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Original Article

Functions, interactions and prognostic role of *POLE*: a bioinformatics analysis

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

ABSTRACT

Objective: To describe *POLE* characteristics and reported mutations in endometrial cancer (EC) and analyze the impact of these mutations on the structure and function of the protein, as well as their relationship with the survival and prognosis of the disease.
Methods: We retrieved reported mutations for *POLE* in EC from Catalogue of Somatic Mutations in Cancer database. We analyzed the most frequent mutations possible impact in the protein using HOPE server. We built a protein-protein network using Network Analyst, Cytoscape, and Network Analyzer plugin for topological analysis, enrichment analysis was performed using Gene Ontology: Biological processes. Clinical data was retrieved from cBioPortal database to compare overall survival between mutated *POLE* (*POLE*mut) and wild-type *POLE*. Relation of mutational status of *POLE* in EC and immune cell infiltration was analyzed using CIBERSORT algorithm in TIMER2.0 server.
Results: Thirty mutations in POLE were retrieved, most reported mutations were p.P286R, p.V411L and p.A456P, these mutations were likely to be pathogenic. Network analysis of POLE showed interaction of this protein in biological processes such as DNA repair, the cell proliferation cycle, and mechanisms of resistance to platinum. Immune infiltration analysis showed that T cell CD8+, T cell memory activated CD4+, T cell follicular helper, T cell gamma delta and macrophage M1 were more infiltrated in EC *POLE*mut tumors.
Conclusion: Mutations in POLE might affect DNA polymerase epsilon function. These mutations also affect interactions with other proteins like proteins involved in different DNA repairing mechanisms. *POLE* mutations may lead to platinum resistance, but they can also trigger an immune response that improves prognosis.

Keywords: Endometrial Cancer; DNA Polymerase Epsilon; Catalytic Subunit; Human; Mutation

Synopsis

POLE mutations may influence the function of DNA polymerase epsilon. DNA repairing mechanisms may be affected. *POLE* mutations might stimulate an immune response that can be related to better patient prognosis.

Author Contributions

Conceptualization: C.V.J., G.C.L.D.; Data curation: C.V.J., G.C.L.D.; Formal analysis: C.V.J., G.M.F., G.C.L.D.; Funding acquisition: G.C.L.D.; Investigation: C.V.J., G.C.L.D.; Methodology: C.V.J.; Project administration: G.C.L.D.; Resources: G.C.L.D.; Supervision: C.V.J., G.C.L.D.; Visualization: C.V.J., G.M.F.; Writing - original draft: C.V.J., G.M.F.; Writing - review & editing: C.V.J., G.M.F., G.C.L.D.

INTRODUCTION

Endometrial cancer (EC) ranks sixth among cancers in women, with approximately 417,367 new cases and 100,000 deaths worldwide in the year 2020 [1]. The incidence rate varies among regions, according to ethnicity [2]. However, despite the higher morbidity rates in developed countries, mortality is higher in less-developed countries [3]. EC was initially classified by Bokhman [4] according to its clinical, endocrine, and epidemiological characteristics [4]. Subsequently, Murali et al. [5] and Gargiulo et al. [6] included the histomorphological characteristics in this model. Type I EC is characterized by a low-grade, endometrioid type, is positive for hormone receptors, and is associated with a better prognosis. On the other hand, type II EC has a high grade, is a non-endometrioid subtype, is negative for hormone receptors, exhibits mutations in the *TP53* gene, and is associated with a poorer prognosis [7,8]. However, the use of these morphological and molecular parameters alone has complicated the classification of this cancer [6]. In the last decade, molecular and pathological studies have provided more information related to endometrial carcinogenesis and molecular characteristics according to different histological subtypes, which has been linked to the response to new treatments, prognosis, and survival of patients [6].

In 2013, The Cancer Genome Atlas (TCGA) consortium proposed a classification based on genomic and transcriptomic characteristics obtained from the analysis of 307 samples of the endometrioid subtype and 66 samples of serous subtype EC [6,9]. This classification is based on the number of mutations per megabase (Mb), copy number alterations, and microsatellite instability (MSI), resulting in 4 groups: The first group is an ultramutated subtype (>100 mutations per Mb) with alterations in the exonuclease domain gene of DNA polymerase ε (Pol ε, encoded by the *POLE*); the second group is a subtype with microsatellite instability, low copy number, and a hypermutated phenotype (>10 mutations per Mb); the third subtype are tumors with low copy number and a low mutation rate, and the fourth group are tumors with high copy number, showing a significant similarity to the serous histological subtype, with a low mutation rate [6].

In 2018, the Proactive Molecular Risk Classifier for Endometrial Cancer complemented the classification by subdividing it into 4 molecular subtypes: exonuclease domain mutant (EDM), mismatch repair deficient, mutant *p53* (abnormal p53), and without a specific molecular profile (wild-type p53) [10]. The EDM group showed high rates of somatic mutations and is referred to as the ultramutated *POLE* group, which has been reported in approximately 10% of ECs. Additionally, tumors of this type are associated with a high rate of infiltrating lymphocytes and are associated with a favorable prognosis [11].

Polymerases are protein complexes that add a deoxyribonucleoside monophosphate to the end of a DNA primer sequence. This polymerization process occurs during DNA replication, repair, and synthesis processes [12]. The B family of DNA polymerases (Pol α, Pol δ, and Pol ε) is actively involved in genomic replication, where Pol δ and Pol ε extend the primers synthesized by Pol α at initiation sites and complete the synthesis of replicated DNA [12]. Pol ε is a heterotetramer composed of subunits POLE1 or POLE (catalytic subunit or exonuclease domain), POLE2 (p59), POLE3 (p12), and POLE4 (p17) [12]. The altered function of the exonuclease domain of the pol ε is one of the factors leading to a cancer hallmark, as the correction of errors introduced during DNA replication by the DNA polymerase enzyme is conducted by the exonuclease domain encoded by the *POLE* gene. This results in the accumulation of errors that lead to genomic instability and, consequently, facilitates cancer

development [13]. Similarly, germinal or somatic mutations in this gene have been associated with various types of cancer, including lung, esophageal, bladder, colorectal, melanoma, and EC [13]. As mentioned earlier, alterations in *POLE* are associated with an ultramutated subtype of EC.

The most frequently reported mutations in *POLE* in EC are C→A transversions, along with mutations in p.P286R and p.V411L [14]. There have also been reports of other mutations associated with the pathology, including TCT→TAT and TCG→TTG substitutions, as well as mutations in the residues p.S297F, p.F367S, A456P, and S459F. These mutations are near the catalytic residues of the exonuclease, leading to a reduction in catalytic activity and an increase in the mutation rate [14].

Currently, the International Federation of Gynecology and Obstetrics (FIGO) includes the mutational status of *POLE* in the staging of EC. Therefore, in this study, we describe the characteristics of the *POLE* gene and the reported mutations in EC and analyze the impact of these mutations on the structure and function of the protein, as well as their relationship with the survival and prognosis of the disease.

MATERIALS AND METHODS

1. Relationship between structure and activity of mutations in the *POLE* gene

Mutations in POLE reported in EC were retrieved from the Catalogue of Somatic Mutations in Cancer (COSMIC) database (cancer.sanger.ac.uk). Mutations were obtained using an advanced filter. The search was filtered by tissue for endometrium, without a specified subsite, and a carcinoma histology. From the identified mutations, those corresponding to the coding segment of the exonuclease domain were filtered. Subsequently, the most frequent mutations were analyzed using the HOPE server (https://www3.cmbi.umcn.nl/hope/) [15] to identify the potential structural effects of mutations on the POLE protein, the pathogenicity score of the mutation is obtained from the results of the analysis in HOPE. In this database, the analysis is conducted using MetaRNN (https://www.liulab.science/MetaRNN) [16], where the score is classified from 0.1 to 1, where 1 represents the highest probability value of pathogenicity.

2. Analysis of POLE interactions

To identify the biological processes that may be impacted by the malfunctioning of DNA polymerase due to its interaction, we generated a protein-protein interaction (PPI) network using the NetworkAnalyst server (https://www.networkanalyst.ca/NetworkAnalyst/home.xhtml) [17]. A second-order PPI network was generated using the STRING Interactome database [18] with a confidence value of 900, filtering the search for endometrial tissue, and the interaction network was then exported to Cytoscape 3.10.1 (https://cytoscape.org/) [19]. Topological analysis was performed using the Network Analyzer plugin [20]. The topological parameters used for the analysis were degree (the number of connections a node has within the network) and betweenness (a measure of how often a node lies on the shortest path between any two nodes in the network). Enrichment analysis was performed using Gene Ontology: Biological processes (GO:BP).

3. Relationship between *POLE* mutations and survival

Analysis of the relationship between the mutational status of the *POLE* gene and survival in patients with EC was conducted using data from studies registered for this cancer in the

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cBioPortal for Cancer Genomics database (https://www.cbioportal.org/) [21]. Six studies reported on EC "endometrial cancer," "endometrial carcinoma," and "uterine corpus endometrial carcinoma." The total number of samples was 2,934. A comparison was conducted between the mutated *POLE* (*POLE*mut) and wild-type *POLE* (*POLE*wt).

4. Relationship between the mutational status of *POLE* and immune cell infiltration

To understand the impact of the mutational status of the *POLE* gene and its relationship with the tumor microenvironment, the infiltration level of cells comprising the microenvironment in biopsies with *POLE*mut or *POLE*wt was analyzed. The TIMER 2.0, server (http://timer.comp-genomics.org/timer/) was utilized [22]. The CIBERSORT algorithm was used to analyze different immune cell types in EC (uterine corpus EC).

RESULTS

1. Structure-activity relationship of mutations in the *POLE* gene

To understand the impact of mutations in the *POLE* gene on protein function, mutations considered pathogenic located in the exonuclease domain (residues 268 to 471) were first extracted [23,24] from the POLE gene mutations associated with endometrial tumors in the COSMIC database (**Table S1**), pathogenic mutations were then extracted. Subsequently, those with the highest frequency of reporting were analyzed using the HOPE server. Thirty mutations in the exonuclease domain were found in this database, and 7 were reported more than twice. The c.857C>G;p.P286R mutation is the most frequent mutation in patients with EC with 83 reports; this mutation results in an amino acid change from proline which is a neutral and rigid amino acid to arginine which is larger and positively charged amino acid (**Fig. 1**). This change affects the hydrophobicity of the protein and causes alterations in electrostatic interactions with other molecules. Additionally, prolines confer rigidity to the protein structure, so the change in this residue represents a destabilization of the spatial conformation of this domain in POLE, according to the analysis in HOPE.

At position 411 of POLE, 2 mutations have been reported: c.1231G>C and c.1231G>T, both resulting in a change in the protein from valine to leucine. These 2 mutations ranked second in frequency in patients with EC, with 22 and 15 reports in this database, respectively. Structural analysis showed that leucine is a larger amino acid than valine and likely affects the core structure of the protein, altering its function, this mutation had a pathogenicity score of 0.88 (**Fig. 1**).

Another common mutation in the *POLE* gene reported in the COSMIC database in samples from patients with EC is the c.1366G>C;p.A456P mutation, which has 12 reports. Analysis of HOPE showed that the change from alanine to proline can cause a shift in the core structure of the protein, as alanine is larger than proline, potentially hindering proper DNA binding and affecting exonuclease function. The pathogenicity analysis of this variant is 0.87 (**Fig. 1**).

The c.890C>T;p.S297F mutation, representing a change from serine to phenylalanine, was reported 11 times in the COSMIC database. Serine is in the core of the protein (position 297), and because of the hydroxyl group in its side chain, it establishes hydrogen-bond-like intermolecular interactions with tyrosine 305, this change has a pathogenicity score of 0.91 (**Fig. 1**). On the other hand, the change from alanine to valine at position 465 (c.1394C>T;p.

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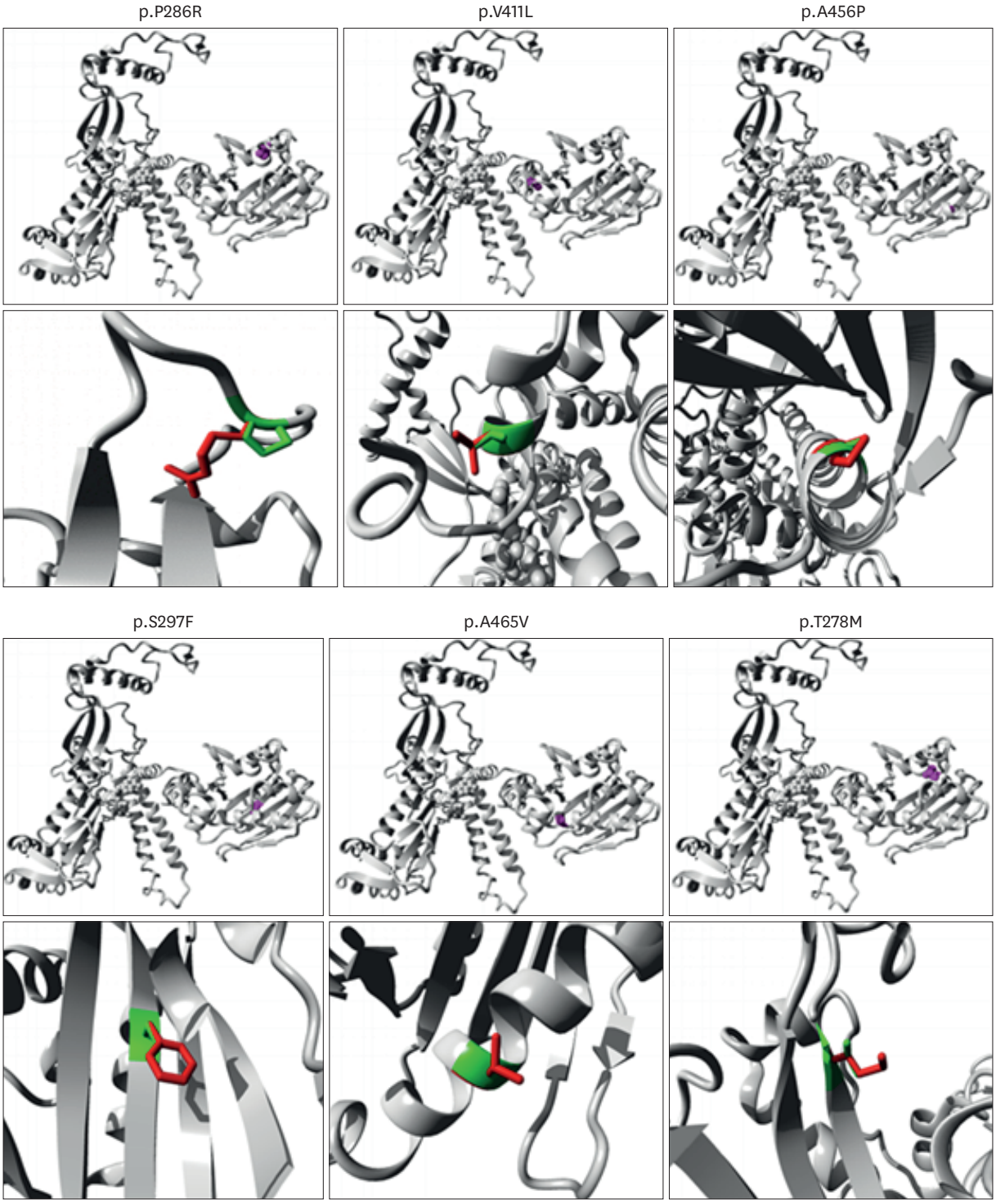

Fig. 1. Structural representation of the most frequent *POLE* mutations in COSMIC database. The magenta color in the upper representations indicates the position of the mutated amino acid. Green indicates the original amino acid, and red indicates the substituted amino acid.
COSMIC, Catalogue of Somatic Mutations in Cancer.

A465V) is reported less frequently (4 times) and the pathogenicity score for this variant is 0.79, and along with the c.833C>T;p.T278M mutation, reported 3 times, they represent changes that alter the nuclear structure of the protein due to the difference in size and charge

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of the residue, respectively (**Fig. 1**). In the case of the threonine-to-methionine change at position 278, the hydrophobic environment caused by the threonine amino acid is affected, and stabilization of the protein through hydrogen bonds decreases, altering the function of the protein. This variant had a pathogenicity score of 0.87.

2. PPI network

The NetworkAnalyst server is a tool that allows visual network analysis of gene expression data. Using this tool, a PPI network was constructed using POLE as the central node (**Fig. 2**). The generated network consisted of 104 nodes (proteins) and 1,528 interactions between the nodes. Topological analyses were performed on this network to identify the relationships between the components. In topological terms, the obtained network had a clustering coefficient of 0.631, diameter of 3, and radius of 2. These values indicate the level of clustering of elements within the network (**Table S2**). Topological analysis of POLE network highlighted different proteins in terms of between within the network. Among the proteins present in this network are the remaining subunits of Pol ε, catalytic subunits of Pol δ, and proteins from chromosomal maintenance and replication factors.

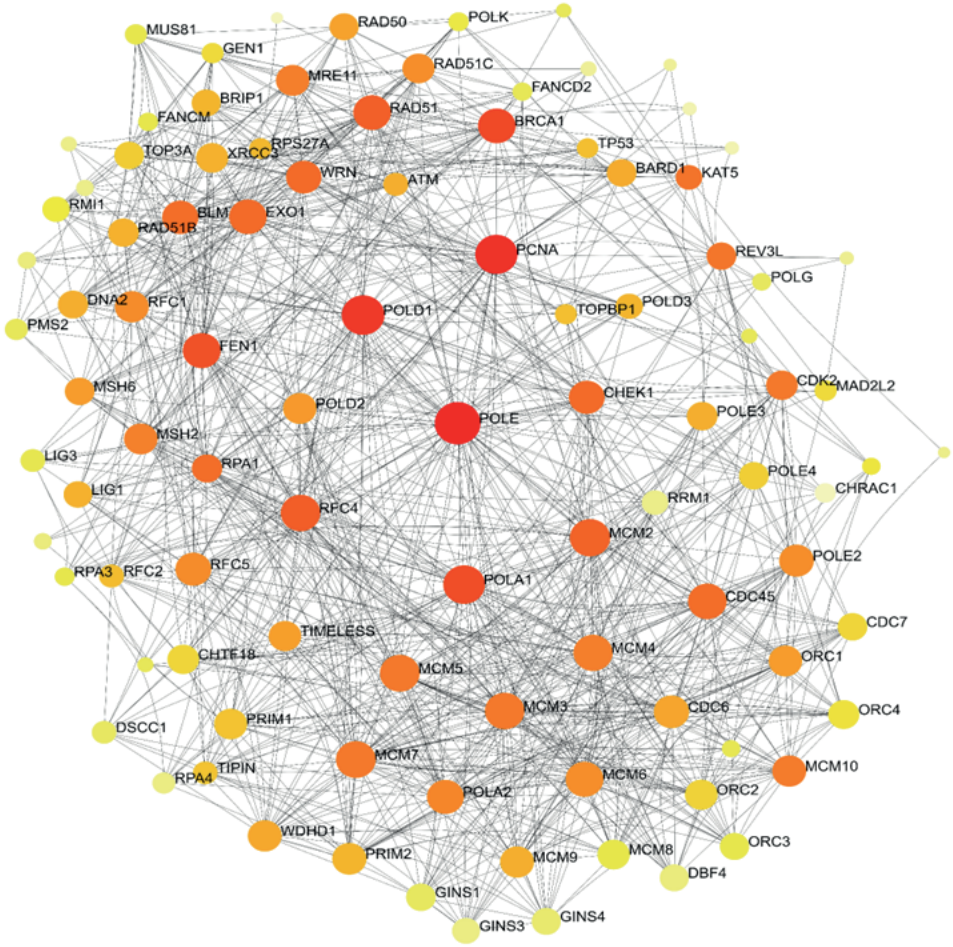

Fig. 2. Protein-protein interaction network of POLE in the endometrium. Proteins are represented as circular nodes; the centrality of the nodes is shown by the color of the node, where the central nodes are in red and the less central nodes are in yellow.

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Table 1. Enrichment analysis of the POLE interaction network

Biological process	p-value*	FDR
DNA replication	4.22E-54	1.34E-51
Mismatch repair	2.19E-30	2.74E-28
Homologous recombination	2.58E-30	2.74E-28
Cell cycle	1.31E-26	1.04E-24
Falconi anemia pathway	2.04E-25	1.30E-23
Nucleotide excision repair	4.95E-23	2.62E-21
Base excision repair	9.89E-15	4.49E-13
Platinum drug resistance	6.49E-7	2.58E-5

FDR, false discovery rate
*Significant p-value ≤0.05.

Through Kyoto Encyclopedia of Genes and Genomes database, a systemic analysis of various biological processes was conducted by associating different components of the PPI network with gene ontologies. Enrichment analysis was performed using the GO:BP database (**Table 1**). Among the obtained results, the proteins in the network were associated with DNA replication, repair mechanisms, cell cycle, and platinum resistance.

3. Relationship between *POLE* mutations and survival

cBioPortal was used for visualization, analysis, and downloading of genomic datasets from studies conducted on endometrial carcinoma and uterine corpus EC. Data from 2,934 samples from the selected projects were retrieved. Of the total studies, 7.56% (222/2,934) of the samples showed microsatellite stability; of these, 5.61% (12/214) had mutations in POLE. In addition, 217 samples were associated with high MSI, and 19.81% (43/217) of these samples corresponded to *POLE*mut. In contrast, 81 samples were classified according to the FIGO staging classification (**Fig. 3**), 59.25% (48/81) belonged to stage IA, of these, 10.42% (5/48) correspond to *POLE*mut.

The overall survival of EC patients with *POLE*mut and *POLE*wt was evaluated using cBioPortal (**Fig. 4**). According to the data recorded in this database, no significant difference was found between patients with EC with or without *POLE* mutations (hazard ratio=0.867; 95% confidence interval=0.398–1.888; p=0.735).

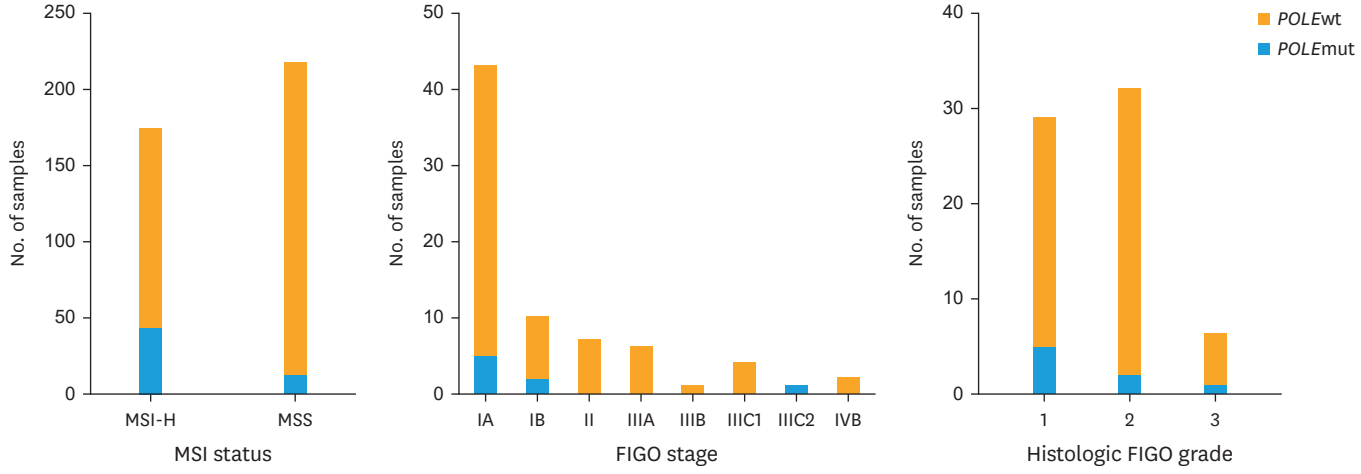

Fig. 3. Analysis of the data extracted from the cBioPortal database. The graphs show the mutational status of *POLE* according to the MSI status, histological FIGO grade, and FIGO stage.
FIGO, International Federation of Gynecology and Obstetrics; MSI, microsatellite instability; *POLE*mut, mutated *POLE*; *POLE*wt, wild-type *POLE*.

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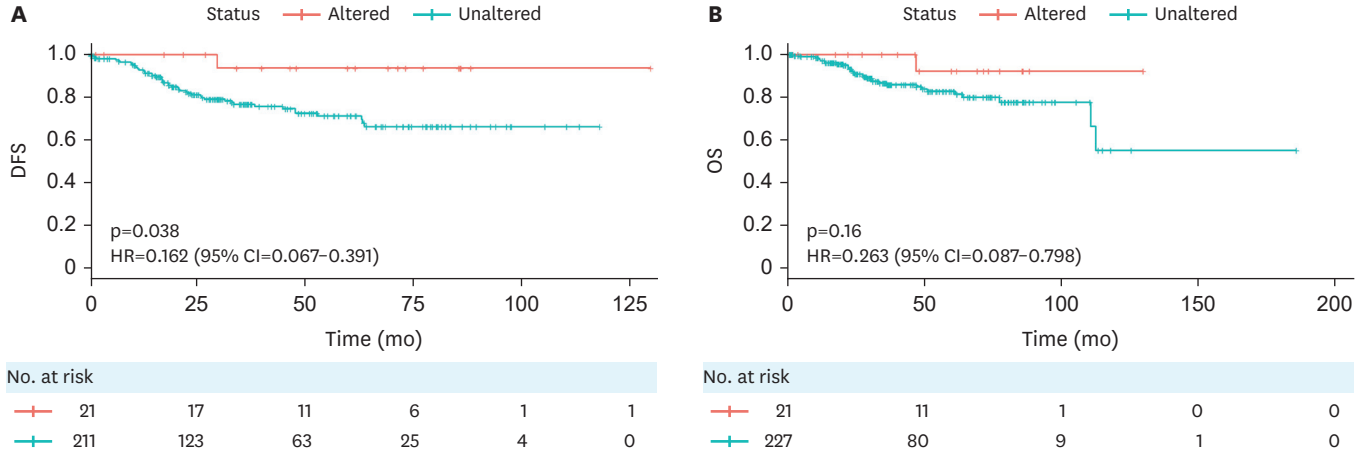

Fig. 4. DFS and OS of endometrial cancer patients according to the data recorded in cBioPortal for *POLE* mutation status and clinical data for this cancer.
CI, confidence interval; DFS, disease-free survival; HR, hazard ratio; OS, overall survival.

To understand the relationship between the immune infiltrate and its association with *POLE*mut in samples with EC, an analysis was conducted using TIMER2.0. Using the TIMER2.0 mutation tool, an analysis was performed to compare the infiltration of immune cell subtypes in samples with *POLE*mut and *POLE*wt. The results that showed a significant correlation between cell subtype and *POLE*mut status (**Fig. 5**) were as follows: T cell CD8+ ($p<0.001$, log2=0.473), T cell memory activated CD4+ ($p<0.001$, log2=0.469), T cell follicular helper ($p<0.001$, log2=0.521), T cell gamma delta ($p=0.006$, log2=1.208), and macrophage M1 ($p<0.001$, log2=0.68), which showed a higher level of infiltration of EC tumors with *POLE*mut. Meanwhile, those that showed a lower level of infiltration in the tumor in the presence of *POLE*mut were CD4+ T cell memory resting ($p=0.006$, log2=−0.422), T cell regulatory ($p=0.004$, log2=−0.422), monocyte ($p=0.006$, log2=−0.505), and myeloid dendritic activated cells ($p<0.001$, log2=−2.16).

DISCUSSION

Currently, there are several biological databases that, along with the development of bioinformatics tools, allow for larger-scale analysis of biological and clinical information generated by different research studies [25]. Biological data, such as genomes, transcriptomes, and proteomes, along with clinical, demographic, and social data, are crucial for a comprehensive understanding of various diseases [26]. However, the information is not sufficient to identify patterns or molecular markers for a given research problem. Therefore, it is necessary to work on the development of data-processing algorithms that facilitate the distinction between emerging properties and molecular patterns within the dataset under investigation [26]. This type of study enables the establishment of relationships between different variables to gain a deeper understanding of the pathology, in this case, the relationship between *POLE* mutations, EC, and the survival of the disease.

According to the findings from the databases cited in this study, the most frequent mutations in the exonuclease domain of Pol ε resulted in physicochemical modifications of the protein. This primarily affects the structural conformation of the active site of the enzyme, thereby reducing its binding affinity for DNA. Several of the reported mutations are associated with highly conserved residues in EXO I and III motifs as well as in the DNA-binding region [23].

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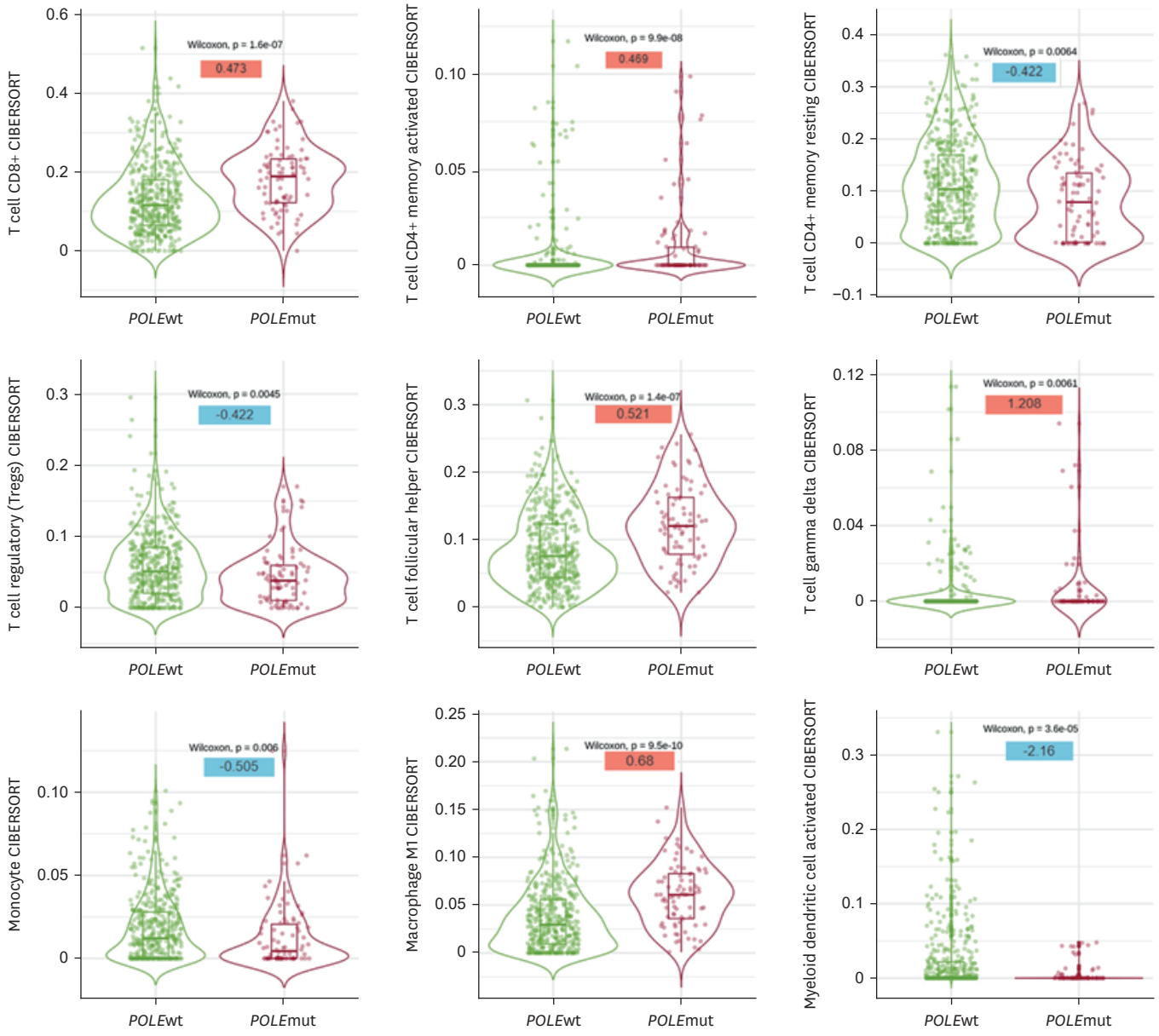

Fig. 5. *POLE* mutations on different immune cell types of infiltration in EC. Violin plots showing the distribution of immune infiltration in tumors with *POLE*mut vs. *POLE*wt. The log-fold change in the level of immune infiltration is color-coded, red indicates an increase infiltration, and blue indicates a decrease in infiltration. Significance level p≤0.05 in the Wilcoxon test.
EC, endometrial cancer; *POLE*mut, mutated *POLE*; *POLE*wt, wild-type *POLE*.

The residue P286 of POLE participates in DNA binding to the enzyme. Therefore, the p.P286R mutation results in a hyperactive polymerase, which introduces numerous errors during DNA synthesis [27]. In mouse and yeast animal models with the p.P286R mutation, EC was induced, and the penetrance was 100%. Therefore, we hypothesized that this is a driver mutation in EC [28]. Similarly, based on a study employing molecular dynamics, it was observed that the p.P286R mutation created steric hindrance, impeding the binding of single-stranded DNA to the enzyme's active site. This results in a physical barrier to exonuclease domain-DNA complex formation. This leads to a decrease in correction speed and strand extension and increases the probability of incorrect extension of primer ends, resulting in a high rate of base substitutions [27].

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In contrast, a study in yeast determined that the V411L mutation did not have a significant impact on reducing the exonuclease activity of the pol ε. Therefore, it is postulated that its association with mutagenesis is related to another mechanism, possibly linked to PPIs, which play a crucial role in replication fidelity rather than its corrective function [29]. This is supported by the high binding affinity of this mutation with certain drugs, such as cladribine [30]; hence, residue 411 (valine) could serve as an anchor point for coenzymes that enhance the catalytic activity of POLE. Additionally, this variant may lead to changes in the proofreading and extension rates of the DNA polymerase, resulting in an increased error rate during nucleotide incorporation. This hypothesis aligns with reports of increased polymerase activity of Pol ε in the presence of the V411L mutation [31], however, the role of this mutation in endometrial carcinogenesis is still not clear.

According to the analysis performed using the HOPE web server, the p.S297F mutation could affect the stability of the enzyme's nucleus. Church et al. [32], conducted a study in which they reported that in an orthologous model of *Saccharomyces cerevisiae*, amino acid 297 interacted with aspartic acid in the active site (D275). Therefore, this mutation not only causes a change in the amino acid size but also alters its polarity and the possibility of interaction for the normal folding of the POLE subunit, disrupting the conformation of the active site [32].

It has been reported that the main signaling pathways associated with *POLE*mut include cell proliferation, evasion of tumor suppression, and evasion of apoptosis in altered cells [33]. In this study, through interaction analysis, the involvement of POLE in biological processes such as DNA repair, the cell proliferation cycle, and mechanisms of resistance to platinum were identified. Additionally, through immune infiltration analysis, it was found that mutations in the *POLE* gene play a significant role in the tumor microenvironment, as significant differences were observed in the infiltration of immune cell subtypes depending on the mutational status of *POLE*. Tian et al. [31], studied the overexpression of the *POLE*wt and POLE with mutations P286R, R375Q, and P452L in HEC-1A and AN3CA EC cell lines and its relation to cisplatin sensitivity. They found that cells expressing *POLE*mut developed resistance to this chemotherapy [31]. Additionally, studies using flow cytometry revealed that *POLE*mut increased the proportion of cells in the G0/G1 phase and decreased both the proportion of cells in the S phase and the expression of proteins inducing the G1/S phase transition. The cell cycle arrest reported by these authors in EC cells could be one of the mechanisms explaining the relationship between the presence of mutations in the *POLE* gene and the increase in the overall survival of patients compared to those carrying other mutations and the *POLE*wt gene [31].

Tumors with high mutation rates have been associated with increased infiltration of immune cells that recognize neoantigens generated from mutations in the genome, which can be either pro-tumoral or anti-tumoral [34]. Tumors with *POLE*mut are characterized by an ultramutated genotype (>100 mutations per Mb), increasing the probability of generating neoantigens and activating immune cells [35]. In this study, the analysis of immune cell infiltration using the TIMER 2.0 tool revealed a positive correlation between the infiltration of CD8+ T cells and activated memory CD4+ T cells in tumors with *POLE*mut. The results are consistent with the study by Bellone et al. [36] in 2015, who evaluated the immune response through the activation of dendritic cells with lysate from autologous tumor cells of EC with or without mutations in the *POLE* gene. The immune response was measured by the activation of CD4+ and CD8+ T lymphocytes and cytokine secretion. They found greater activation

of CD4+ T lymphocytes in the presence of EC cells with *POLE*mut, as well as increased expression of interferon-γ [36]. These results indicate that, in EC tumors with *POLE*mut, there is an inflammatory response due to the presence of tumor neoantigens.

*POLE*mut EC tumors have been associated with a good prognosis, as the 5-year survival is higher compared to those classified in the other TCGA subtypes [37,38]. In the present study, we observed that analysis of the data retrieved from the cBioPortal database showed the same trend (**Fig. 4**). However, we did not find any statistically significant differences. In relation to the above, the high rate of lymphocytic infiltration in *POLE*mut EC tumors has been associated with a good response to immunotherapy and a good prognosis for the disease [39]. Fan et al. [40] conducted a retrospective study in a cohort of 237 patients with EC in which they analyzed the presence of infiltrating T lymphocytes in the tumor tissue. They found a significant relationship between disease-free survival and lymphocyte infiltration in high-grade EC tumors [40].

In conclusion, POLE plays a significant role in EC development. The most frequent mutations in this gene cause alterations in the resulting protein, affecting the corrective function of Pol ε. These mutations also affect POLE interactions with other proteins, which may interfere with repair mechanisms. Despite the possible relationship between *POLE* mutations and the development of platinum resistance due to signaling pathway alterations, the ultramutated genotype is also associated with high immune infiltration cells, triggering an adequate response that leads to a favorable prognosis for the disease.

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SUPPLEMENTARY MATERIALS

Table S1

Mutations in POLE were retrieved from the COSMIC database

Table S2

Topological analysis of the protein-protein interaction network of POLE

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