cancers (N=16) had an improved recurrence-free survival (10-year recurrence-free survival 100% vs. 80.1% for *POLE*-wild-type; HR, 0.143; 95% confidence interval, 0.001-0.996; *P*=0.049). *Pole* mutations did not increase sensitivity to radiotherapy nor to chemotherapeutics in mES cells. In contrast, *Pole*-mutant cells displayed significantly increased sensitivity to cytarabine and fludarabine (IC_{50} *Pole* P286R-mutant vs. wild-type: 0.05 vs. 0.17 μM for cytarabine, 4.62 vs. 11.1 μM for fludarabine; *P*<0.001 for both comparisons).

Conclusions

The favorable prognosis of *POLE*-mutant cancers cannot be explained by increased sensitivity to currently used adjuvant treatments. These results support studies exploring minimization of adjuvant therapy for early-stage *POLE*-mutant cancers, including endometrial and colorectal cancers. Conversely, *POLE* mutations result in hypersensitivity to nucleoside analogs, suggesting the use of these compounds as a potentially effective targeted treatment for advanced-stage *POLE*-mutant cancers.

INTRODUCTION

Somatic mutations in the proofreading (exonuclease) domain of DNA polymerase ε (encoded by the *POLE* gene) are found in a wide variety of human malignancies, including 7-12% of endometrial cancers, ~1% of colorectal cancers and sporadically in cancers of the brain, stomach, breast, and pancreas.[1-4] During DNA replication, the proofreading activity of polymerase ε recognizes and excises misincorporated nucleotides, thereby increasing replication fidelity. Pathogenic *POLE* proofreading domain mutations (hereafter referred to as '*POLE* mutations') are associated with an exceptionally high number of single nucleotide variants: cancers with such *POLE* mutations often harbor more than 100 mutations/Mb.[2,5] Despite this so-called 'ultramutated' phenotype, patients with *POLE*-mutant cancers have a favorable prognosis. This has been shown in early-stage endometrial cancer, where impact was particularly strong in high-grade endometrioid tumors, stage II/III colorectal cancer, and was suggested for glioblastomas.[1,4,6-12] The majority of patients in these studies received additional treatment after surgery. Therefore, we evaluated in an endometrial cancer series and in a cell-based model whether the favorable prognosis of *POLE*-mutant cancers might be caused by increased sensitivity to adjuvant treatment. *POLE* mutations can also be found in advanced-stage disease, where the prognostic impact remains uncertain, and in cancer types with poor prognosis such as glioblastomas. Because these *POLE* mutations reside in the proofreading domain that excises non-matched nucleotides, we investigated the efficacy of nucleoside analogs as potential targeted therapies for advanced-stage *POLE*-mutant cancers.

MATERIALS AND METHODS

Endometrial cancer patient cohort

Clinical data of women with endometrial cancer who did not receive adjuvant treatment (N=369) were collected from the randomized PORTEC-1 trial, which compared external beam radiotherapy with observation in stage I endometrial cancer.[13] *POLE* mutational status was determined as described previously[7] with the addition of screening *POLE* exon 14 using Sanger sequencing (primers available upon request). For 119 endometrial cancers, *POLE* mutational status could not be determined, because screening of exons 9, 13 and 14 failed or because no material was available. Twenty-three cases were designated '*POLE*-wild-type' despite failure of exon 14 sequencing, based on screening of *POLE* exons 9 and 13. Women with endometrial cancers harboring a *POLE* mutation but also another relevant molecular alteration (i.e. p53 mutation or mismatch repair deficiency, based on previous analyses[14]) were excluded (*POLE*-mutant & p53-mutant, n=3; *POLE*-mutant, p53-mutant, mismatch repair-deficient, n=2), in accordance with our

previous publication.[14] This resulted in 245 endometrial cancer patients for analysis. Details on the specific *POLE* mutations identified in the endometrial cancers can be found in Supplementary Table 1.

Generation of the cell-based model

Different *POLE* proofreading domain mutations (P286R, S297F, V411L, and D275A/E277A) were recreated in wild-type mouse-derived embryonic stem (mES) cell lines using CRISPR-Cas9. All cell lines in this study were derived from wild-type primary diploid 129/OLA mouse-derived embryonic stem cell line E14.[15] Cell lines were cultured for three to six weeks from thawing to use in experiments. Cell culture was tested every other month for mycoplasma contamination using MycoAlert Myocplasma Detection Kit (LT07-318; Lonza, Switzerland). As this study is limited to experiments with mES cells, no live animals were used, an Institutional Animal Care and Use Committee approval was not required.

In order to make these mES cells *Pole*-mutant, a CRISPR guide RNA was designed for each mutation using the web resources available at http://crispr.mit.edu. Pairs of these oligonucleotides (Supplementary Table 2) were ordered from Integrated DNA Technologies (Leuven, Belgium), annealed, and, after BbsI digestion (New England Biolabs), cloned into a modified expression plasmid pX330 (Addgene #42230; now containing the CRISPR-Cas9 system and a puromycin-resistance marker). Plasmids were isolated using an endotoxin-free Midiprep kit (Qiagen), and gRNA inserts were verified by Sanger sequencing. As template for homologous recombination, PCR fragments (~2.4 kbp) containing the specific *Pole* proofreading domain mutations as well as silent mutations in the PAM sequences were generated as described previously (oligonucleotides in Supplementary Table 3).[16] Introduction of the specific substitutions was confirmed by Sanger sequencing.

Wild-type mES cells were transfected with the mutation-specific Cas9- and CRISPR guideRNA-expressing plasmid and PCR-based repair template using Lipofectamine 2000 (#11668019, Thermo Fisher Scientific). For this transfection, the manufacturer's protocol was followed with minor alterations: use of knockout DMEM, more Lipofectamine for each transfection (14.4µl in 125µl medium), a higher DNA input (3-4µg), and a 20 minute incubation of diluted DNA in Lipofectamine at room temperature. Puromycin (1.5 µg/ml for two days; A11138-03, Thermo Fisher Scientific) was added to the medium for positive selection of transfected cells. Drug-resistant cells were seeded at a low density. Independent clones were manually picked, expanded and reseeded as single cells. Isolated clones were again manually picked to ensure purity of the resultant cell line. To identify targeted clones, DNA and RNA were isolated from drug-resistant clones and

regions were amplified by PCR (oligonucleotides sequences in Supplementary Table 4). *Pole* proofreading domain mutations were designed to yield a restriction site; therefore, PCR products amplified from genomic DNA were subjected to restriction site digestion to verify knockin (BtgZI for D275A/E277A, BstUI for P286R, MboII for S297F, and AcuI for V411L; all New England Biolabs). Amplified genomic DNA and cDNA products of clones with an introduced restriction site were purified, and mutations were confirmed by Sanger sequencing analysis.

For each *Pole*-mutant mES cell line, metaphase spreads were made, and fluorescence in situ hybridization was performed using biotin-16-dUTP-labelled or digoxigenin-11-dUTP-labeled (#11093070910 and #11093088910 respectively, Roche) bacterial artificial chromosome clones RP24-154A19 and RP24-388K6 (BACPAC Resource Center, Chori). Chromosome numbers were equal for all cell lines used in subsequent experiments, and all cell lines contained two copies of the *Pole* allele (Supplementary Figure 1). Spontaneous mutant frequencies of the *Pole*-mutant mES cell lines were determined at the Hypoxanthine Phosphorybosyl Transferase (*Hprt*) gene as described previously.[17] Additional details on these experiments can be found in the Supplementary Methods.

Treatment sensitivity of mES cells

Pole proofreading domain-mutant and wild-type mES cells were treated with 5-fluorouracil (F6627, Sigma Aldrich), methotrexate (S1210, Selleckchem), paclitaxel (S1150, Selleckchem), cytarabine (S1648, Selleckchem), gemcitabine (S1714, Selleckchem), fludarabine (S1229, Selleckchem), cladribine (S1199, Selleckchem), clofarabine (S1218, Selleckchem), doxorubicin, cisplatin, or etoposide. To determine the sensitivity to these compounds, mES cells were seeded in 96-well tissue culture plates (5000 cells/well). The following day, cells were treated with increasing concentrations of each compound and incubated for 24-72 hours (24 hours: cytarabine, gemcitabine, clofarabine, paclitaxel, doxorubicin; 48 hours: cisplatin, cladribine, fludarabine, methotrexate, etoposide; 72 hours: 5-fluorouracil). Seventy-two hours after treatment was initiated, cells were detached with 0.05% trypsin-EDTA and resuspended in PBS containing 2% FCS. Adherent cells were quantified using flow cytometry. Sensitivity to ionizing radiation was determined similarly: mES cells were seeded in 6-well tissue culture plates (50,000 cells/well). The following day, cells were irradiated with a single dose of up to 8 Gy. Seventy-two hours after irradiation, cells were detached with 0.05% trypsin-EDTA and manually counted. In each experiment, for every cell line, cell counts were normalized to counts of vehicle-treated cells. At least three independent experiments were conducted per cell line for each treatment.

Statistical analyses

For PORTEC-1 participants in the observation arm, recurrence-free survival (RFS) was calculated, defined as the time from randomization to recurrence, with censoring at date of last contact or death in patients without recurrence. RFS was evaluated using the Kaplan-Meier method and compared between *POLE*-mutant and *POLE*-wild-type endometrial cancers using a Cox proportional hazards model with Firth's correction, owing to the absence of events in the *POLE*-mutant group.[18] To analyze treatment sensitivity in the cell-based model, IC_{50} values of mES cell lines were calculated for each experiment by non-linear regression analysis (variable slope) of the normalized cell counts. Outliers in IC_{50} values were identified with Grubbs test for each cell line. For each *Pole*-mutant cell line, IC50's were compared to IC50's of the isogenic wild-type cell line using independent-samples t-tests (with Holm-Bonferroni correction when indicated). Levene's test for equality of variances was used to determine when to assume equal variances. All statistical tests were two-sided, and a *P* value of less than 0.05 was considered statistically significant; this was adjusted downward in case of Holm-Bonferroni correction. All analyses were performed in R (3.3.0 version, package 'coxphf'), Graphpad Prism 7, or SPSS 23.0 software.

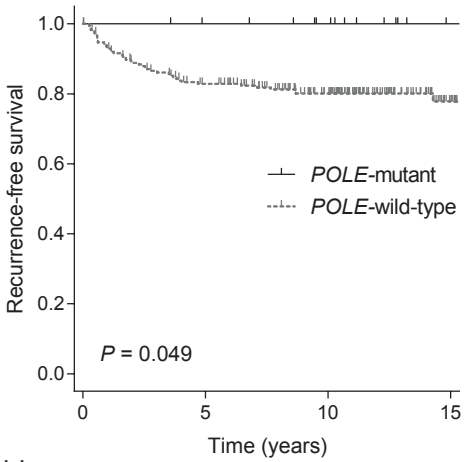
RESULTS

Prognosis of patients with *POLE*-mutant endometrial cancers who did not receive adjuvant treatment

The prognostic significance of *POLE* mutations was evaluated in the observation arm of the randomized PORTEC-1 trial, which included 245 stage I endometrial cancers for analysis (Figure 1).[7,13,14] None of the women with *POLE*-mutant endometrial cancers (0/16) developed a recurrence, compared to 44 recurrences (19.2%) in 229 patients with *POLE*-wild-type tumors; this difference in RFS was statistically significant (10-year RFS 100% vs. 80.1%; HR, 0.143; 95% confidence interval (CI), 0.001-0.996; *P*=0.049 based on Cox regression with Firth's correction). These results suggest a favorable prognosis of early-stage *POLE*-mutant cancers in the absence of adjuvant treatment, indicating that the increased sensitivity to adjuvant treatment does not explain the good clinical outcome of these tumors.

Treatment sensitivity conferred by *POLE* mutations in a model system

To support the PORTEC-1 data, we investigated the effect of *POLE* mutations on treatment sensitivity in a model system. To this end, we recreated three common somatic, cancer-associated *POLE* proofreading domain mutations (P286R, S297F, and V411L),[2] as well as a proofreading-deficient positive control (double mutation of exonuclease active sites, D275A/E277A)[19] in isogenic mouse-derived embryonic stem (mES) cell lines using



Number at risk				
POLE-mutant	16	14	10	1
POLE-wild-type	229	172	122	22

Figure 1. Recurrence-free survival of endometrial cancer patients in the observation arm of the PORTEC-1 study according to *POLE* mutational status.
Among endometrial cancer patients who did not receive adjuvant treatment, none of the women with *POLE*-mutant endometrial cancers developed a recurrence (0/16, 10-year RFS 100%), as compared to 44 recurrences in 229 women with *POLE*-wild-type tumors (19.2%, 10-year RFS 80.1%). This difference in recurrence-free survival was statistically significant (HR, 0.143; 95% CI, 0.001-0.996; *P*=0.049).

CRISPR-Cas9. For each *Pole* mutation, a minimum of two independent homozygous cell lines was used for subsequent experiments. Consistent with the mutational burden in *POLE*-mutant cancers, these *Pole* mutations gave rise to a mutator phenotype in mES cells with the severity of the phenotype depending on the substitution (P286R ~8x higher than wild-type, D275A/E277A, S297F, V411L ~4x; Supplementary Figure 2).

Sensitivity to adjuvant treatments used for cancer types that harbor *POLE* mutations was determined in the mES cells. *Pole* mutations did not result in increased sensitivity to ionizing radiation or to selected chemotherapeutic agents (i.e. 5-fluorouracil, cisplatin, paclitaxel, doxorubicin, etoposide and methotrexate; Table 1, Figures 2 and 3, Supplementary Table 5).

Based on the role of the polymerase ε proofreading domain in the recognition and excision of mispaired nucleotides, we investigated nucleoside analogs as potential novel targeted therapies for *POLE*-mutant cancers. Indeed, *Pole* mutations resulted in increased sensitivity to cytarabine and fludarabine: sensitivity to both compounds was significantly increased for the *Pole* D275A/E277A-mutant cell lines, but was even more pronounced in cells with somatic *Pole* hotspot mutations (Figure 4, Table 1, Supplementary Tables 5 and 6). Because somatic *POLE* mutations in cancers are heterozygous, sensitivity to

Table 1. Sensitivity to adjuvant treatment strategies conferred by *POLE* proofreading domain mutations in a cell-based model.

	Wild-type	***Pole* D275A/E277A***		***Pole* P286R***		***Pole* S297F***		***Pole* V411L***	
Treatment	**IC_{50} (mean ± SD)**	**IC_{50} (mean ± SD)**	***P* (95% CI)**	**IC_{50} (mean ± SD)**	***P* (95% CI)**	**IC_{50} (mean ± SD)**	***P* (95% CI)**	**IC_{50} (mean ± SD)**	***P* (95% CI)**
Ionizing radiation (Gy)	2.96 ± 1.28	2.94 ± 1.02	0.976 (-1.63, 1.67)	3.51 ± 1.36	0.553 (-2.53, 1.45)	2.19 ± 1.21	0.390 (-1.16, 2.71)	2.82 ± 0.893	0.866 (-1.70, 1.98)
5-Fluorouracil(μM)	0.25 ± 0.04	0.23 ± 0.07	0.513 (-0.06, 0.11)	0.31 ± 0.02	0.057 (-0.12, 0.002)	0.30 ± 0.01	0.109 (-0.11, 0.01)	0.25 ± 0.01	0.895 (-0.06, 0.06)
Cisplatin (μM)	0.39 ± 0.17	0.28 ± 0.09	0.265 (-0.10, 0.32)	0.31 ± 0.07	0.403 -0.12, 0.28)	0.36 ± 0.09	0.825 (-0.22, 0.27)	0.19 ± 0.06	0.051 (-0.001, 0.40)
Doxorubicin (nM)	8.92 ± 4.04	10.7 ± 2.92	0.468 (-7.01, 3.49)	9.21 ± 0.23	0.909 (-5.85, 5.28)	12.0 ± 1.11	0.237 (-8.76, 2.51)	7.70 ± 1.73	0.586 (-3.66, 6.11)
Etoposide (nM)	27.2 ± 15.7	32.7 ± 7.09	0.515 (-23.0, 12.0)	32.8 ± 7.39	0.513 (-23.1, 12.0)	27.5 ± 7.05	0.971 (-15.9, 15.4)	26.6 ± 10.6	0.937 (-15.6, 16.9)
Methotrexate (nM)	60.1 ± 8.48	70.7 ± 28.1	0.401 (-38.0, 16.9)	85.6 ± 24.6	0.043 (-50.1, -0.97)	74.2 ± 11.1	0.069 (-29.6, 1.46)	75.7 ± 28.2	0.228 (-43.1, 12.0)
Paclitaxel (nM)	9.57 ± 2.94	13.7 ± 3.41	0.084 (-9.04, 0.70)	10.9 ± 3.00	0.490 (-5.53, 2.86)	6.64 ± 0.59	0.039 (0.19, 5.68)	9.14 ± 0.70	0.719 (-2.30, 3.15)
Cytarabine (μM)	0.17 ± 0.02	0.13 ± 0.03	0.027‡ (0.006, 0.07)	0.05 ± 0.01	<0.001‡ (0.09, 0.15)	0.05 ± 0.01	<0.001‡ (0.09, 0.15)	0.04 ± 0.004	<0.001‡ (0.10, 0.16)
Fludarabine (μM)	11.1 ± 3.98	6.82 ± 0.69	0.012‡ (1.23, 7.40)	4.62 ± 1.50	0.001‡ (3.35, 9.68)	3.40 ± 0.51	<0.001‡ (4.66, 10.8)	6.29 ± 0.43	0.006‡ (1.77, 7.92)

*Sensitivity to treatment of mouse embryonic stem cell lines with different homozygous *Pole* proofreading domain mutations (D275A/E277A, P286R, S297F, V411L, respectively) is shown. For each *Pole* mutation, mean IC_{50} and standard deviation (SD) of one cell line are provided (results of additional cell lines in Supplementary Table 5). IC_{50}s of the *Pole*-mutant cell line are compared to IC_{50}s of the *Pole*-wild-type cell line; the corresponding *P*-values and 95% confidence intervals (CI) are shown.
‡Difference remains significant after Holm-Bonferroni correction for multiple comparisons.

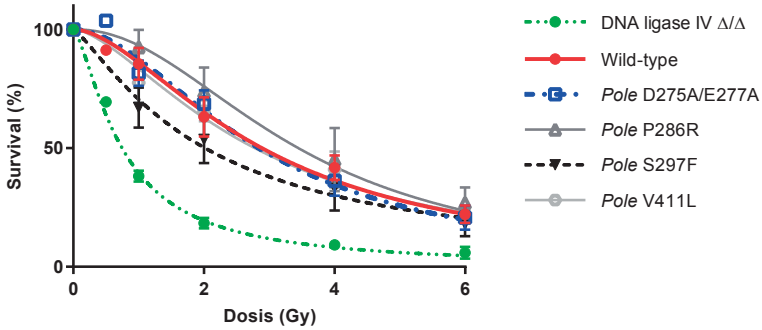


Figure 2. Sensitivity to ionizing radiation in *Pole*-mutant and *Pole*-wild-type cell lines.
Non-linear regression of relative survival after ionizing radiation is shown for homozygous *Pole*-mutant and *Pole*-wild-type mouse embryonic stem cells. The *Pole* D275A/E277A double mutation results in proofreading deficiency.[19] The *Pole* P286R, S297F, and V411L mutations are somatic *POLE* hotspot mutations.[2] As a radiosensitive positive control cell line, a DNA ligase IV knockout mouse-derived embryonic stem cell line (DNA ligase IV Δ/Δ) was used. Sensitivity to ionizing radiation did not significantly differ between *Pole*-mutant and *Pole*-wild-type cells (for details on these comparisons see also Table 1). Dots and error bars indicate mean and standard error of the mean respectively.

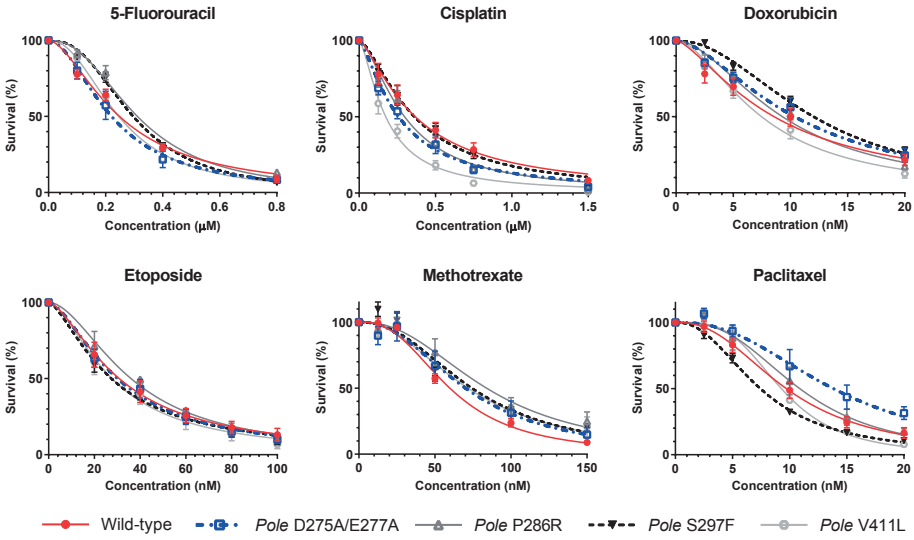


Figure 3. Sensitivity to different chemotherapeutic agents in *Pole*-mutant and *Pole*-wild-type cell lines.
Non-linear regression of relative survival after treatment with chemotherapeutic agents is shown for homozygous *Pole*-mutant and *Pole*-wild-type mouse embryonic stem cells. The *Pole* D275A/E277A double mutation results in proofreading deficiency.[19] The *Pole* P286R, S297F, and V411L mutations are somatic *POLE* hotspot mutations.[2] Sensitivity to 5-fluorouracil, cisplatin, doxorubicin, and etoposide did not significantly differ between *Pole*-mutant and *Pole*-wild-type cells. For methotrexate, a significant difference was found between IC_{50}s of the *Pole* P286R-mutant cell line and the *Pole*-wild-type cell line. For paclitaxel, a significant difference was found between IC_{50}s of the *Pole* S297F-mutant and the *Pole*-wild-type cell lines. These differences in both methotrexate and paclitaxel sensitivity did not remain significant after correction for multiple comparisons (for details on these comparisons, see also Table 1). Dots and error bars indicate mean and standard error of the mean, respectively.

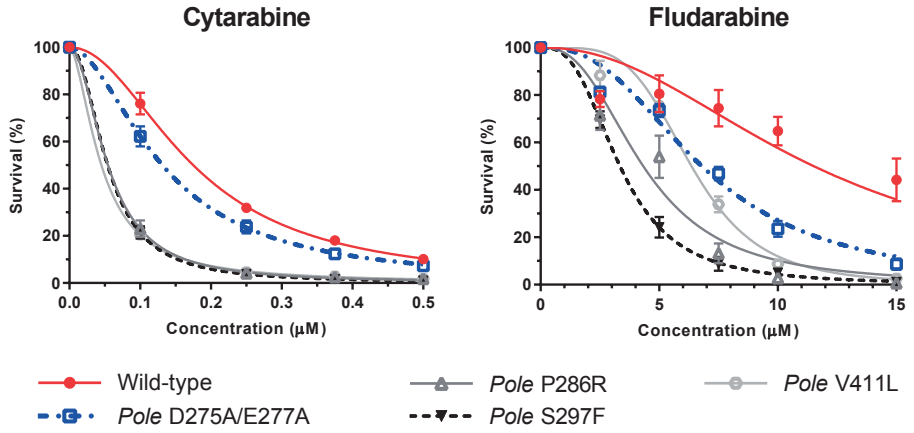


Figure 4. Cytarabine and fludarabine sensitivity in *Pole*-mutant and *Pole*-wild-type cell lines.
Non-linear regression of relative survival after nucleoside analog treatment is shown for *Pole*-mutant and *Pole*-wild-type mouse embryonic stem cells. The *POLE* D275A/E277A double mutation results in proofreading deficiency.[19] The *Pole* P286R, S297F, and V411L mutations are somatic *POLE* hotspot mutations.[2] All homozygous *Pole*-mutant cell lines were significantly more sensitive to cytarabine and fludarabine than the isogenic *Pole*-wild-type cell line (also after multiple comparisons correction, see Table 1). Dots and error bars indicate mean and standard error of the mean, respectively.

cytarabine and fludarabine was also assessed in a heterozygous *Pole* S297F-mutant cell line. This resulted in sensitivity lower than in the homozygous *Pole*-mutant lines, but higher than in the wild-type, reaching significance for cytarabine (mean IC_{50} 0.14μM vs. 0.17μM; P=0.025 for cytarabine; mean IC_{50} 7.58μM vs. 11.1μM; P=0.169 for fludarabine; Supplementary Figure 3).

DISCUSSION

In this study, we present two independent lines of evidence to show that the prognostic benefit of *POLE* proofreading domain mutations is independent of adjuvant treatment. First, we provide clinical data from the PORTEC-1 endometrial cancer trial to show that the favorable outcome associated with *POLE* mutations is also observed in women who did not receive adjuvant therapy. Second, in an *in vitro* model we demonstrate that *POLE* mutations confer increased sensitivity neither to ionizing radiation, nor to commonly used chemotherapeutics. We also show that *Pole* mutations result in significantly increased sensitivity to specific nucleoside analogs currently used to treat hematological malignancies.

This study is limited by the composition of the endometrial cancer cohort: the relatively

favorable outcome of stage I disease made it more challenging to confirm the prognostic effect of *POLE* mutations. Its composition also precluded the joined examination of *POLE* mutational status and other prognostically relevant, clinical factors: this should be addressed in future research. Because *POLE*-mutant cancers are characterized by an antitumor immune response,[20-22] it should be noted that in the cell-based model, an interaction between the immune system and radiotherapy or chemotherapy could not be assessed. However, the results of the endometrial cancer cohort and the *in vitro* findings are consistent regarding the prognosis of *POLE*-mutant tumors and its independence of adjuvant therapy. These results are supported by previous studies on endometrial and colorectal cancer. McConechy et al. observed no endometrial cancer-related deaths or evidence of recurrent or progressive disease among 14 patients with *POLE*-mutant cancers who did not receive adjuvant treatment.[9] Moreover, among 23 patients with stage II *POLE*-mutant colorectal cancers, no recurrences were found irrespective of adjuvant chemotherapy.[4] Our *in vitro* findings are in line with another preclinical study in which no significant difference in sensitivity to paclitaxel was found between *POLE*-mutant and *POLE*-wild-type endometrial cancer cell lines.[22] This study reported increased resistance to carboplatin in the *POLE*-mutant cell lines. However, the cell lines used in this study were, contrary to those in our study, non-isogenic, and one carried a *POLE* polymerase domain variant of unknown pathogenicity. Finally, considering the mechanisms of action of the commonly used anticancer treatments and the presumed lack of a role herein of DNA polymerase epsilon's proofreading activity, a *POLE* mutation is unlikely to alter sensitivity to these therapies.

We are the first to study cancer-associated *POLE* proofreading domain mutations in mammalian cells. Remarkably, sensitivity to nucleoside analogs cytarabine and fludarabine was much greater in the cancer-associated *Pole* mutants than in the *Pole* proofreading-deficient cell lines,[23] supporting the presence of defects besides proofreading alone.[2,5] In contrast, these *POLE* mutations resulted in only a four- to eight-fold increase in the spontaneous mutation frequency at the *Hprt* gene, which seems low compared with the ultramutated phenotype found in *POLE*-mutant cancers.[2,5] Our mutation data are supported by forward mutation assays with mammalian proteins.[5] The mutation frequencies in this study were, however, lower than those previously observed in yeast,[24] which may be due to different mechanisms of mutability between yeast and mammals. Future studies are needed to unravel the mechanisms behind the ultramutated phenotype of *POLE*-mutant cancers.

In conclusion, these findings suggest that the favorable prognosis of *POLE*-mutant cancers cannot be explained by increased sensitivity to adjuvant therapy but could be due to increased immunogenicity as described in previous studies or to the accumulation of

deleterious mutations ('error catastrophe').[20-22,25] These results support clinical trials such as the PORTEC-4A trial, which prospectively evaluates observation alone for early-stage *POLE*-mutant cancers; this may provide an opportunity to decrease overtreatment and may spare these patients unnecessary side effects.[26] In patients with advanced-stage or aggressive tumors, *POLE* mutations identify candidates for targeted therapies. Recent results with immune checkpoint inhibitors for *POLE*-mutant cancers are promising.[27,28] This study proposes nucleoside analogs cytarabine and fludarabine as targeted treatment option for *POLE*-mutant cancers, potentially as an alternative to or in combination with immune checkpoint inhibition, which should be the subject of future research. As the prognostic value and therapeutic implications of *POLE* mutations seem generalizable across cancer types, the results of this study support further individualization of treatment for a wide variety of malignancies.

ACKNOWLEDGEMENTS

We thank all members of the PORTEC study group and all patients who participated in the PORTEC trials. We would also like to thank Henri van de Vrugt (dept. of Clinical Genetics, VU University Medical Center, Amsterdam, NL) for providing the modified expression plasmid used for CRISPR-Cas9; Joost Schimmel (dept. of Human Genetics, Leiden University Medical Center) for providing the radiosensitive DNA ligase IV knockout cell line; Sjaak Neefjes (dept. of Chemical Immunology, Leiden University Medical Center, Leiden, NL) for providing chemotherapeutic agents doxorubicin, cisplatin, and etoposide; Karoly Szuhai and Daniëlle de Jong (dept. of Molecular Cell Biology, Leiden University Medical Center, Leiden, NL) for assistance with the FISH experiments; and Jelle Goeman en Hein Putter (dept. of Medical Statistics and Bio-informatics, Leiden University Medical Center, Leiden, NL) for assistance with statistical analysis.

This work was supported by the Dutch Cancer Society (KWF-UL2012-5719 to I.C. Van Gool, E.M. Osse, R.A. Nout, C.L. Creutzberg, V.T.H.B.M. Smit, and T. Bosse; and KWF -Young Investigator Grant – 10418 to T. Bosse); the Oxford NIHR Biomedical Research Centre and by core funding to the Wellcome Centre for Human Genetics from the Wellcome Trust (090532/Z/09/Z). D.N. Church is funded by a Health Foundation/Academy of Medical Sciences Clinician Scientist Fellowship, and has received funding from the Oxford Cancer Research Centre.

REFERENCES

1. The Cancer Genome Atlas Research Network. Integrated genomic characterization of endometrial carcinoma. Nature 2013;497(7447):67-73.
2. Rayner E, van Gool IC, Palles C, Kearsey SE, Bosse T, Tomlinson I, et al. A panoply of errors: polymerase proofreading domain mutations in cancer. Nat Rev Cancer 2016;16(2):71-81.
3. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2(5):401-404.
4. Domingo E, Freeman-Mills L, Rayner E, Glaire M, Briggs S, Vermeulen L, et al. Somatic POLE proofreading domain mutation, immune response, and prognosis in colorectal cancer: a retrospective, pooled biomarker study. Lancet Gastroenterol Hepatol 2016;1(3):207-216.
5. Shinbrot E, Henninger EE, Weinhold N, Covington KR, Goksenin AY, Schultz N, et al. Exonuclease mutations in DNA polymerase epsilon reveal replication strand specific mutation patterns and human origins of replication. Genome Res 2014;24(11):1740-1750.
6. Stelloo E, Bosse T, Nout RA, MacKay HJ, Church DN, Nijman HW, et al. Refining prognosis and identifying targetable pathways for high-risk endometrial cancer; a TransPORTEC initiative. Mod Pathol 2015;28(6):836-844.
7. Church DN, Stelloo E, Nout RA, Valtcheva N, Depreeuw J, ter Haar N, et al. Prognostic significance of POLE proofreading mutations in endometrial cancer. J Natl Cancer Inst 2015;107(1):402.
8. Talhouk A, McConechy MK, Leung S, Li-Chang HH, Kwon JS, Melnyk N, et al. A clinically applicable molecular-based classification for endometrial cancers. Br J Cancer 2015;113(2):299-310.
9. McConechy MK, Talhouk A, Leung S, Chiu D, Yang W, Senz J, et al. Endometrial Carcinomas with POLE Exonuclease Domain Mutations Have a Favorable Prognosis. Clin Cancer Res 2016;22(12):2865-2873.
10. Meng B, Hoang LN, McIntyre JB, Duggan MA, Nelson GS, Lee CH, et al. POLE exonuclease domain mutation predicts long progression-free survival in grade 3 endometrioid carcinoma of the endometrium. Gynecol Oncol 2014;134(1):15-19.
11. Billingsley CC, Cohn DE, Mutch DG, Hade EM, Goodfellow PJ. Prognostic Significance of POLE Exonuclease Domain Mutations in High-Grade Endometrioid Endometrial Cancer on Survival and Recurrence: A Subanalysis. Int J Gynecol Cancer 2016;26(5):933-938.
12. Erson-Omay EZ, Caglayan AO, Schultz N, Weinhold N, Omay SB, Ozduman K, et al. Somatic POLE mutations cause an ultramutated giant cell high-grade glioma subtype with better prognosis. Neuro Oncol 2015;17(10):1356-1364.
13. Creutzberg CL, Nout RA, Lybeert ML, Warlam-Rodenhuis CC, Jobsen JJ, Mens JW, et al. Fifteen-year radiotherapy outcomes of the randomized PORTEC-1 trial for endometrial carcinoma. Int J Radiat Oncol Biol Phys 2011;81(4):e631-638.
14. Stelloo E, Nout RA, Osse EM, Jurgenliemk-Schulz IJ, Jobsen JJ, Lutgens LC, et al. Improved Risk Assessment by Integrating Molecular and Clinicopathological Factors in Early-stage Endometrial Cancer-Combined Analysis of the PORTEC Cohorts. Clin Cancer Res 2016;22(16):4215-4224.
15. Wakayama T, Rodriguez I, Perry AC, Yanagimachi R, Mombaerts P. Mice cloned from embryonic stem cells. Proc Natl Acad Sci USA 1999;96(26):14984-14989.
16. Drost M, Zonneveld JB, van Hees S, Rasmussen LJ, Hofstra RM, de Wind N. A rapid and cell-free assay to test the activity of lynch syndrome-associated MSH2 and MSH6 missense variants. Hum Mutat 2012;33(3):488-494.
17. Drost M, Lutzen A, van Hees S, Ferreira D, Calleja F, Zonneveld JB, et al. Genetic screens to identify

pathogenic gene variants in the common cancer predisposition Lynch syndrome. Proc Natl Acad Sci USA 2013;110(23):9403-9408.
18. Heinze G, Schemper M. A solution to the problem of monotone likelihood in Cox regression. Biometrics 2001;57(1):114-119.
19. Derbyshire V, Freemont PS, Sanderson MR, Beese L, Friedman JM, Joyce CM, et al. Genetic and crystallographic studies of the 3',5'-exonucleolytic site of DNA polymerase I. Science 1988;240(4849):199-201.
20. van Gool IC, Eggink FA, Freeman-Mills L, Stelloo E, Marchi E, de Bruyn M, et al. POLE Proofreading Mutations Elicit an Antitumor Immune Response in Endometrial Cancer. Clin Cancer Res 2015;21(14):3347-3355.
21. Howitt BE, Shukla SA, Sholl LM, Ritterhouse LL, Watkins JC, Rodig S, et al. Association of Polymerase e-Mutated and Microsatellite-Instable Endometrial Cancers With Neoantigen Load, Number of Tumor-Infiltrating Lymphocytes, and Expression of PD-1 and PD-L1. JAMA Oncol 2015;1(9):1319-1323.
22. Bellone S, Bignotti E, Lonardi S, Ferrari F, Centritto F, Masserdotti A, et al. Polymerase epsilon (POLE) ultra-mutation in uterine tumors correlates with T lymphocyte infiltration and increased resistance to platinum-based chemotherapy in vitro. Gynecol Oncol 2017;144(1):146-152.
23. Tsuda M, Terada K, Ooka M, Kobayashi K, Sasanuma H, Fujisawa R, et al. The dominant role of proofreading exonuclease activity of replicative polymerase epsilon in cellular tolerance to cytarabine (Ara-C). Oncotarget 2017;8(20):33457-33474.
24. Kane DP, Shcherbakova PV. A common cancer-associated DNA polymerase epsilon mutation causes an exceptionally strong mutator phenotype, indicating fidelity defects distinct from loss of proofreading. Cancer Res 2014;74(7):1895-1901.
25. Fox EJ, Loeb LA. Lethal mutagenesis: targeting the mutator phenotype in cancer. Semin Cancer Biol 2010;20(5):353-359.
26. The Cancer Genome Atlas Research Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487(7407):330-337.
27. Santin AD, Bellone S, Buza N, Choi J, Schwartz PE, Schlessinger J, et al. Regression of chemotherapy-resistant polymerase epsilon (POLE) ultra-mutated and MSH6 hyper-mutated endometrial tumors with nivolumab. Clin Cancer Res 2016;22(23):5682-5687.
28. Mehnert JM, Panda A, Zhong H, Hirshfield K, Damare S, Lane K, et al. Immune activation and response to pembrolizumab in POLE-mutant endometrial cancer. J Clin Invest 2016;126(6):2334-2340.

SUPPLEMENTARY METHODS

Preparation of metaphase spreads

For each *Pole* proofreading domain-mutant mouse embryonic stem cell line, cells were treated with colcemid (0.21µg/ml for 2.5 hours; #15210040, Thermo Fisher Scientific) to arrest cells in metaphase. After treatment, cells were washed with PBS, detached with 0.05% trypsin-EDTA (Thermo Fisher Scientific), collected and centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in hypotonic solution (0.2% sodiumcitrate and 0.2% KCL). After centrifugation, the hypotonic solution was removed. Cells were resuspended carefully in fixative solution (4:1, methanol:acetic acid) and centrifuged again. This fixation was performed three times before the cells were spread onto glass slides. Slides were mounted with DAPI-containing mounting medium (#H-1200, Vectashield). At least fifty metaphases were examined for each cell line. Chromosome numbers were equal for all cell lines used in subsequent experiments.

Fluorescence in situ hybridization

Bacterial artificial chromosomes (BAC) were ordered from the BACPAC Resource Center (Chori, USA): RP24-154A19 containing the *Pole* gene (5qF), and RP24-388K6 more proximal of the CRISPR/Cas9 target sequences (5qA3). DNA from the BAC clones was isolated using the Nucleobond Xtra Midi kit (MN740410.10, Macherey Nagel). To use the BAC clones as FISH probes, BAC clones were labeled with either biotin-16-dUTP (#11093070910, Roche) or digoxigenin-11-dUTP (#11093088910, Roche) using a nick translation kit (Roche and Enzo Life Sciences) as described previously.[1] Prior to precipitation, Yeast tRNA (#15401011, Invitrogen; ratio yeast tRNA:BAC is 5:1), fish sperm DNA (#1146740001, Roche; ratio fish sperm:BAC is 5:1) and Cot-I (#18440016, Invitrogen; ratio Cot-I:BAC is 10:1) was added to the probes. Probes were then diluted in hybridization mix (50% formamide, 10% dextran sulphate, 2x SSCP; 0.3M NaCl, 0.03M sodium citrate, 0.04M sodium phosphate).

Metaphase spreads were treated with RNase (10µg per slide, diluted in 2xSSC; 0.3M NaCl, 0.03M sodium citrate, pH7) for 10 minutes at 37°C, washed three times in 2xSSC, incubated with 0.005% pepsin/0.02M HCl (#10108057001, Roche) at 37°C for 5 minutes, and washed twice in PBS. Slides were then fixed in 1% formaldehyde/PBS, washed twice in PBS and dehydrated in a graded ethanol series. Ten µl of the probe mix (100ng per probe) was added to each slide, after which slides were placed at 80°C for 75 seconds for denaturation. Hybridization was performed overnight at 37°C in a moist chamber. Slides were washed in stringent wash buffer (K5799, Dako) for 5 minutes at 60°C, twice for 3 minutes in wash buffer, and 3 times 5 minutes in Dig-buffer (0.1M Tris, 0.15M NaCl, pH7.5) with 0.05% Tween20 (TNT) at room temperature. For detection, slides were

incubated with Cy3-labeled streptavidin (1:500, Sigma Aldrich) and FITC-labeled mouse anti-digoxigenin antibody (1:250, Sigma Aldrich) for 10 minutes at 37°C. After dehydration, slides were mounted with mounting medium containing DAPI (Vectashield). A minimum of twenty metaphases was examined for each cell line. All cell lines contained two copies of the *Pole* allele (Supplementary Figure 1).

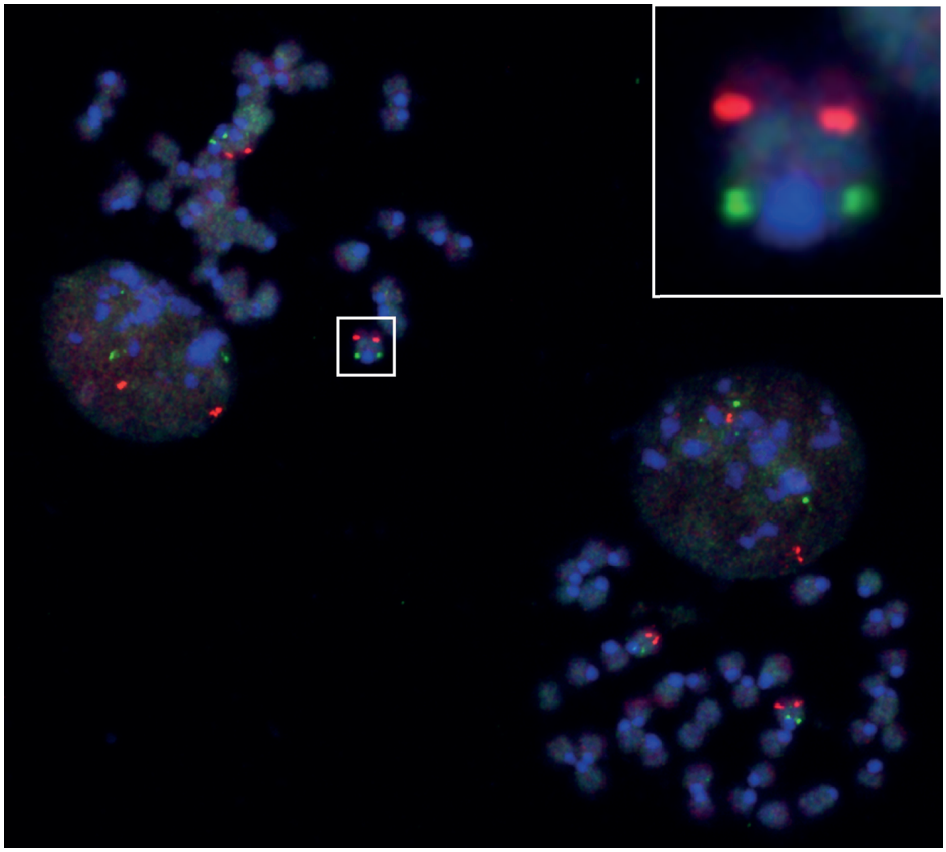
Assessment of mutator phenotypes at the *Hypoxanthine Phosphorybosyl Transferase (Hprt)* gene

Spontaneous mutagenesis at the *Hprt* gene was determined as described previously.[2] In brief, cells were cultured in medium containing HAT supplement (#21060-017, Gibco) for two consecutive passages to eliminate preexisting *Hprt*-deficient cells. Subsequently, cells were cultured in HT-supplemented medium (#41065-012, Gibco) for one passage, followed by two passages in medium without HAT- or HT-supplement, during which spontaneous *Hprt* mutations can accumulate. Afterwards, cells were continuously grown in medium with 40 μM 6-thioguanine to select for *Hprt*-deficient cells. After approximately ten days, colonies were fixed, stained with methylene blue and counted. These counts were corrected for cloning efficiencies.

As controls for this assay, a wild-type and a mismatch repair-deficient mouse embryonic stem cell line were used. The mismatch repair-deficient cell line resulted from a knockout of *Msh6* using CRISPR-Cas9 (guide RNA forward 5'-caccgGGAGCCTCCGCTTCCCGCGG-3', reverse 5'-aaacCCGCGGGAAGCGGAGGCTCC-3', plasmid Addgene #42230), generated similarly to the *Pole*-mutant cell lines. Msh6 deficiency of the resultant cell line was verified by Sanger sequencing of a PCR product amplified from the CRISPR/Cas9 targeted locus (forward primer 5'-AAAGCACCTTGTACAGCTTC-3', reverse primer 5'-GCTTGCCCAATACTCCGAAG-3'), Western Blot for Msh6 (antibody Abcam #14204) and by verifying its resistance to low doses of 6-thioguanine (A4882, Sigma Aldrich; 4hr 40 μM pulse treatment).

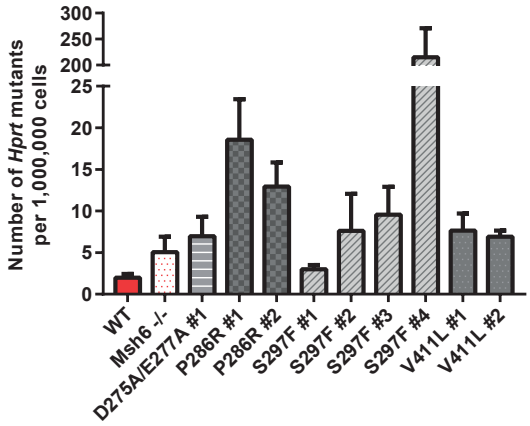
REFERENCES

1. Rossi S, Szuhai K, Ijszenga M, et al. EWSR1-CREB1 and EWSR1-ATF1 fusion genes in angiomatoid fibrous histiocytoma. Clin Cancer Res 2007;13(24):7322-7328.
2. Drost M, Lutzen A, van Hees S, et al. Genetic screens to identify pathogenic gene variants in the common cancer predisposition Lynch syndrome. Proc Natl Acad Sci USA 2013;110(23):9403-9408.

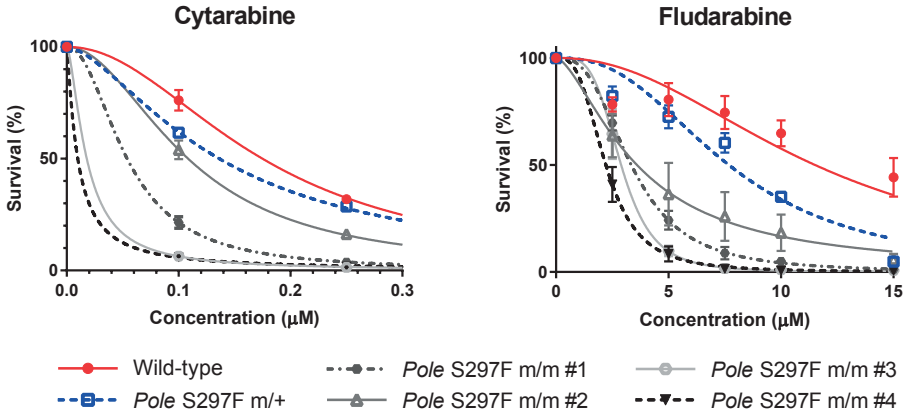


Supplementary Figure 1. Fluorescent In Situ Hybridization of *Pole* on metaphase spreads of *Pole*-mutant cell line.

FISH was performed using a probe containing the *Pole* gene (5qF; biotin-labeled probe detected with Cy3-labeled streptavidin [red]) and a probe proximal of the CRISPR-Cas9 target sequences on the same chromosome (5qA3; digoxigenin-labeled probed detected with FITC-labeled anti-digenoxigenin [green]) with DAPI nuclear counterstaining. For each mouse-derived embryonic stem cell line, a minimum of twenty metaphases was analyzed: all cell lines showed normal, comparable intensity of the probes on two chromosome pairs (example of *Pole*-mutant cell line shown, inset of chromosome pair shown).



Supplementary Figure 2. Spontaneous mutant frequency of *Pole*-mutant cell lines.
The spontaneous mutant frequency of different homozygous *Pole* proofreading domain-mutant mouse-derived embryonic stem cells at the Hypoxanthine Phosphorybosyl Transferase (*Hprt)* gene was determined. Wild-type (WT) and *Msh6*-deficient (Msh6 -/-) mouse-derived embryonic stem cell lines were used as controls. D275A/E277A is a *Pole* proofreading-deficient cell line due to double D275A and E277A mutations. P286R, S297F and V411L indicate cell lines with the cancer-associated *POLE* P286R, S297F, or V411L mutations, respectively. For these *Pole* hotspot mutations, a minimum of two independent cell lines was tested (#1 up to #4). The mean and standard error of the mean based on at least two experiments are shown.



Supplementary Figure 3. Sensitivity to nucleoside analogs cytarabine and fludarabine in *Pole* S297F-mutant and *Pole*-wild-type cell lines.
Non-linear regression of relative survival after treatment with nucleoside analogs cytarabine and fludarabine is shown for *Pole* S297F-mutant and *Pole*-wild-type mouse embryonic stem cells. With regard to the *Pole* S297F-mutant cell lines, one heterozygous *Pole* S297F-mutant (m/+) and four homozygous *Pole* S297F-mutant (m/m #1 - #4) cell lines were tested. Sensitivity for the homozygous *Pole* S297F-mutant cell lines was significantly higher compared to *Pole*-wild-type (for details on these comparisons see also Table 1 and Supplementary Table 5). A heterozygous *Pole* S297F mutation resulted in sensitivity lower than the homozygous mutant cell lines, but higher than wild-type, reaching significance for cytarabine (mean IC_{50} 0.14μM vs. 0.17μM; *P*=0.025 for cytarabine; mean IC_{50} 7.58μM vs. 11.1μM; *P*=0.169 for fludarabine). Dots and error bars indicate mean and standard error of the mean, respectively.

Supplementary Table 1. *POLE* mutations identified in endometrial cancers in the observation arm of the PORTEC-1 trial.

***POLE* proofreading domain mutation**	**Frequency (n=16)**
No. (%) with P286R	7 (44)
No. (%) with V411L	5 (31)
No. (%) with S297F	3 (19)
No. (%) with S459F	1 (6)

Supplementary Table 2. Oligonucleotide guide RNA sequences to introduce *Pole* proofreading domain mutations through CRISPR-Cas9.

***Pole* mutation**	**Forward/Reverse**	**Sequence 5'-3'**
D275A / E277A	Forward	TCTGATCGGTCTCAGCATCA
	Reverse	TGATGCTGAGACCGATCAGA
P286R	Forward	GGAGATCATCATGATCTGAT
	Reverse	ATCAGATCATGATGATCTCC
S297F	Forward	CTCCTATATGATTGATGGCC
	Reverse	GGCCATCAATCATATAGGAG
V411L	Forward	GATTATGACTGCCCACAGGA
	Reverse	TCCTGTGGGCAGTCATAATC

Supplementary Table 3. Oligonucleotide primer sequences to generate a template for homologous recombination for transfection*.

Mutation-specific primers‡		
***Pole* mutation**	**Forward/ Reverse**	**Sequence 5'-3'**§
D275A / E277A	Forward	CGACCAAACTGCCTCTCAAATTTCCTGATGCTGAGACCGATCAG
	Reverse	GAAATTTGAGAGGCAGTTTGGTCGTG**G**CGATG**G**CAAATGCCAAAACCACAGGGTC
P286R	Forward	CAAATTCC**GC**GATGCTGAGACAGATCAGATCATG
	Reverse	CTGATCTGTCTCAGCATC**GC**GGAATTTGAGAGG
S297F	Forward	GATTGATGGCCAAGTGAACAGAATCTC
	Reverse	GGCCATCAATCATATAG**A**AGATCATCATGATCTGATCGGTCTCAGC
V411L	Forward	GG**C**TGAAGAGGGACAGTTATCTTCCTGTGGGCAGTCATAATC
	Reverse	GATAACTGTCCCTCTTCA**G**CCACCTGGAAAAAACACAG
Common primers‡		
External oligo exon 9‖	Forward	TCTGCAAGGTGGCAGTGTAATTAC
	Reverse	GGACCAGCCTCATCTGACTT
External oligo exon 13‖	Forward	GGCCTCTAAGAAACGGCTTTGG
	Reverse	CTTCTCGACTCGTTGCAGCAGG
Joining PCR exon 9	Forward	CCAATTGGACAACATAGTGGAC
	Reverse	GTACATGCTGAGGCCATGAATTG
Joining PCR exon 13	Forward	GGCTTTGGGTCATAGGGATTTG
	Reverse	CTAGGATAAGACACCACAGGGC

*Primers were used to generate a template for homologous recombination, to be used with CRISPR-Cas9 in cell transfection, containing *Pole* proofreading domain mutations as well as silent mutations in the PAM sequences. This template was made using two-stage PCR amplification.

‡The mutation-specific primers together with the common external oligonucleotides were used to generate overlapping PCR fragments with the different substitutions. Fragments were pooled and amplified with the joining primers in a subsequent PCR.

§*Pole* proofreading domain mutations are shown in bold and underlined. Silent mutations in the PAM sequence are underlined.

‖Exon 9 contains the D275, E277, P286 and S297 amino acids. Exon 13 contains the V411 amino acid.

Supplementary Table 4. Oligonucleotide sequences to identify targeted colonies after transfection.

Material	***Pole* proofreading domain exon**	**Forward/ Reverse**	**Sequence 5'-3'**	**Protocol**
gDNA	Exon 9 (D275A/E277A, P286R, S297F)	Forward	GTCCCTTGCTAGTGCTGTCC	(95° 30 s, 65° 30 s, 72° 2 min) 34x
		Reverse	TACCTGGGATGCCTACTTGC	
gDNA	Exon 13 (V411L)	Forward	CCCCACGTAGTAGGCAGCTC	(95° 30 s, 63° 30 s, 72° 2 min) 34x
		Reverse	GAGCCAATGGGCTTACGTGCC	
cDNA	Spanning exons 7-14	Forward	GAATCCATGTGGCCCACTGG	(95° 30 s, 59° 30 s, 72° 1 min) 34x
		Reverse	AGTCTGGGGCTGTTCAGTGG	

Supplementary Table 5. Sensitivity to adjuvant treatment strategies conferred by *Pole* proofreading domain mutations in a cell-based model.

Treatment	WT	*Pole* D275A/E277A		*Pole* P286R		*Pole* S297F						*Pole* V411L	
		#2*		#2*		#2*		#3*		#4*		#2*	
	IC_{50} (mean ± SD)	IC_{50} (mean ± SD)	*P*	IC_{50} (mean ± SD)	*P*	IC_{50} (mean ± SD)	*P*	IC_{50} (mean ± SD)	*P*	IC_{50} (mean ± SD)	*P*	IC_{50} (mean ± SD)	*P*
Ionizing radiation (Gy)	2.96 ± 1.28	2.61 ± 0.41	0.654	3.02 ± 1.61	0.938	2.30 ± 1.07	0.446	1.48 ± 0.07	0.013	2.99 ± 0.71	0.973	3.05 ± 0.68	0.920
5-Fluorouracil (μM)	0.25 ± 0.04	0.22 ± 0.03	0.316	0.20 ± 0.03	0.127	0.26 ± 0.01	0.596	0.28 ± 0.04	0.372	0.18 ± 0.03	0.046	0.25 ± 0.03	0.945
Cisplatin (μM)	0.39 ± 0.17	0.30 ± 0.11	0.353	0.20 ± 0.04	0.061	0.27 ± 0.03	0.290	0.34 ± 0.13	0.645	0.22 ± 0.06	0.089	0.29 ± 0.03	0.308
Doxorubicin (nM)	8.92 ± 4.04	9.47 ± 1.94	0.786	7.95 ± 1.97	0.666	8.77 ± 2.61	0.955	10.3 ± 1.20	0.585	6.16 ± 2.03	0.240	9.35 ± 3.02	0.859
Etoposide (nM)	27.2 ± 15.7	26.3 ± 4.65	0.912	21.1 ± 6.25	0.416	26.4 ± 11.3	0.906	29.9 ± 8.92	0.710	22.8 ± 5.54	0.550	27.3 ± 12.3	0.999
Methotrexate (nM)	60.1 ± 8.48	94.7 ± 22.2	0.047	84.9 ± 28.4	0.179	81.8 ± 11.9	0.015	75.0 ± 21.2	0.346	66.0 ± 27.6	0.623	78.5 ± 29.2	0.174
Paclitaxel (nM)	9.57 ± 2.94	7.60 ± 1.87	0.324	11.1 ± 2.06	0.385	6.78 ± 1.13	0.160	7.09 ± 1.14	0.206	9.20 ± 2.64	0.859	11.1 ± 3.72	0.465
Cytarabine (μM)	0.17 ± 0.02	0.10 ± 0.01	0.001‡	0.05 ± 0.01	<0.001‡	0.11 ± 0.02	0.002‡	0.02 ± 0.01	<0.001‡	0.01 ± 0.01	<0.001‡	0.09 ± 0.02	0.001‡
Fludarabine (μM)	11.1 ± 3.98	5.29 ± 2.13	0.004‡	3.90 ± 0.81	0.001‡	4.76 ± 2.86	0.005‡	2.59 ± 0.31	<0.001‡	2.29 ± 0.72	<0.001‡	5.10 ± 1.53	0.008‡

*For each *Pole* proofreading domain mutation (D275A/E277A, P286R, S297F, V411L, respectively) at least two homozygous cell lines were used to determine the treatment sensitivity. In addition to Table 1, this table shows the sensitivity of the other *Pole*-mutant lines (mean IC_{50} and standard deviation, SD, in table) compared to the sensitivity of the *Pole*-wild-type (WT) mouse embryonic stem cell line; the corresponding *P*-values are shown.

‡Difference remains significant after Holm-Bonferroni correction for multiple comparisons.

Supplementary Table 6. Sensitivity to nucleoside analogs gemcitabine, cladribine and clofarabine conferred by *Pole* proofreading domain mutations in a cell-based model.

Cell line	Treatment*					
	Gemcitabine (nM)		Cladribine (μM)		Clofarabine (μM)	
	IC_{50} (mean ± SD)	*P* (95% CI)	IC_{50} (mean ± SD)	*P* (95% CI)	IC_{50} (mean ± SD)	*P* (95% CI)
WT	16.7 ± 5.74	NA	0.16 ± 0.03	NA	0.23 ± 0.08	NA
Pole D275A/E277A						
#1	19.6 ± 6.90	0.379 (-9.69, 3.92)	0.19 ± 0.04	0.049 (-0.06,-0.0002)	0.23 ± 0.06	0.936 (-0.12, 0.13)
#2	18.5 ± 4.12	0.594 (-9.03, 5.46)	0.19 ± 0.05	0.218 (-0.07, 0.02)	0.21 ± 0.05	0.664 (-0.09, 0.14)
Pole P286R						
#1	19.2 ± 4.35	0.465 (-9.81, 4.82)	0.22 ± 0.07	0.197 (-0.16, 0.05)	0.22 ± 0.09	0.893 (-0.13, 0.15)
#2	17.2 ± 0.37	0.901 (-8.20, 7.32)	0.21 ± 0.06	0.046 (-0.09, -0.01)	0.19 ± 0.04	0.384 (-0.07, 0.16)
Pole S297F						
#1	15.9 ± 0.55	0.820 (-6.97, 8.57)	0.19 ± 0.03	0.112 (-0.07, 0.01)	0.15 ± 0.05	0.172 (-0.05, 0.22)
#2	15.3 ± 0.94	0.685 (-6.34, 9.23)	0.13 ± 0.03	0.126 (-0.01, 0.06)	0.12 ± 0.04	0.067 (-0.01, 0.23)
#3	16.5 ± 1.94	0.962 (-7.71, 8.06)	0.13 ± 0.01	0.047 (0.0005, 0.07)	0.13 ± 0.02	0.068 (-0.01, 0.22)
#4	15.4 ± 0.52	0.661 (-5.24, 7.90)	0.13 ± 0.02	0.082 (-0.004, 0.06)	0.15 ± 0.05	0.135 (-0.04, 0.20)
Pole V411L						
#1	17.8 ± 3.12	0.725 (-8.09, 5.83)	0.21 ± 0.03	0.015 (-0.08, -0.01)	0.21 ± 0.06	0.695 (-0.10, 0.14)
#2	17.1 ± 3.88	0.914 (-7.53, 6.81)	0.17 ± 0.03	0.566 (-0.04, 0.02)	0.19 ± 0.09	0.547 (-0.11, 0.19)

*Sensitivity to nucleoside analogs gemcitabine, cladribine and clofarabine was determined for all *Pole* proofreading domain-mutant cell lines and compared to the sensitivity of the *Pole*-wild-type (WT) mouse embryonic stem cell line; the corresponding *P*-values are shown. None of the *P*-values remained statistically significant after Holm-Bonferroni correction for multiple comparisons.

Abbreviations: SD, standard deviation; CI, confidence interval; NA, not applicable.