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POLE and *POLD1* pathogenic variants in the proofreading domain in papillary thyroid cancer

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Abstract

Thyroid cancer is the most frequent endocrine cancer with an increasing incidence rate worldwide and is the second most common malignancy among females in Saudi Arabia. Papillary thyroid cancer (PTC) is the most common subtype. Germline pathogenic variants in the proofreading domain of the *POLE* and *POLD1* genes predispose to several types of cancers. However, the role of pathogenic variants of these two genes in PTC remains unknown. Capture sequencing, Sanger sequencing and immunohistochemistry were performed on 300 PTC cases from the Middle Eastern region. One germline pathogenic variant each of *POLE* (1/300, 0.33%) and *POLD1* (1/300, 0.33%) genes was identified. Low expression of POLD1 was detected in 46.5% (133/286) of cases and was significantly associated with the follicular variant of PTC (P = 0.0006), distant metastasis (P = 0.0033) and stage IV tumours (P = 0.0081). However, no somatic pathogenic variant was detected in *POLE* gene. Furthermore, low expression of POLE was noted in 61.7% (175/284) of cases with no significant clinicopathological associations. Our study shows that pathogenic variant in the *POLE* and *POLD1* proofreading domain is a cause of PTC and low expression of POLD1 is associated with poor prognostic markers in the Middle Eastern population. Further studies from different geographic populations are needed to determine the frequency and spectrum of proofreading domain pathogenic variants in *POLE* and *POLD1* genes and in PTC from different ethnicities.

Key Words

- papillary thyroid cancer
- IHC
- pathogenic variants
- *POLE*
- *POLD1*

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Introduction

Thyroid cancer is the most frequently occurring endocrine cancer with an increasing incidence rate worldwide and with a frequency among the younger age group (1, 2). It is also the second most common malignancy, after breast cancer, among females in Saudi Arabia, with a significantly higher incidence rate when compared to other countries, such as the USA (2, 3). Amongst the different subtypes, papillary thyroid cancer (PTC) is the most common and accounts for 80–90% of all thyroid cancers (4). PTC is well differentiated and curable with thyroidectomy (often followed by radioiodine treatment); however, the clinical behaviour of PTC varies widely and many patients suffer disease recurrence (5, 6). Although differentiated PTC is usually sporadic, cases of the familial form that is thought to be more aggressive in nature have also been reported (7, 8).

Faithful DNA replication and error control are imperative for the maintenance of genomic stability and repression of mutagenesis (9). The acquisition and accumulation of deleterious mutations can trigger

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carcinogenesis; therefore, a rigorous system to regulate DNA synthesis is essential. There are multiple mechanisms in place to ensure accurate replication of DNA; these include highly selective integration of nucleotides in the daughter strand; exonucleolytic proofreading to remove mismatched nucleotides; and post-replication mismatch repair (MMR) (10, 11). The fidelity of eukaryotic DNA replication is primarily governed by the DNA polymerases ε (Pol ε) and δ (Pol δ); the catalytic and proofreading domains of which are encoded by *POLE* and *POLD1*, respectively (10). The leading strand of the replication fork is synthesised by Pol ε whilst Okazaki fragments of the lagging strand are synthesised by Pol δ (in tandem with DNA polymerase α). Both polymerases have an error rate of ~10^{-4} to 10^{-5} which is further reduced to ~10^{-9} to 10^{-10} via proofreading at the 3′–5′ exonuclease domain as well as post-replication repair by the MMR apparatus (9, 10, 11, 12).

Given that several mutations are needed for tumourigenesis, and DNA replication is extremely meticulous, it can be postulated that mutant polymerases and erroneous DNA synthesis play a key role in cancer development. Indeed, studies have reported an increase in the rate of spontaneous mutations in yeast and mice harbouring mutations in the 3′–5′ exonuclease domain of Pol ε and Pol δ (13, 14, 15). Furthermore, inactivation of the proofreading domain by a point mutation (*POLD1* Asp400Ala) has been shown to increase the rate of oncogenesis in a recessive manner in mice (16, 17). Similarly, studies in humans using whole-genome and targeted sequencing have identified germline pathogenic variants in the exonuclease domain of *POLE* and *POLD1* which predispose, with a high penetrance, to multiple and/or large colorectal adenomas, early-onset colorectal cancer (CRC) as well as endometrial cancer in female carriers (18, 19, 20). In addition, somatic pathogenic variants of *POLE* have also been identified in different types of cancers with various incidence previously, most commonly reported in endometrial cancer (up to 10%) (19, 21, 22), followed by CRC (3–4%) (23, 24). However, among 560 PTC cases characterised by TCGA, only one case harboured *POLE* somatic pathogenic variant (25).

Whilst the effect of mutant *POLE* and *POLD1* on susceptibility to colorectal cancer and endometrial cancer has been widely reported in the literature; the role of *POLE* and *POLD1* in PTC remains unknown. Identification of genetic risk factors can help with screening and early detection and have important implications with respect to genetic counselling and implementation of treatment guidelines.

In our study, we investigated the prevalence and clinical relevance of germline and somatic variants in *POLE* and *POLD1* proofreading domain and their protein expression in PTC cases from the Middle Eastern region.

Materials and methods

Sample selection

Archival samples from 300 PTC patients diagnosed at King Faisal Specialist Hospital and Research Centre (Riyadh, Saudi Arabia) between 1999 and 2013 were included in the study. Detailed clinicopathological data were noted from case records and have been summarised in Table 1. All samples were obtained from patients with approval from the Institutional Review Board of the hospital. For the study, waiver of consent was obtained for archived paraffin tissue blocks from Research Advisory Council (RAC) under project RAC # 2110 031.

DNA extraction

DNAs were isolated from formalin-fixed, paraffin-embedded (FFPE) PTC tumour and corresponding non-tumour tissues using Gentra DNA isolation kit (Gentra, Minneapolis, MN, USA) following the manufacturer's recommendations as described previously (26, 27).

PCR and Sanger sequencing for detection of germline and somatic variants in the proofreading domain of POLE and POLD1 genes

Initially, direct sequencing of the entire coding/splicing region of the proofreading domains of *POLE* and *POLD1* genes was performed on DNA samples from 50 cases both at somatic and germline level. Primer 3 software was used to design the primers for all coding exons and their flanking intronic sequences of proofreading domain of *POLE* and *POLD1* genes (available upon request). PCR was performed in a total volume of 25 µL using 20 ng of genomic DNA, 2.5 µL 10X Taq buffer, 2.3 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit Taq polymerase (all reagents were from Qiagen Inc), 0.2 µM of each primer, and water. The efficiency and quality of the amplified PCR products were confirmed by running the PCR products on a 2% agarose gel.

For Sanger sequencing, the PCR products were subsequently subjected to direct sequencing with BigDye terminator V 3.1 cycle sequencing reagents and analysed on an ABI 3730XL DNA analyser (Applied Biosystems). The reference sequence was downloaded from NCBI

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Table 1 Clinicopathological variables for the patient cohort (n = 300).

	n (%)
Age	
Median	39.0
Range (IQR)	29.0–50.0
Gender	
Female	233 (77.7)
Male	67 (22.3)
Histopathology	
Classical variant	145 (48.4)
Follicular variant	60 (20.0)
Tall cell variant	43 (14.3)
Others	52 (17.3)
Extra-thyroidal extension	
Absent	172 (57.3)
Present	118 (39.4)
Unknown	10 (3.3)
pT	
T1	89 (29.7)
T2	60 (20.0)
T3	131 (43.7)
T4	18 (6.0)
Unknown	2 (0.6)
pN	
N0	175 (58.3)
N1	122 (40.7)
Unknown	3 (1.0)
pM	
M0	286 (95.4)
M1	13 (4.3)
Unknown	1 (0.3)
Stage	
I	205 (68.3)
II	20 (6.7)
III	42 (14.0)
IV	31 (10.4)
Unknown	2 (0.6)

IQR, Interquartile range.

GenBank. Sequencing results were compared with the reference sequence by Mutation Surveyor V4.04 (Soft Genetics, LLC, State College, PA, USA).

Whole exome sequencing of germline and somatic mutations in the proofreading domain of POLE and POLD1 genes

Furthermore, whole exome sequencing (WES) was performed on 250 PTC cases as described previously (28). The tumour and corresponding normal DNA samples with A260/A280 ratio between 1.8 and 2.0 were processed for library construction. The sequencing library was prepared by random fragmentation of the DNA, followed by 5′ and 3′ adapter ligation. Adapter-ligated fragments were then PCR amplified and gel purified. Clusters were generated by loading the library into a flow cell where fragments were captured on a lawn of surface-bound oligos complementary to the library adapters. Each fragment was then amplified into distinct, clonal clusters through bridge amplification. Raw data was generated utilising HCS (HiSeq control software v3.3) and RTA (real-time analysis. v2.5.2).

The BCL (base calls) generated by Illumina Hiseq 4000 were converted into FASTQ files by bcl2fastq (v2.16). The sequence reads in fastq format from each sample were aligned to the reference human genome (GRCh37/hg19) using Burrows–Wheeler aligner (BWA) (29). BAM file generation, PCR duplicates and local realignment was performed using Picard-tools and genome analysis toolkit (GATK) (30).

The variant calling was performed by GATK, subsequently, the variants were annotated by ANNOVAR (31), with dbSNP138, 1000 Genomes, ESP6500, Exome Aggregation Consortium (ExAC), Clinvar and other genome databases. The variants detected by WES were further confirmed by Sanger sequencing.

Assessment of pathogenicity of variants

ACMG/AMP 2015 guideline was utilised first for interpretation of sequence variants. All the uncertain significant variants by ACMG/AMP 2015 guideline were further analysed using five *in silico* pathogenicity prediction tools: CADD, MutationAssessor, PolyPhen-2, SIFT and Mutation Taster (32). The variants predicted as damaging or possibly damaging by three or more *in silico* prediction tools were classified as potentially pathogenic variant and further investigated.

Tissue microarray construction and immunohistochemistry

All samples were analysed in a tissue microarray (TMA) format. TMA construction was performed as described earlier (33). Briefly, tissue cylinders with a diameter of 0.6 mm were punched from representative tumour regions of each donor tissue block and brought into a recipient paraffin block using a modified semiautomatic robotic precision instrument (Beecher Instruments, Woodland, WI). Two cores of PTC were arrayed from each case.

Standard protocol was followed for IHC staining. For antigen retrieval, Dako (Dako Denmark A/S) Target Retrieval Solution pH 9.0 (Catalog number S2367) was used, and the slides were placed in a Pascal pressure cooker for 8 min at 1200°C. The slides were then incubated with primary antibodies against POLE

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(ab-134941, Abcam) and POLD1 (ab-186407, Abcam) at a dilution of 1:1000 (pH 9.0). The Dako Envision Plus System kit was used as the secondary detection system with 3,3'-diaminobenzidine as chromogen. All slides were counterstained with haematoxylin, dehydrated, cleared and mounted. Negative controls included omission of the primary antibody. Normal tissues of different organ system were also included in the TMA to serve as control. An array of normal thyroid tissue ($n=242$) was also stained. Only fresh cut slides were stained simultaneously to minimise the influence of slide ageing and maximise the reproducibility of the experiment.

Scoring and interpretation were performed as described previously (34). Briefly, each TMA spot was assigned an intensity score from 0 to 3 (I0–I3) corresponding to no, weak, moderate and strong staining, and the proportion of tumour staining for that intensity was recorded as 5% increments from a range of 0 to 100 (P0–P3). A final H score (range, 0–300) was obtained by adding the products of scores obtained for each intensity and proportion of area stained (H score = I1XP1 + I2xP2 + I3xP3). Using X-tile version 3.6.1 (35), we defined the optimal cut-off point for POLE and POLD1 expression as H = 130 and H = 125, respectively. Based on H scores, PTC cases were classified into two subgroups: those below the cut-off score were defined as low expression, whereas those above the cut-off score were defined as overexpression.

Staining and evaluation of mismatch repair proteins (MLH1, MSH2, MSH6 and PMS2) were performed as described previously (36).

Statistical analysis

Contingency table analysis and Chi square tests were used to study the relationship between clinicopathological variables and protein expression. Disease-free survival curves were generated using the Kaplan–Meier method, with significance evaluated using the Mantel–Cox log-rank test. The limit of significance for all analyses was defined as P value of < 0.05; two-sided tests were used in these calculations. The JMP11.0 (SAS Institute, Inc., Cary, NC, USA) software package was used for data analyses.

Results

Sample characteristics

A total of 300 unselected PTC cases were included in the study cohort. Majority of the patients were females (77.7%) and had the classical variant of PTC (48.4%). 68.3% of patients had stage I tumour and 4.3% of patients had distant metastasis (Table 1).

Identification of germline and somatic variants in proofreading domain of *POLE* and *POLD1* genes

Initially, we performed Sanger sequencing of 50 PTC samples to look for germline and somatic mutations in proofreading domain of *POLE* and *POLD1* genes. We found one germline variant (c.1370C>T: p.Thr457Met) of the *POLE* gene which was interpreted as of uncertain significance by ACMG/AMP 2015 guideline. Further analysis showed that this variant was potentially pathogenic as predicted by three out of five *in silico* pathogenicity prediction tools. To further analyse the frequency of *POLE* and *POLD1* pathogenic variants in PTC, we expanded our study to include another 250 PTC cases, which were analysed by WES. In this cohort of 250 cases, one germline variant (c.1006C>T:p. Gln336Ter) of *POLD1* gene was detected and interpreted as pathogenic by ACMG/AMP 2015 guideline. However, no pathogenic variants were identified at somatic level in our cohort. Altogether, two germline variants (0.66%), one pathogenic and one potentially pathogenic, were identified in the entire cohort of 300 PTC cases.

The *POLD1* p. Gln336Ter heterozygous pathogenic variant was detected in a 36-year-old female who had a mixed classical and follicular PTC. The tumour was stage I with no extra-thyroidal extension. The *POLE* p.Thr457Met heterozygous variant was identified in an early-onset PTC patient (diagnosed at age of 18 years). The patient with *POLE* p.Thr457Met variant had stage I classical variant of PTC. This variant is conserved in four out of six species and absent in the population database of ExAC (Table 2). Both the cases were MMR proficient by immunohistochemistry.

Since germline mutations in the proofreading domains of POLE and POLD1 are associated with an increased risk

Table 2 Characteristics of *POLE* p.Thr457Met identified.

Gene	*POLE*
Mutation	c.1370C>T: p Thr457Met
PolyPhen-2	Probably damaging
Mutation assessor	Low
SIFT	Tolerated
CADD	25.1
Mutation taster	Disease causing
Family history	Negative
Conservation between species	4 out of 6
Age at diagnosis	18
Frequency in ExAC	Absent

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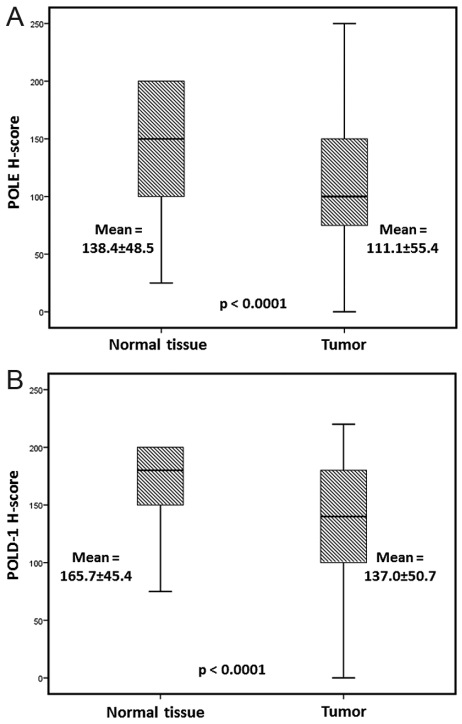

Figure 1
Boxplot showing difference in expression between normal and tumour tissue for POLE and POLD1. (A) POLE expression was significantly higher in normal tissue compared to tumour (mean 138.4 ± 48.5 vs 111.1 ± 55.4; $P < 0.0001$). (B) POLD1 expression was significantly higher in normal tissue compared to tumour (mean 165.7 ± 45.4 vs 137.0 ± 50.7; $P < 0.0001$).

of colorectal cancer, endometrial cancer and giant cell glioblastoma (37, 38, 39), we looked for the co-existence of these malignancies in our cohort. One patient had developed rectal cancer prior to PTC, whereas another patient had endometrial cancer before developing PTC. However, neither of these patients harboured pathogenic variants of POLE or POLD1.

POLE and POLD1 expression in PTC and their association with clinicopathological features

Next, we evaluated the expression of POLE and POLD1 by immunohistochemistry in 300 PTC cases and 242 normal thyroid tissues using tissue microarray. The tumour tissues showed a significantly lower expression of POLE and POLD1 proteins compared to normal thyroid tissue (Fig. 1). POLE immunohistochemical expression was interpretable in 284 cases. Low expression of POLE was noted in 61.7% (175/284) of cases (Fig. 2A and B). No significant clinicopathological associations were noted (Table 3). The single *POLE* mutant case also showed low expression of POLE by IHC. POLD1 expression was interpretable in 286 cases. Low expression of POLD1 was noted in 46.5% (133/286) of cases (Fig. 2C and D) and was significantly associated with the follicular variant of PTC ($P = 0.0006$), distant metastasis ($P = 0.0033$) and stage IV tumours ($P = 0.0081$) (Table 4). The *POLD1* mutant case showed over-expression of POLD1 protein. Both POLE

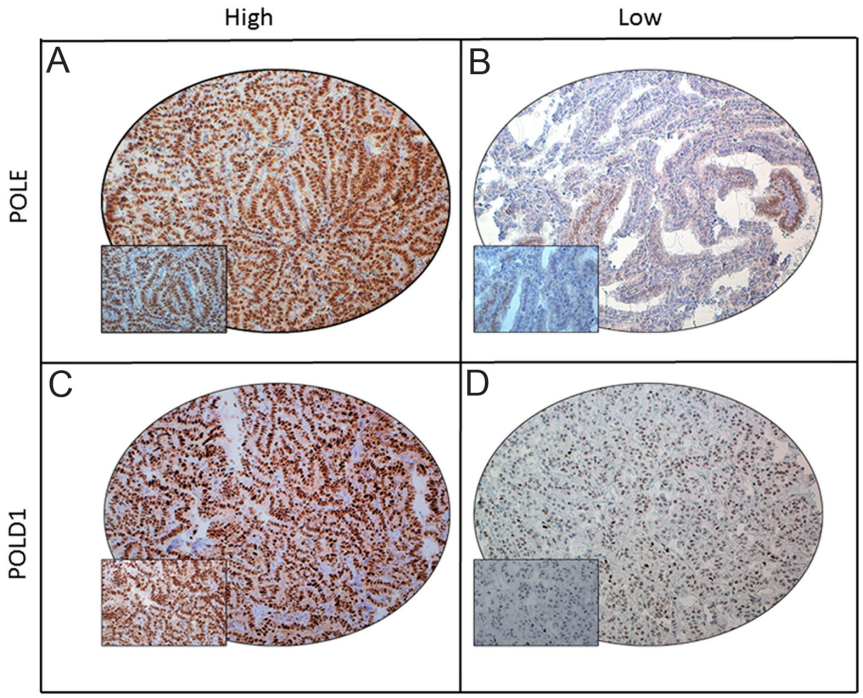

Figure 2
Tissue microarray-based immunohistochemistry analysis of POLE and POLD1 in PTC patients. PTC TMA spots showing overexpression of POLE (A) and POLD1 (C). In contrast, another set of TMA spots showing reduced expression of POLE (B) and POLD1 (D). 20X/0.70 objective on an Olympus BX 51 microscope with the inset showing a 40X/0.85 aperture magnified view of the same TMA spot.

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Table 3 Clinicopathological associations of POLE protein expression in papillary thyroid carcinoma.

	Total		POLE overexpression		POLE low expression		*P* value
	No.	%	No.	%	No.	%	
No. of patients	284		109	38.3	175	61.7	
Age (years)							
≤45	176	62.2	67	38.1	109	61.9	0.8427
>45	107	37.8	42	39.3	65	60.7	
Sex							
Female	222	78.2	91	41.0	131	59.0	0.0825
Male	62	21.8	18	29.0	44	71.0	
Extra-thyroidal extension							
Absent	164	59.6	60	36.6	104	63.4	0.4175
Present	111	40.4	46	41.4	65	58.6	
pT							
T1	85	30.1	27	31.8	58	68.2	0.2926
T2	58	20.6	24	41.4	34	58.6	
T3	122	43.3	53	43.4	69	56.6	
T4	17	6.0	5	29.4	12	70.6	
pN							
pN0	166	59.1	65	39.2	101	60.8	0.7647
pN1	115	40.9	43	37.4	72	62.6	
pM							
pM0	272	96.1	107	39.3	165	60.7	0.1364
pM1	11	3.9	2	18.2	9	81.8	
Stage							
I	195	69.1	73	37.4	122	62.6	0.2346
II	17	6.0	5	29.4	12	70.6	
III	40	14.2	21	52.5	19	47.5	
IV	30	10.7	10	33.3	20	66.7	
Histology type							
Classical variant	137	48.2	49	35.8	88	64.2	0.5692
Follicular variant	58	20.5	22	37.9	36	62.1	
Tall-cell variant	43	15.1	16	38.1	27	61.9	
Other variants	46	16.2	22	47.8	24	52.2	
Disease-free survival							
5 years				84.3		76.4	0.1590

and POLD1 protein expression were not associated with disease-free survival (P=0.1590 and 0.5197, respectively; Fig. 3).

Discussion

During the past decades, there has been a significant increase in the incidence of PTC worldwide (1, 2, 3, 4). Hence, further research is necessary to identify the cellular and genetic changes which trigger oncogenesis in order to administer appropriate targeted therapies as well as improve the prevention and assessment of cancer risk. Knowledge of mutation distribution can also be used to plan cost-effective genetic testing strategies on a national scale.

Pathogenic variants of the proofreading domains in POLE and POLD1 genes are widely known to be associated with different cancers including endometrial cancer, colorectal cancer, lung cancer, and glioblastoma (19, 22, 24, 39, 40, 41, 42, 43, 44). However, there are very limited current literature regarding these variants in PTC, with TCGA reporting only a single POLE somatic pathogenic variant among 560 cases (25). To the best of our knowledge, we are the first to report the frequency of proofreading domain germline pathogenic variant of the *POLE* and *POLD1* genes in PTC cases. In our cohort, one pathogenic variant in the proofreading domain of *POLD1* and one potentially pathogenic variant in the proofreading domain of *POLE* were identified, accounting for 0.66% of all PTC cases. Interestingly, these two variants were not reported previously. The variant of *POLD1* p. Gln336Ter caused a stop gain and was identified in an early onset patient, indicating a functional loss and predisposition to PTC. Although the *POLE* p.Thr457Met variant was classified as uncertain significance by ACMG, it was partially conserved and completely absent in the population database of ExAC.

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Table 4 Clinicopathological associations of POLD1 protein expression in papillary thyroid carcinoma.

	Total		POLD1 overexpression		POLD1 low expression		*P* value
	No.	%	No.	%	No.	%	
No. of patients	286		153	53.5	133	46.5	
Age (years)							
≤ 45	179	62.8	102	57.0	77	43.0	0.1085
> 45	106	37.2	50	47.2	56	52.8	
Sex							
Female	223	78.0	123	55.2	100	44.8	0.2900
Male	63	22.0	30	47.6	33	52.4	
Extrathyroidal extension							
Absent	165	57.7	85	51.5	80	48.5	0.2488
Present	111	42.3	65	58.6	46	41.4	
pT							
T1	85	29.9	43	50.6	42	49.4	0.1013
T2	59	20.8	28	47.5	31	52.5	
T3	123	43.3	75	61.0	48	39.0	
T4	17	6.0	6	35.3	11	64.7	
pN							
pN0	166	58.7	82	49.4	84	50.6	0.1112
pN1	117	41.3	69	59.0	48	41.0	
pM							
pM0	272	95.4	151	55.5	121	44.5	0.0033
pM1	13	4.6	2	15.4	11	84.6	
Stage							
I	195	68.6	109	55.9	86	44.1	0.0081
II	19	6.7	9	47.4	10	52.6	
III	40	14.1	26	65.0	14	35.0	
IV	30	10.6	8	26.7	22	73.3	
Histology type							
Classical variant	138	48.3	75	54.3	63	45.7	0.0006
Follicular variant	59	20.6	20	33.9	39	66.1	
Tall-cell variant	43	15.0	32	74.4	11	25.6	
Other variants	46	16.1	26	56.5	20	43.5	
Disease-free survival							
5 years				81.3		77.9	0.5197

Furthermore, the carrier of this variant developed PTC at a very early age of 18 years. The early onset of PTC suggests that *POLE* p.Thr457Met might have strong cause-effect on pathogenesis of PTC. Interestingly, this variant was also identified in one CRC patient previously by us (45). Our data indicate that this variant might predispose to not only PTC but also CRC. However, further functional analysis is required to investigate the effect of this variant on the function of the *POLE* gene. In our cohort, we did not identify any germline pathogenic variants which were common in Western populations. These data imply the unique spectrum of proofreading domain pathogenic variants of *POLE* and *POLD1* in our population. Despite the large number of cases screened, no somatic pathogenic variants were identified in our cohort, indicating that somatic proofreading domain pathogenic variants of POLE and POLD1 might not play important role in the development of sporadic PTC in our population.

We have previously studied the expression of POLE and POLD1 in endometrial carcinomas and their clinicopathological associations (34). However, to the best of our knowledge, this is the first study to report the clinicopathological associations of POLE and POLD1 protein expression in PTC. Low expression of POLD1 was associated with poorer prognostic markers such as distant metastasis and stage IV tumours. We also found low expression of POLE and POLD1 to be associated with poor prognostic factors such as lymph node involvement, grade 3 tumours and stage III tumours in CRC (45). However, no significant association with disease-free survival was noted with both biomarkers. Interestingly, while the case harbouring *POLE* p.Thr457Met showed a low expression of POLE protein, another case harbouring the pathogenic variant of *POLD1* p. Gln336Ter showed high expression of POLD1. This discrepancy between protein expression and mutation has been highlighted previously by us (34) and others (12) in different organ sites. It might be

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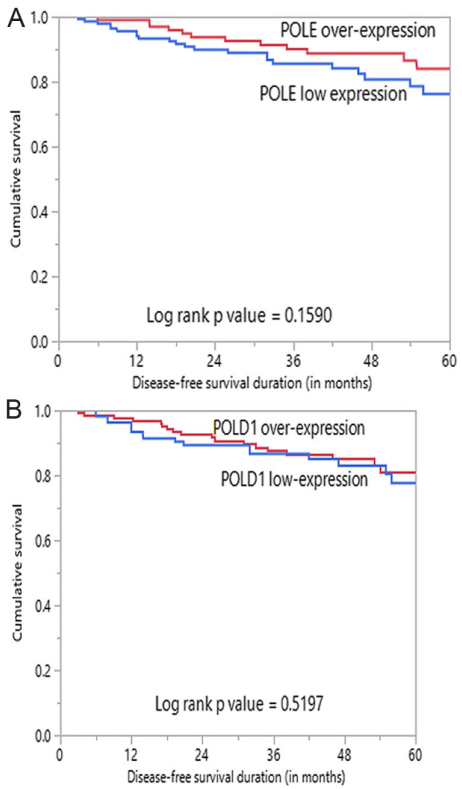

Figure 3
Kaplan–Meier survival analysis for the prognostic significance of POLE and POLD1 expression in PTC. (A) There was no significant difference in disease-free survival with POLE expression (P = 0.1590). (B) There was no significant difference in disease-free survival with POLD1 expression (P = 0.5197).

partly explained by the fact that only pathogenic variants in proofreading region were investigated. In addition, a previous study also demonstrated that there were more truncated variants detected in the region outside of proofreading domain in POLE and POLD1 genes (46) and these truncated variants could also cause loss of POLE or POLD1 expression. Further studies are warranted to validate our findings.

Conclusions

In conclusion, this study presents data on the frequency of *POLE* and *POLD1* proofreading domain somatic and germline pathogenic variants and association of POLE and POLD1 expression with prognostic biomarkers in a cohort of patients with PTC; more specifically, in the Middle East. Our data suggest that pathogenic variants in the proofreading domain of *POLE* and *POLD1* is a cause of PTC and low expression of POLD1 is found to be associated with poor prognostic markers. Nonetheless, it may be of interest to include these two genes in the gene panel for future studies.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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