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DNA Polymerase Delta 1 Catalytic Subunit (POLD1) as a Prognostic Factor in Clear Cell Renal Cell Carcinoma Patients

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Abstract. *Background/Aim: DNA polymerase delta 1 catalytic subunit (POLD1 or POLD1/p125) plays a crucial role in DNA synthesis and proofreading during the semiconservative genome replication. Mutations of POLD1 are associated with abnormal cell division in various human tumors. However, the significance of altered POLD1 expression in malignant diseases and its usefulness as a prognostic factor is not fully understood. This study aimed to determine POLD1 immunoexpression levels in paired sections of tumor and normal kidney derived from 56 patients with clear cell renal cell carcinoma (ccRCC) and evaluate the significance of POLD1 protein as a potential prognostic factor in ccRCC. Materials and Methods: Tissue samples were collected from 56 patients (27 females and 29 males, mean age 62.6, range=27-83 years) who underwent nephrectomy due to ccRCC. Paired tissue samples were obtained from the tumor and unchanged part of the kidney. The expression of POLD1 protein was assessed by immunohistochemistry. Clinical and pathological data of patients were also collected. Patients were followed-up and the median time of observation period was 39.3 months. Results: The study revealed a significantly higher POLD1 nuclear expression in ccRCC tumor tissue samples and this was correlated with longer survival rates (better prognosis) of ccRCC patients. Conclusion: POLD1 immunoreactivity in ccRCC postoperative material could be helpful as a prognostic marker in the ccRCC patient group.*

Clear cell renal cell carcinoma (ccRCC) is the most frequent subtype of renal cancer characterized by worst prognosis among the most common subtypes of kidney tumors (1). Despite remarkable progress that has been made in the treatment of kidney tumors in recent years, the outcomes of patients with ccRCC have not significantly improved (2). Clinical decisions concerning patients diagnosed with ccRCC and implementation of adjuvant therapy are based on cancer staging evaluation and the assessment of the patient's general condition. Pathomorphological analysis of postoperative material (kidney tumor) consists of several features which complement preoperative TNM classification and mainly comprise: the size of the tumor (pT), cancer invasion to the renal fibrous capsule and perinephric fat or/and renal sinus fat as well as cancer extension into a renal vein (3). Histological type of tumor, microscopic markers of tumor aggressiveness (*e.g.* nuclear pleomorphism, visibility of nucleoli, presence of multinucleated tumor giant cells, sarcomatoid or rhabdoid differentiation, tumor necrosis) and microvascular invasion are also substantial because they could predict the course of illness (4). However, the current classification systems of ccRCC tumors have only limited utility when not supported by new markers that include molecular features of the tumor (2, 5). The current state of knowledge recommends the use of diagnostic IHC staining to determine the histological RCC subtype. For this purpose, the panel of several antibodies (CD10, CK7, AMACR, CD117) has been proposed (6-8). Numerous biomarkers (*e.g.* CaIX, VEGF, HIF, Ki67,

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PTEN, p53, p21, CD44, osteopontin, CXCR4 and E-cadherin) and their prognostic significance in RCC were investigated so far, however, there are currently no recommendations to implement the routine evaluation of these markers to the clinical practice (9). Therefore, the identification of new reliable immunohistochemical prognostic markers in ccRCC could improve the postoperative surveillance of patients and help to implement the decision for the adjuvant therapy on time.

The development of cancer, its progression and aggressive clinical course is closely related to the uncontrolled proliferation of cancer cells. The potential indicators of intensive cell divisions could be enzymes that participate in DNA replication (S phase of the cell cycle). DNA polymerase delta (Polδ) is the main replicative polymerase which complementary synthesizes the lagging strand of the DNA template. It may also participate in the replication of the DNA leading strand (10). Polδ is a heterotetrameric enzyme and its DNA polymerase delta 1 catalytic subunit (POLD1 or POLD1/p125) exhibits both, polymerase and 3'-5' exonuclease enzymatic activity. The rest of the Polδ subunits (POLD2, POLD3 and POLD4) are regulatory proteins (11).

Germline and sporadic *POLD1* mutations and gene polymorphisms were shown to contribute to the malignant phenotype of several human tumors including colorectal cancer (CRC), endometrial cancer, glioblastoma and lung cancers (12, 13). In addition to genetic alterations, disordered expression of *POLD1* at the mRNA and/or protein levels could also associate with the progression of breast tumors, lung tumors and acute lymphoblastic leukemia (14-16). However, the significance of POLD1 immunoexpression as a prognostic marker in ccRCC and associations between POLD1 immunoreactivity in cancer cells and clinicopathological parameters of the patients have not been analyzed so far. Therefore, the aim of the study was the evaluation of POLD1 protein expression in cancer tissues compared to tissue samples collected from the unaltered part of the kidney.

To analyze the significance of POLD1 protein as a possible marker of patient survival, postoperative follow-up data were assessed. The results of the study allow to consider POLD1 as a molecular marker of the ccRCC course and facilitate recognizing high-risk patients of cancer progression or recurrence.

Materials and Methods

Patients and collection of tissue samples. The study was approved by the Bioethical Commission of the University of Warmia and Mazury in Olsztyn, Poland (approval no. 4/2010), and written consents were obtained from all participants. The kidney tissue samples for this analysis were collected from patients who underwent nephrectomy due to the diagnosis of kidney tumor. Shortly (up to 5 min) after organ resection, two specimens (each ca. 1.5 cm in size) were collected, one from the peripheral part of the tumor and the next one from the macroscopically unchanged part of the kidney. Both samples were fixed in 4% buffered (pH 7.4) paraformaldehyde. The postoperative tissue samples from histopathologically confirmed ccRCCs were included in the study. The tissue material was collected from 56 patients with ccRCC (27 females and 29 males, mean age 62.6 years, range 27-83) between years 2010 and 2012. Demographic and clinical data of all evaluated patients were collected and they are presented in Table I. The median time of follow-up was 39.3 months. For the clinical use, the cancer staging according to the American Joint Committee on Cancer (AJCC) (17) and grading by Fuhrman scale (18) were evaluated by the pathologist.

Table I. *Association of clinicopathological features of clear cell renal cell carcinoma (ccRCC) patients and DNA polymerase delta 1 catalytic subunit (POLD1) nuclear immunoreactivity, determined by immunohistochemistry.*

Qualitative parameters	Number of cases n (%)	POLD1 nuclear immunoreactivity in ccRCC cells		
		IRS 0-6 n (%)	IRS>6 n (%)	*p*-Value
Total	56	38 (68)	18 (32)	
Men	29	18 (62)	11 (38)	0.3990
Women	27	20 (74)	7 (26)	
Age				
≤61 years old	29	19 (66)	10 (34)	0.7789
>61 years old	27	19 (70)	8 (30)	
Fuhrman grade				
G1+G2	41	25 (63)	15 (38)	0.1064
G3+G4	15	13 (87)	2 (13)	
Tumor size				
≤7 cm	33	21 (64)	12 (36)	0.5628
>7 cm	23	17 (74)	6 (26)	
Primary tumor status				
T1+T2	27	18 (67)	9 (33)	1.0000
T3	29	20 (69)	9 (31)	
Distant metastasis (M)				
M0	40	25 (63)	15 (38)	0.2183
M1	16	13 (81)	3 (19)	

POLD1: DNA polymerase delta 1 catalytic subunit; ccRCC: clear cell renal cell carcinoma; IRS: Immunoreactive score of Remmele and Stegner; *p*-values were calculated using the Fisher's exact test.

Immunohistochemistry. Fixed tissue samples of paired tumor and unchanged kidney tissues of ccRCC patients were processed according to the routine histologic protocol. Subsequent immunohistochemical staining with the use of antibodies directed against POLD1 protein on deparaffined microscopic slides was performed. Initially, the sections were subjected to antigen retrieval procedure by microwaving for 6 min in Retrieval Solution Buffer, pH 9.0 (Leica, Wetzlar, Germany) followed by the incubation in 3% H_2O_2 in methanol for 10 min and in 2.5% normal horse serum (Vector Laboratories, Burlingame, CA, USA) for 30 min respectively. The sections were incubated overnight at 4˚C with rabbit anti-human antibody against POLD1 (HPA046524; Sigma-Aldrich, St. Louis, MO, USA), diluted 1:2000 in PBS. After washing with PBS, the sections were treated with HRP-conjugated secondary antibody (ready-to-use dilution; ImmPRESS Universal reagent Anti-Mouse/Rabbit Ig; Vector Laboratories) for 30 min. The sections were

immersed in diaminobenzidine (DAB; Dako, Glostrup, Denmark), counterstained with hematoxylin (Sigma-Aldrich), dehydrated in ethanol, cleared in xylene and mounted with DPX (Sigma-Aldrich). The negative controls were performed for every set of staining by omitting the primary antibody. In order to assess the morphology of paired pathological and control samples from all analyzed patients, additional staining of microscopic slides with hematoxylin and eosin (H&E) was performed and the tissue characteristics were determined.

Evaluation of immunohistochemical reactions. The labelled sections were examined using an Olympus BX 41 microscope equipped with a photo collecting system: camera Olympus XC50 with an appropriate computer Cell* software (all: Olympus, Tokyo, Japan). Immunoreactivity for POLD1 in ccRCC tumors and corresponding control kidney tissue samples (epithelial cells of proximal convoluted tubules – PCTs) were evaluated by a pathologist who had no access to the patients' clinical data. The scoring system for POLD1 nuclear reaction was applied according to the immunoreactive score system of Remmele and Stegner (IRS) (19) which is based on multiplication of the percentage of immunoreactive cells (1 point: 1-10%, 2-points: 11-50%, 3 points: 51-80%, 4 points: >80% cells) and reaction intensity (1 point: low, 2 points: moderate, 3 points: intense reaction). The scores ranged from 0 to 12 points.

Statistical analysis. Statistical analysis was carried out using Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and Statistica 13 (TIBCO Software, Inc., Palo Alto, CA, USA) software. The significance of differences between expression levels of analyzed proteins in ccRCC cells and PCT epithelial cells were tested by the Wilcoxon matched-pairs signed rank test. The ratios of POLD1 scores of paired ccRCC tumor cells and PCTs of corresponding unchanged kidney sections were calculated. Correlations between the levels of protein expression and clinicopathological data were evaluated using Fisher exact test. The univariate associations of clinicopathological data with patients' overall survival were plotted using the Kaplan-Meier method and the differences between the patient cohorts were assessed by log-rank test. Differences were considered statistically significant for $p<0.05$.

Results

Increased POLD1 immunoreactivity in clear cell RCC cell does not correlate with clinicopathological characteristics of patients. POLD1 protein immunoreactivity was found in the nuclei of both PCT epithelial cells (Figure 1A) and ccRCC cells (Figure 1B, C). POLD1 immunoexpression levels were low (absent or weak) in 38 and high (moderate and strong) in 18 out of 56 tumor sections (Figure 2A). The average immunoreactivity of POLD1 was significantly increased in the nuclei of ccRCC cells as compared to the PCT cells of unchanged renal tissue ($p=0.003$; Figure 2B). POLD1 expression levels in the tumor cells did not correlate with demographic and clinicopathological data of patients with ccRCC (Table I).

Clear cell RCC patient OS is associated with the level of POLD1 immunoexpression in tumor cells. Strong nuclear immunoreactivity of POLD1 in ccRCC cells correlated with better prognosis in patients with ccRCC (HR=0.35; $p=0.0436$; Table II and Figure 3A) compared to those with weak or negative POLD1 immunoexpression. Of the analyzed demographic and clinicopathological parameters, higher Fuhrman grade, greater tumor size, higher primary tumor status and the presence of distant metastasis were significantly associated with worse prognosis in ccRCC patients (Table II and Figure 3D-G, respectively), while the sex and age did not correlate with the OS (Table II and Figure 3B and C).

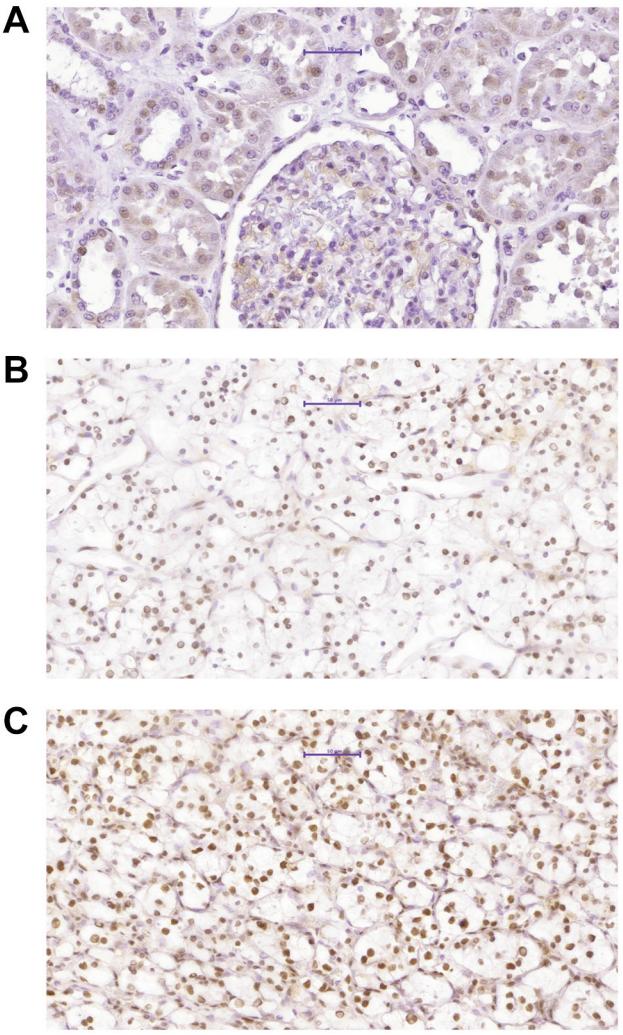

Figure 1. *Immunoreactivity of DNA polymerase delta 1 catalytic subunit (POLD1) in sections of unaltered kidney tissue and clear cell renal cell carcinoma (ccRCC). (A) The normal structure of the kidney's cortex, nuclear staining is visible in the tubular epithelium. Week POLD1 immunoreactivity (1 point) in part of epithelial cells' nuclei (2 points) gives 2 points of IRS scale; (B) POLD1 immunostaining of ccRCC section; moderate (2 points) POLD1 immunoreaction in prevalence (3 points) of cancer cells (IRS-6); (C) Strong nuclear immunoreactivity (3 points) is present in all cancer cells (4 points) which gives 12 points in IRS scale. Immunohistochemistry was performed as described in the Materials and Methods. Scale bar=50 μm.*

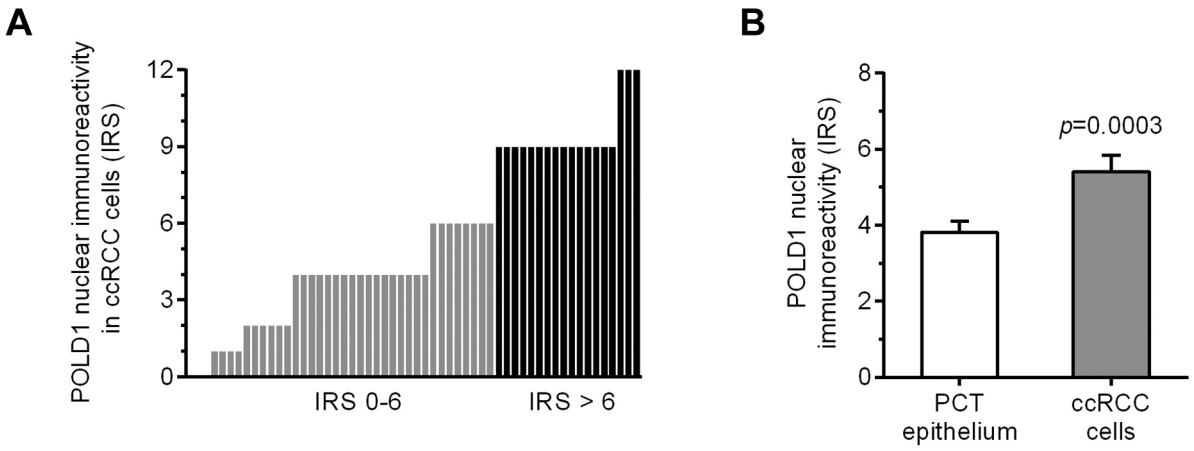

Figure 2. *Evaluation of DNA polymerase delta 1 catalytic subunit (POLD1) immunoexpression in the clear cell renal cell carcinoma (ccRCC) and unaltered renal tissues by immunohistochemistry. (A) POLD1 nuclear immunoreactivity in the tumor sections of individual patients with ccRCC is shown. Grey bars represent patients with low POLD1 immunoreactivity, black bars represent patients with high levels of POLD1 immunoreactivity (B). The average nuclear immunoreactivity of POLD1 in tumor cells is shown in relation to POLD1 levels in epithelial cells of proximal convoluted tubules (PCT) of unaltered kidneys. Data are presented as the means±SEMs (n=56). The p-value was calculated using the Wilcoxon matched-pair test.*

Discussion

The present study provides a novel insight into the significance of POLD1 as a putative prognostic factor in ccRCC. Using the IHC method we demonstrated an increased immunoreactivity of POLD1 in ccRCC specimens. Survival analysis revealed a positive correlation between the increased immunoexpression of POLD1 in the nuclei of cancer cells and longer OS of patients with ccRCC. To the best of our knowledge, the present study is the first to investigate the potential utility of POLD1 expression level as a prognostic factor in ccRCC.

POLD1 provides 5'-3' DNA polymerase and 3'-5' exonuclease activities of Polδ that are essential for DNA replication and DNA repair in eukaryotic cells, respectively (12). Germline and sporadic *POLD1* mutations, especially those within the proofreading (exonuclease) domain, impair replication fidelity control, contributing to genomic instability, mutator phenotype and malignant transformation (10, 12). Most previous studies linking POLD1 and neoplastic diseases focused on POLD1 pathologic variants that harbor exonuclease domain mutation (13), while the prognostic significance of *POLD1* mRNA and/or protein expression in human tumors have not been extensively investigated to date. Siraj *et al.* (20) analyzed POLD1 protein expression in 300 papillary thyroid carcinoma cases, demonstrating that a low level of POLD1 correlates with a higher stage of the disease and the presence of distant metastases. Another study that included 1,069 CRC cases revealed an association between the low expression of POLD1 and markers of worse prognosis such as higher primary tumor status and higher tumor stage (21). The results of two latter studies seem to be in line with our findings, suggesting that a high level of POLD1 may associate with lower progression and/or better prognosis in the thyroid, colorectal and renal tumors. However, the results of other studies reveal the potential oncogenic role of POLD1 in several human malignancies, reporting that *POLD1* mRNA or protein overexpression correlates with the progression and/or worse prognosis in acute

Table II. *Overall survival of clear cell renal cell carcinoma (ccRCC) patients in relation to DNA polymerase delta 1 catalytic subunit (POLD1) nuclear immunoreactivity and their clinico-pathological characteristics.*

Parameter	Log-rank test	
	HR (95% CI)	*p*-Value
POLD1 nuclear immunoreactivity in ccRCC cells (IRS *vs.* IRS 0-6)	0.35 (0.18-0.97)	**0.0436**
Sex (men *vs.* women)	0.83 (0.37-1.88)	0.6577
Age (>61 *vs.* ≤61 years old)	1.21 (0.53-2.73)	0.6562
Fuhrman grade (G3 *vs.* G1+G2)	2.98 (1.55-11.73)	**0.0056**
Tumor size (>7 *vs.* ≤7 cm)	2.55 (1.15-6.18)	**0.0230**
Primary tumor status (T3 *vs.* T1+T2)	4.36 (1.70-8.80)	**0.0013**
Distant metastasis (M1 *vs.* M0)	3.52 (1.90-12.97)	**0.0012**

Median follow-up time: 39.3 months; HR: hazard ratio; CI: confidence interval; POLD1: DNA polymerase delta 1 catalytic subunit; ccRCC: clear cell renal cell carcinoma; IRS: immunoreactivity score of Remmele and Stegner; Significant *p*-values (<0.05) are given in bold.

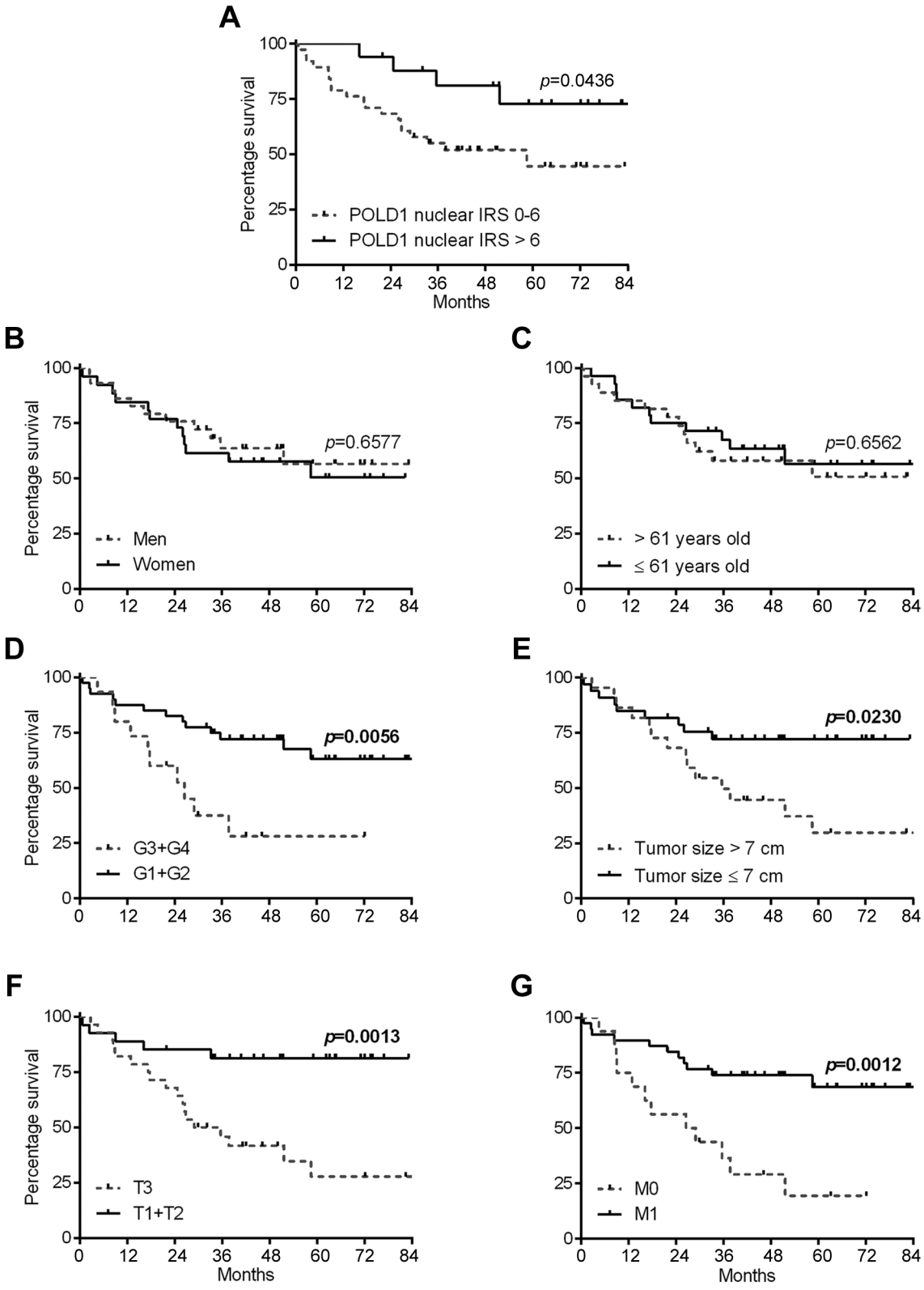

Figure 3. *Kaplan-Meier diagrams of overall survival of 56 patients with clear cell renal cell carcinoma (ccRCC) in correlation with (A) immunoexpression levels of DNA polymerase delta 1 catalytic subunit (POLD1), (B-C) demographic and (D-G) clinicopathological characteristics. Significant p-values (<0.05) for corresponding log-rank tests are given in bold.*

lymphoblastic leukemia (14), endometrial carcinoma (22), triple negative breast cancer (16) and lung adenocarcinoma (15). Those discrepancies suggest that the potential oncogenic or suppressive role of POLD1 in various human malignancies could be considered as a cancer-specific feature.

Alterations of catalytic subunits of proofreading DNA polymerases delta and epsilon (Polε), POLD1 and POLE, respectively, were shown to have prognostic significance in several types of cancers (23). *POLD1* and *POLE* mutations or their abnormal expression can be useful for the prediction of clinical outcomes in the oncological patients treated with immune checkpoint inhibitors such as programmed death-1 (PD-1) and its ligand PD-L1 (23, 24). The recent study on ccRCC disclosed that elevated *POLE* expression correlates with immune-suppressive tumor microenvironment and worse outcomes of patients (25). These observations suggest that *POLE* expression could be a useful predictive marker in advanced renal tumors treated with immunotherapy (25). Since both POLE and POLD1 are hub proteins in the protein-protein interaction networks related to ccRCC progression (25), it is likely that evaluation of *POLD1* expression status could also be considered as potential prognostic and/or predictive factor in ccRCC. Interestingly, in the present study we demonstrated potential survival benefits in patients exhibiting increased POLD1 immunoreactivity, while findings of Wu *et al*. imply an oncogenic role of POLE in ccRCC (25). This dissimilarity may result partially from different properties of DNA polymerases which POLD1 and POLE contribute to, Polδ and Polε, respectively (11). The model of replication fork and DNA polymerases arrangement assumes that Polδ operates on the lagging and leading strand and proofreads errors on both strands, while Polε is the helicase-associated leading strand DNA polymerase and it is excluded from the lagging strand (13). Moreover, Polδ can substitute for missing helicase-associated Polε and the machinery controlling the cell cycle can accommodate for loss of this polymerase (13). Bioinformatic analysis of The Cancer Genome Atlas (TCGA) ccRCC datasets revealed that *POLE* expression may regulate transcription of key immune checkpoint genes (25). However, without further functional studies, the exact molecular mechanisms underlaying the role of proofreading DNA polymerases in ccRCC remain largely unknown.

In our study, we did not disclose the correlation of POLD1 expression and any of clinicopathological factors in ccRCC such as nuclear grade, tumor size and T-status of the primary tumor or presence of distant metastasis but nuclear immunoreactivity of POLD1 was associated with better prognosis. While the elevated immunoexpression of POLD1 in cancer cells could be attributed to their higher proliferative potential (11), the positive correlation of POLD1 immunoreactivity with longer overall survival of the ccRCC patients remains apparently controversial. TCGA data (26, 27) reveal that *POLD1* is mutated in 0.39% of ccRCC cases. Relatively low frequency of *POLD1* somatic mutations in ccRCC (12) suggests that the POLD1 immunoexpression observed in ccRCC cells comprises a wild-type POLD1 protein with intact proofreading activity. Therefore, elevated level of POLD1 protein in cancer cells might be considered not only as the S-phase related marker of cell proliferation but also as a protective, genome-instability limiting factor (10, 12).

Conclusion

The results of the study, demonstrating altered levels of POLD1 in cancer tissue in correlation to patient survival data, suggest that IHC POLD1 evaluation, could be considered as a supplementary marker helpful for risk stratification in patients with ccRCC. However, further molecular studies are essential to elucidate the role of POLD1 in ccRCC development.

Conflicts of Interest

The Authors declare no competing interests related to this study.

Authors' Contributions

Conceptualization, JG and PS; methodology, JK and BK; validation, JG, PS, JK and BK; formal analysis, JG, PS, JK and BK; investigation, JK; writing—original draft preparation, JG, PS and JK; writing—review and editing, JG and BK; visualization, JK and BK; supervision, JG; project administration, JG; funding acquisition, JG All authors have read and agreed to the published version of the manuscript.

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