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Białko POLD1 jako czynnik prognostyczny w raku jelita grubego i w raku nerki

POLD1 protein as a prognostic factor in colorectal cancer and renal cancer

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Prace oryginalne

1. Godlewski J*, **Stefaniak P***, Kiezun J, Krazinski B E. DNA polymerase delta 1 catalytic subunit (POLD1) as a prognostic factor in clear cell renal cell carcinoma patients. *In Vivo*. 2022; 36(3):1188-1194. DOI:10.21873/invivo.12818.

   *oznaczone osoby są równoważnymi pierwszymi autorami tej pracy

   IF: **2,3** i **40** punktów MNiSW;

2. **Stefaniak P,** Krazinski B E, Kiezun J, Majewska H, Godlewski J. Altered immunoexpression of DNA polymerase delta1 catalytic subunit (POLD1) in colorectal cancer. *Contemp Oncol (Pozn)* 2023; 27(3):147-154. DOI:10.5114/wo.2023.133505.

   IF: **1,8** i **70** punktów MNiSW

Prace przeglądowe

3. **Stefaniak P,** Majewska H, Zadrozny D, Godlewski J. Cancer in polyp of the large intestine - an interdisciplinary decision problem. *Gastroenterology Rev* 2021; 16(4):306-310. DOI:10.5114/pg.2021.111481.

   40 punktów MNiSW

4. Gola M, **Stefaniak P,** Godlewski J, Jereczek-Fossa B A, Starzyńska A. Prospects of POLD1 in human cancers: a review. *Cancers (Basel)*. 2023; 15(6):1905. DOI: 10.3390/cancers15061905.

   IF: **5,2** i **200** punktów MNiSW

Łącznie: IF=9,3 i 350 punktów MNiSW

Wykaz skrótów

AJCC – Amerykański Wspólny Komitet ds. Raka (ang. *American Joint Commitee on Cancer*)

ccRCC – rak jasnokomórkowy nerkowokomórkowy (ang. *clear cell renal cell carcinoma*)

CDE/CHR – element zależny od cyklu komórkowego/region homologii genów cyklu komórkowego (ang. *the cell cycle-dependent element/ the cell cycle genes homology region*)

CRC – rak jelita grubego (ang. *colorectal cancer*)

CTCF – czynnik transkrypcyjny CTFC (ang. *CCTC-binding factor*)

E2F1 – czynnik transkrypcyjny E2F1 (ang. *E2F transcription factor 1*)

GEP-NENs – nowotwory neuroendokrynne przewodu pokarmowego (ang. *gastroenteropancreatic neuroendocrine neoplasms*)

GIST – nowotwór podścieliskowy przewodu pokarmowego (ang. *gastrointestinal stromal tumor*)

IHC – immunohistochemią (ang. *immunohistochemistry*)

Ki-67 – marker proliferacji Kiel 67 (ang. *marker of proliferation Kiel 67*)

MCM2-10 – kompleks białek podtrzymujących minichromosomy MCM 2-10 (ang. *minichromosome maintenace protein complex*)

OS – czas przeżycia całkowitego (ang. *overall survival*)

p21 – białko p21, inhibitor kinaz zależnych od cyklin 1A (ang. *cyclin-dependent kinase inhibitor 1A*)

p53 – białko p53, supresor nowotoworwy p53 (ang. *tumor protein p53*)

PCNA – jądrowy antygen proliferującej komórki (ang. *proliferating cell nuclear antigen*)

PCTs – kanaliki kręte bliższe (ang. *proximal convoluted tubules*)

PFS – czas przeżycia wolny od progresji (ang. *progression-free survival*)

POLD1 – podjednostka katalityczna polimerazy DNA delta (ang. *DNA polymerase delta catalytic subunit*)

POLE – podjednostka katalityczna polimerazy DNA epsilon (ang. *DNA polymerase epsilon catalytic subunit*)

Polδ –polimeraza DNA delta (ang. *DNA polymerase delta*)

PPAP – polipowatość związana z funkcją korektorską polimerazy (ang. *polymerase proofreading-associated polyposis*)

Skala G – skala biologicznej złośliwości raka (ang. *cancer grade*)

Sp1 i Sp3 – białka specyficzności 1 i 3 (specificity factors 1 and 3)

TCGA – Atlas genomu raka (ang. *The Cancer Genome Atlas)*

Tis – rak przedinwazyjny (ang. *tumor in situ*)

TNM – klasyfikacja zaawansowania klinicznego raka (T- guz, N- węzeł chłonny, M- przerzut) (ang. *tumor, node, metastasis*)

Streszczenie

Celem niniejszej pracy było ustalenie czy immunoekspresja podjednostki katalitycznej polimerazy DNA delta (POLD1) ma znacznie prognostyczne w raku jasnokomórkowym nerki (ccRCC) i raku jelita grubego (CRC). Skrawki tkankowe pozyskane z materiału pooperacyjnego, tkanek guza i niezmienionego fragmentu nerki lub jelita grubego, były poddane analizie immunohistochemicznej (IHC) w celu określenia poziomu ekspresji POLD1 w komórkach guza i odpowiadających im niezmienionych nowotworowo komórkach nabłonka. Oceny lokalizacji i poziomu immunoreaktywności POLD1 i jej poziomu dokonywał patomorfolog. Do oceny korelacji pomiędzy immunoekspresją POLD1, a danymi kliniczno-patologicznymi i przeżyciem pacjentów zastosowano metody statystyczne oraz analizę przeżycia. Do badania włączono także poziomy ekspresji mRNA *POLD1* u 599 pacjentów z CRC pochodzące z baz danych programu The Cancer Genome Atlas (TCGA). W grupie pacjentów z ccRCC stwierdzono istotnie wyższy poziom immunoreaktywności POLD1 w komórkach raka w porównaniu do komórek nabłonka kanalików krętych bliższych. Wysoki poziom immunoekspresji POLD1 w komórkach ccRCC korelował dodatnio z całkowitym przeżyciem, wskazując na potencjalną przydatność POLD1 jako markera związanego z dobrym rokowaniem u pacjentów z tym nowotworem. Z kolei u pacjentów z CRC stwierdzono, że średni poziom immunoekspresji POLD1 w komórkach raka był niższy niż w enterocytach niezmienionej nowotworowo części jelita grubego. Poziom ekspresji POLD1 w CRC nie korelował z danymi kliniczno-patologicznymi ani z okresem przeżycia pacjentów. W rozprawie dokonano także przeglądu aktualnego stanu wiedzy dotyczącego roli POLD1 w nowotworach złośliwych człowieka i możliwości wykorzystania tego czynnika jako markera w prognozowaniu i optymalizacji terapii przeciwnowotworowej. Dodatkowo w nawiązaniu do tematyki raka jelita grubego, omówiono istotny problem decyzyjny jaki powstaje po stwierdzeniu nacieku raka w obrębie polipa wyciętego w technice kolonoskopowej.

Słowa kluczowe

POLD1, polimeraza DNA, rak jasnokomórkowy nerki, rak jelita grubego, rokowanie, rak w polipie jelita grubego

Abstract

This study aimed to determine prognostic significance of catalytic subunit of DNA polymerase delta (POLD1) expression in clear cell renal cell carcinoma (ccRCC) and colorectal cancer (CRC). Paired tissue sections of the tumoral and unchanged tissues were obtained from postoperative material and subjected to immunohistochemical (IHC) analysis to determine the level of POLD1 expression in cancer cells and matched unchanged epithelia. POLD1 immunoreactivity localization and score in the analyzed sections was determined by the pathologist. The study included also analysis of *POLD1* mRNA expression in 599 patients with CRC derived from The Cancer Genome Atlas (TCGA) databases. POLD1 immunoreactivity was significantly higher in ccRCC cells as compared to the proximal convoluted tubule epithelial cells. High level of POLD1 immunoexpression in ccRCC cells was correlated with longer overall survival, indicating potential usefulness of POLD1 as a marker associated with better prognosis in the patients with this malignancy. In CRC patients the average immunoexpression of POLD1 was lower in cancer cells as compared to the enterocytes of unchanged large intestine. However, the expression levels of POLD1 in CRC did not correlate with clinicopathological nor survival of the patients. In the summary, the results of our experimental studies and previously published articles on the potential role of POLD1 in human malignancies as prognostic and predictive biomarker were reviewed. In addition, the 'cancer in a polyp' of the large intestine decision problem was described and the review of relevant literature was included.

Key words

POLD1, DNA polymerase, clear cell renal carcinoma, colorectal cancer, prognosis, cancer in large intestine polyp

Wstęp

Na świecie, w tym także w Polsce, odnotowuje się wysoką zapadalność na nowotwory złośliwe ze stałym trendem wzrostu w większości typów nowotworów. Stanowi to bardzo istotny problem natury zdrowotnej, społecznej i ekonomicznej. Według Krajowego Rejestru Nowotworów, w 2020 r. odnotowywano w Polsce około 146 tysięcy nowych przypadków nowotworów, a liczba zgonów, będących konsekwencją chorób nowotworowych, była bliska 100 tysięcy, stanowiąc w przybliżeniu 25% ogólnej liczby wszystkich zgonów w Polsce (Wojciechowska i in., 2020). Niemal 4,5 tysiąca nowo zdiagnozowanych nowotworów u Polek i Polaków stanowiły guzy złośliwe nerki, którego najczęściej występującym typem morfologicznym jest rak jasnokomórkowy nerki (ccRCC, ang. *clear cell renal cell carcinoma*). W tym samym roku zarejestrowano także ponad 16 tysięcy nowych przypadków raka jelita grubego (CRC, ang. *colorectal cancer*). Procentowy udział raka nerki w grupie nowo zdiagnozowanych nowotworów wynosił około 2,4% u kobiet i 3,8% u mężczyzn, a z kolei w przypadku raka jelita grubego, nowe przypadki choroby stanowiły 8,5% w grupie kobiet i aż 11,2% w grupie mężczyzn (Wojciechowska i in., 2020). Odnotowano 2.380 zgonów pacjentów z rakiem nerki oraz 12.501 zmarłych, którzy chorowali na raka jelita grubego. Podsumowując dostępne statystyki zapadalności i śmiertelności, można stwierdzić, że guzy złośliwe nerki i jelita grubego charakteryzują się wysokimi współczynnikami śmiertelności, które według Krajowego Rejestru Nowotworów wynoszą 0,53 dla raka nerki i 0,70 w przypadku raka jelita grubego. Jednocześnie należy mieć na uwadze w odniesieniu do aktualnych danych z roku 2020, że warunki pandemii COVID-19 wpłynęły na obniżenie zgłaszalności nowych przypadków (obniżenie o około 15% w stosunku do lat poprzednich), a zakażenie wirusem SARS CoV-2 znacząco podwyższało śmiertelność w grupie pacjentów onkologicznych (Wojciechowska i in., 2020).

Proces nowotworzenia (także: karcynogenezy lub kancerogenezy) zdefiniować można jako zaburzenie budowy i funkcji komórek, zakłócenie procesu dojrzewania i różnicowania się komórek, wynikające m.in. ze współwystępowania mutacji genów zaliczanych do grup protoonkogenów, genów supresorowych guzów i genów mutatorowych (Faeron, Vogelstein, 1990). Zmiany na poziomie molekularnym przekładają się na zaburzenia dotyczące budowy i funkcjonowania komórek, a sam proces przechodzi przez kolejne punkty zwrotne obejmujące inicjację, promocję i progresję zmiany nowotworowej. Nieprawidłowa struktura i fizjologia komórek przekładają się na zmiany na poziomie organizacji tkanek i narządów. Wśród

głównych cech nowotworów złośliwych zdefiniowanych (ang. *The Hallmarks of Cancer*) przez Hanahan i Weinberga (2000) wymienić można: nadmierne i niekontrolowane podziały komórkowe z równoczesnym zahamowaniem dojrzewania komórkowego, nabycie przez komórki nowotworowe zdolności do naciekania podścieliska, tworzenia mikrośrodowiska guza sprzyjającego progresji choroby, powstawanie ognisk przerzutowych prowadzących do niewydolności wielonarządowej (Abbas i in. 2019). Zaburzenia proliferacji komórkowej (nadmierna) jak i zaburzenie dojrzewania komórkowego (niedobór lub brak różnicowania) przekładają się na szybsze tempo wzrostu nowotworu i jego większą złośliwość (Hanahan, Weinberg, 2000). Te cechy biologicznej złośliwości komórek nowotworowych stanowią w praktyce klinicznej podstawę klasyfikacji w czterostopniowej (guzy nerki) (Fuhrman i in. 1982) lub trójstopniowej (guzy jelita grubego) skali G (ang. *cancer grade*, G) (Abbas i in. 2019). Czynniki decydujące o tempie wzrostu komórek nowotworowych przekładają się na przebieg choroby i wpływają na dobór sposobu leczenia i sposób monitorowania terapii. Ponadto mają kluczowe znaczenie w określeniu rokowania pacjentów z choroba nowotworową, oraz wpływają na częstotliwość wizyt kontrolnych podczas obserwacji/nadzoru pacjentów po przebytym leczeniu.

Do tej pory nie ustalono jednego uniwersalnego wskaźnika proliferacji komórkowej, który byłby przyjęty dla wszystkich typów nowotworów. Jak dotychczas klasyczna diagnostyka mikroskopowa (w ramach analizy patomorfologicznej) odnosi się do stwierdzenia komórek w trakcie podziału (indeks mitotyczny - liczba figur podziałowych w kolejnych 50 polach widzenia). Wskaźnik ten okazał się skuteczny tylko w przypadku oceny niektórych nowotworów złośliwych pochodzenia mezenchymalnego: żeńskiego układu rozrodczego, piersi (guz liściasty) i tkanek miękkich i kości oraz nowotworów podścieliska i nowotworów neuroendokrynnych przewodu pokarmowego. W nowotworach GIST (ang. *gastrointestinal stromal tumour*) (Paganini i in. 2022) i GEP-NENs (ang. *gastroenteropancreatic neuroendocrine neoplasms*) (Takayanagi i in. 2022) indeks mitotyczny został włączony do oceny stopnia złośliwości nowotworu (wysoce zróżnicowany vs nisko zróżnicowany), co przekłada się na klasyfikację zaawansowania nowotworu, rekomendacje co do sposobu leczenia i ocenę ryzyka nawrotu choroby (WHO Classification of Tumours, 2020). Natomiast w nowotworach złośliwych pochodzenia nabłonkowego zastosowanie znalazła metoda immunohistochemicznej detekcji białek - markerów proliferacji komórkowej. Markerami proliferacji określa się białka, których stopień ekspresji przekłada się na intensywność podziałów komórkowych. Takim markerem jest białko Ki-67 (marker proliferacji Kiel 67, ang.

marker of proliferation Kiel 67), które występuje na powierzchni chromosomów dzielącej się komórki, a jego ekspresja koreluje z ilością komórek znajdujących się w fazie M cyklu komórkowego (Scholzen, Gerdes, 2000). Wartość prognostyczną markera Ki-67 potwierdzono w u pacjentek z rakiem piersi (Gonzales-Hurtado i in. 2023), a marker ten jest określany jako czynnik predykcyjny leczenia systemowego niektórych typów raka piersi (Finkelman i in. 2023). Badanie ekspresji Ki-67 jest obecnie rekomendowane i powinno się znaleźć w protokole histopatologicznym raka piersi (Jassem i in. 2020). Ponadto immunoekspresja białka Ki-67 brana jest pod uwagę w ocenie histopatologicznej rozrostów nabłonkowych szyjki macicy (Nasierowska-Guttmejer i in. 2016) oraz nowotworów neuroendokrynnych przewodu pokarmowego (Nadler i in. 2013) i płuca (Pelosi, Travis, 2021; Luchini i in. 2022).

Innym markerem, którego obecność w komórkach związana jest z ich podziałami, jest białko PCNA (jądrowy antygen proliferującej komórki, ang. *proliferating cell nuclear antigen*). Założenia teoretyczne wynikające ze znajomości przebiegu replikacji (faza S cyklu komórkowego) i roli PCNA w tym procesie, mogłyby wskazywać na użyteczność oznaczenia ekspresji tego białka jako markera podziałów komórkowych (Choe, Moldovan, 2017). PCNA jest białkiem pomocniczym, które w postaci pierścienia wokół nici DNA umożliwia połączenie się polimeraz DNA z nicią DNA podczas replikacji (Gonzales-Magana i in. 2020). Jak dotychczas wiadomo, część badań wskazuje na powiązanie pomiędzy poziomem ekspresji antygenu PCNA a natężeniem proliferacji komórkowej (Dworakowska i in. 2002; Cappello i in. 2003; Jurikova i in. 2016). Jednocześnie te wyniki nie są na tyle przekonywujące aby ocenę stopnia ekspresji PCNA w pooperacyjnym materiale histopatologicznym wprowadzono do rekomendacji klinicznych jako marker proliferacji komórkowej.

Kompleks białkowy MCM2-10 (ang. *minichromosome maintenace protein complex*, MCM), podobnie jak PCNA, jest związany z procesem replikacji DNA, a jego rolą jest rozplatanie nici DNA i tworzenie widełek replikacyjnych (Bochman, Schwacha, 2009) Dotychczas stwierdzono podwyższoną ekspresję niektórych podjednostek tego kompleksu w liniach komórkowychi materiale tkankowym różnych typów nowotworów: raka płaskonabłonkowego płuca, raka nerki, raka gruczołu krokowego, raka piersi, raka wątroby i raka jelita grubego (Yu i in. 2020). Ponadto obecność białek MCM w warstwach nabłonka proliferującego, może mieć znaczenie w ocenie zmian przednowotworowych w narządach wyścielonych nabłonkiem wielowarstwowym płaskim, czyli w szyjce macicy, w przełyku i jamie ustnej. Co więcej w przebiegu niektórych z wymienionych powyżej typów nowotworów

poziom ekspresji podjednostek kompleksu MCM koreluje ze stopniem złośliwości nowotworu, zaawansowaniem klinicznym i wielkością guza (Nowińska, Dzięgiel, 2010).

Prowadzone dotychczas badania naukowe, których celem było ustalenie przydatnych klinicznie biomarkerów związanych z proliferacją komórek nie przełożyło się na powszechne ich zastosowanie we wczesnej diagnozie i różnicowaniu nowotworów, rokowaniu i wyborze optymalnej ścieżki terapeutycznej. Równocześnie, m.in. ze względu na coraz bardziej powszechny dostęp do zróżnicowanych terapii celowanych konieczne jest poszukiwanie i walidacja nowych markerów molekularnych, których zastosowanie umożliwiłoby lepszą stratyfikację ryzyka u pacjentów nowotworowych, wspomagało wybór sposobu leczenia i monitorowanie efektów terapii przeciwnowotworowej.

Polimeraza DNA delta (Polδ) jest podstawowym enzymem replikacyjnym odpowiedzialnym przede wszystkim za syntezę nici opóźnionej DNA. Katalityczną podjednostką Polδ stanowi białko POLD1 (lub POLD1/p125), które jest dużym białkiem o masie 125 kDa. Niezależnie od aktywności polimerazy DNA (replikacyjnej), POLD1 posiada także aktywność 3'-5' egzonukleazy, czyli enzymu wycinającego błędnie sparowane nukleotydy. Obecność w POLD1 domeny egzonukleazy wiążę się z istotną rolą tego białka w procesach korekty i naprawy DNA, przekładając się na wysoką zgodność procesu replikacji (Morrison i in. 1991; Prindle, Loeb, 2012) . Dotychczas stwierdzono, że aktywność promotora genu POLD1 jest stymulowana czynnikami transkrypcyjnymi: Sp1, Sp3, E2F1 i CTCF (Zhao, Chang, 1997; Hou i in. 2021), natomiast supresja genu następuje pod wpływem białek p53 i p21, które mają zdolność do inhibicji Sp1 zależnej aktywacji promotora *POLD1* (Li, Lee, 2001; Song i in. 2009). Zależna od kontekstu fizjologicznego naprzemienna aktywacja i inhibicja promotora genu POLD1 przekłada się na zmienny poziom ekspresji transkryptów POLD1 w trakcie cyklu komórkowego ze szczytem przypadającym na przejście z fazy G1 do S (Mjelle i in. 2015). Zmienna aktywność transkrypcji genu *POLD1* jest efektem obecności w obrębie promotora genu zależnych od cyklu komórkowego składników CDE/CHR, które są miejscem bezpośredniego i/lub pośredniego oddziaływania na transkrypcję *POLD1* m.in. czynnika transkrypcyjnego E2F1 oraz białek p21 i p53 (Song i in. 2009; Fischer i in. 2016).

Germinalne i somatyczne mutacje *POLD1* powodują zniesienie jej korektorskiej funkcji i możliwości naprawy genu (błędnie sparowanych nukleotydów) co przekłada się na niestabilność genomu i promocję procesu nowotworzenia (Nicolas i in. 2016). Stwierdzono, że rzadko występująca dziedziczna mutacja *POLD1* skutkuje zespołem klinicznym –

polipowatością związaną z funkcją korektorską polimerazy (PPAP; ang. *polymerase proofreading-associated polyposis*) co u nosicieli jest podłożem rozwoju mnogich polipów jelita grubego i w konsekwencji raka jelita grubego. Co więcej, to obciążenie genetyczne także jest stwierdzane w rozwoju innych nowotworów jak raka endometrium, raka piersi i rak żołądka (Mur i in. 2020). Mutacja somatyczna *POLD1* może także leżeć u podłoża rozwoju raka jelita grubego, ale jednocześnie określane jest to jako bardzo rzadko zachodzące zdarzenie, częściej w wyniku mutacji *POLE* niż mutacji *POLD1*. Obecnie proponowana jest nazwa zespół guza związanego z *POLD1/POLE* (ang. *POLE/POLD1-associated tumor syndrome*) określająca zarówno PPAP, jaki pojedynczy guz jelita grubego powstały w wyniku mutacji somatycznej (Schubert i in. 2020). Obserwacje mikroskopowe raka jelita grubego związanego z mutacją *POLE/POLD1* wskazują na obecność nacieków limfocytarnych, co może być związane ze zwiększoną immunogennością tego typu guza, ale w konsekwencji może mieć implikacje terapeutyczne, sugerując predykcyjną rolę zmian genetycznych *POLE* i *POLD* w CRC (Keshinro i in. 2021) .

Pomimo dość dobrze udokumentowanej roli patologicznych wariantów genu *POLD1* w procesach nowotworzenia oraz stwierdzenia ich potencjalnej przydatności w rokowaniu i doborze terapii niektórych nowotworów złośliwych, tylko nieliczne prace miały na celu określenie poziomu ekspresji POLD1 w tkankach zmienionych nowotworowo i zbadanie przydatności immunoekspresji tego czynnika jako biomarkera.

Hipoteza badawcza

Przyjęto założenie, że poziomy ekspresji POLD1 w raku nerki i raku jelita grubego są związane z natężeniem proliferacji komórkowej i przez to mogą korelować z progresją choroby i przeżyciem pacjentów.

Cele pracy

1. Analiza poziomu ekspresji białka POLD1 w skrawkach mikroskopowych raka jasnokomórkowego nerki i w niezmienionej morfologicznie części nerki (nabłonek kanalików bliższych).

2. Zbadanie korelacji występujących pomiędzy poziomem immunoekspresji POLD1 w skrawkach raka nerki, a danymi demograficznymi i kliniczno-patologicznymi pacjentów w tej grupie.

3. Określenie przydatności immunohistochemicznej oceny poziomu ekspresji białka POLD1 jako czynnika prognostycznego pacjentów z rakiem jasnokomórkowym nerki.

4. Analiza immunoekspresji białka POLD1 w preparatach histologicznych: raka jelita grubego i w niezmienionego morfologicznie części jelita grubego (komórki nabłonka błony śluzowej).

5. Analiza statystyczna zależności pomiędzy poziomem immunoekspresji POLD1 w preparatach raka jelita grubego w odniesieniu do danych demograficznymi i kliniczno-patologicznymi pacjentów z rakiem jelita grubego.

6. Ocena przydatności immunohistochemicznej analizy ekspresji białka POLD1 jako czynnika prognostycznego w raku jelita grubego.

7. Zbadanie zależności pomiędzy poziomami ekspresji mRNA POLD1 a danymi demograficznymi, kliniczno-patologicznymi oraz ogólnym przeżyciem i czasem wolnym od symptomów choroby u pacjentów z rakiem jelita grubego w oparciu o dane sekwencjonowania dostępne w bazach danych projektu TCGA (ang. *The Cancer Genome Atlas*).

8. Przegląd aktualnego stanu wiedzy na temat funkcji POLD1 i jego roli w przebiegu nowotworów złośliwych człowieka.

9. Omówienie problemu decyzyjnego, który powstaje po stwierdzeniu nacieku raka w polipie wyciętym w technice kolonoskopowej i podanie kryteriów wyboru ścieżki terapeutycznej.

Zakres badań wchodzących w skład rozprawy doktorskiej

W skład rozprawy doktorskiej wchodzi cykl czterech publikacji: dwóch prac oryginalnych i dwóch prac poglądowych. Dwie prace oryginalne przedstawiają wyniki badań eksperymentalnych wraz z dyskusją i przeglądem aktualnego stanu wiedzy z zakresu problematyki zdefiniowanej w celach pracy. Dotyczą analizy ekspresji potencjalnego markera proliferacji komórek – białka POLD1 w materiale pooperacyjnym pozyskanym od chorych na ccRCC i CRC. W badaniach wykorzystano metodę immunohistochemiczną (IHC), analizę danych bioinformatycznych pochodzących z sekwencjonowania oraz metody statystyki biomedycznej, w tym analizę przeżycia. Podsumowaniem cyklu prac eksperymentalnych dotyczących przydatności POLD1 jako markera prognostycznego i/lub predykcyjnego w nowotworach człowieka stanowi opublikowana po zakończeniu badan eksperymentalnych praca przeglądowa. Pracą uzupełniającą ten cykl jest publikacja, której celem był przegląd aktualnego stanu wiedzy w odniesieniu do problemu decyzyjnego pojawiającego się po rozpoznaniu „rak w polipie” usuniętym podczas badania kolonoskopowego.

Materiały i metody

Do realizacji celów obu prac oryginalnych (eksperymentalnych) posłużył materiał tkankowy zarchiwizowany w postaci bloczków parafinowych w Katedrze Histologii i Embriologii Człowieka i pozyskane wcześniej dane pacjentów (demograficzne, kliniczne i obserwacja prowadzona co do przebiegu choroby) w grupie z ccRCC i w grupie z CRC. Pobieranie materiału tkankowego oraz gromadzenie danych kliniczno-patologicznych pacjentów obywało się na podstawie odpowiednich zgód lokalnej Komisji Bioetycznej Uniwersytetu Warmińsko-Mazurskiego w Olsztynie (zgody nr 3/2010, 4/2010 i 21/2017). Każdy z pacjentów był szczegółowo informowany o prowadzonym badaniu, zgoda była dobrowolna, a od każdego pacjenta pobierano pisemną świadomą zgodę na uczestnictwo w badaniu, pobraniu materiału tkankowego pooperacyjnego i przetwarzaniu zakodowanych danych osobowych i klinicznych.

Materiałem badawczym poddanym analizie w pierwszej pracy oryginalnej były tkanki pooperacyjne guzów ccRCC oraz niezmienionych fragmentów nerki pochodzące z zabiegów nefrektomii. Do badanej grupy byli włączeni tylko chorzy z potwierdzonym badaniem patomorfologicznym ccRCC, a stopień zaawansowania klinicznego choroby był określany w oparciu o dane kliniczne i dane pochodzące z badania patomorfologicznego określane według Amerykańskiego Wspólnego Komitetu ds. Raka (ang. *American Joint Committee on Cancer*; AJCC) i skalę Fuhrmana (Fuhrman i in. 1982). Grupę badaną stanowiło 56 pacjentów z ccRCC (27 kobiet i 29 mężczyzn, ze średnią wieku 62,6 lat) leczonych operacyjnie w latach od 2010 do 2012. Krótko po amputacji nerki z narządu były wycinane dwa małe fragmenty tkankowe, jeden bezpośrednio z guza nowotworowego nerki, a drugi z niezmienionej części narządu. Po operacji pacjenci objęci byli obserwacją, której wyniki stanowiły podstawę do analizy przeżycia. Mediana okresu obserwacji chorych z rakiem nerki wynosiła 39,3 miesiące.

Grupę badaną w drugiej pracy oryginalnej stanowiło 78 pacjentów z CRC (44 mężczyzn i 34 kobiety, ze średnią wieku 67,5 lat). Materiał do badania był pobrany od pacjentów, którzy byli poddani operacji wycięcia części jelita grubego z rakiem. Pacjenci mieli wcześniej rozpoznanie histopatologiczne jako rak gruczołowy jelita grubego pochodzące z wykonanej kolonoskopii diagnostycznej. Z badania wyłączeni zostali pacjenci po radioterapii, chemioterapii lub z objawami podniedrożnościowymi. Bezpośrednio po wycięciu odcinka jelita pobierany był jeden wycinek tkankowy z guza nowotworowego oraz drugi, będący fragmentem niezmienionego morfologicznie, odległego o co najmniej 5-7 cm od nacieku CRC. Materiał

pooperacyjny był analizowany przez patomorfologa, który określał stopień zaawansowania nowotworu w oparciu o wytyczne AJCC. Po zakończeniu leczenia operacyjnego pacjenci podlegali obserwacji (analiza przeżycia), a mediana jej okresu wynosiła w tej grupie chorych 52,2 miesiące.

Pobrane w obu badaniach wycinki guzów i tkanek niezmienionych utrwalano w 4% buforowanym paraformaldehydzie i poddawano rutynowym procedurom w celu przygotowania skrawków mikroskopowych i przeprowadzenia w nich reakcji IHC, pozwalającej ocenić poziom ekspresji białka POLD1. Zastosowano królicze przeciwciała pierwszorzędowe skierowane przeciwko POLD1 oraz następnie przeciwciała drugorzędowe, sprzężone z peroksydazą chrzanową. Do uzyskania reakcji barwnej zastosowano diaminobenzydynę jako substrat oraz hematoksylinę, jako barwnik kontrastujący. W każdej serii wykonywano kontrole negatywne (pomijając przeciwciało pierwszorzędowe) oraz barwienia hematoksyliną-eozyną w celu potwierdzenia obecności komórek raka lub prawidłowej morfologii analizowanych skrawków tkankowych. Oceny immunoekspresji POLD1 w jądrach komórkowych komórek ccRCC lub komórek CRC i odpowiadających im komórkach nienowotworowych dokonywał patomorfolog, który nie posiadał wglądu do danych klinicznych pacjentów. Do oceny immunoreaktywności POLD1 w skrawkach zastosowano metodę Remmele i Stegner (Remmele, Stegner, 1987), uwzględniającą procentowy udział pozytywnych komórek (od 0 do 4 punktów) i intensywność reakcji (od 0 do 3 puktów). Poziom immunoekspresji POLD1 wyrażano w skali punktowej, stanowiącej iloczyn obu powyższych punktów (skala od 0 do 12 punktów).

Korelację ekspresji POLD1 z danymi kliniczno-patologicznymi oraz przeżyciem pacjentów z CRC badano także na poziomie mRNA, wykorzystując profile ekspresji uzyskane na materiale pobranym z 599 pacjentów z rakiem jelita grubego, które udostępnione są w portalu danych projektu TCGA.

Wyniki ekspresji POLD1analizowano za pomocą pakietu narzędzi statystycznych dostępnych w programach Statistica 13 i GraphPad Prism 6,07. Do określenia różnic pomiędzy ekspresją białka POLD1 w komórkach raka i komórkach nabłonkowych niezmienionej tkanki zastosowano test Wilcoxona dla par obserwacji. Korelację pomiędzy poziomami ekspresji POLD1, a danymi klinicznymi i patomorfologicznymi badano za pomocą testu dokładnego Fishera. Analizę przeżycia pacjentów przeprowadzono stosując metodę Kaplan-Meier, a okresy przeżycia w poszczególnych grupach pacjentów porównywano za pomocą testu log-rank.

Wyniki

Omówienie pracy oryginalnej nr 1

Godlewski J*, **Stefaniak P***, Kiezun J, Krazinski B E. DNA Polymerase Delta 1 Catalytic Subunit (POLD1) as a Prognostic Factor in Clear Cell Renal Cell Carcinoma Patients. *In Vivo*. 2022; 36(3):1188-1194. DOI:10.21873/invivo.12818.

*oznaczone osoby są równoważnymi pierwszymi autorami pracy.

Metoda IHC wykazała immunoreaktywność POLD1 w jądrach komórek ccRCC i w jądrach komórek nabłonka kanalików krętych bliższych (ang. *proximal convoluted tubules*; PCTs). Średnie wartości immunoekspresji były istotnie wyższe w komórkach raka niż w komórkach nabłonka PCTs. Wysoki poziom immunoekspresji POLD1 w raku korelował dodatnio z całkowitym przeżyciem (ang. *overall survival*; OS) pacjentów (HR=0,35; p=0,0436), wskazując na potencjalną przydatność POLD1 jako markera związanego z dobrym rokowaniem u pacjentów z ccRCC. Badania potwierdziły także istotna negatywną korelację OS pacjentów z wyższym stopniem złośliwości komórek raka w skali Fuhrman, średnicą guza, statusem T, oraz obecnością przerzutów odległych pacjentów, podczas gdy pozostałe parametry takie jak wiek i płeć pacjentów nie korelowały istotnie z OS pacjentów.

Omówienie pracy oryginalnej nr 2

Stefaniak P, Krazinski B E, Kiezun J, Majewska H, Godlewski J. Altered immunoexpression of DNA polymerase delta1 catalytic subunit (POLD1) in colorectal cancer. *Contemp Oncol (Pozn)* 2023; 27(3):147-154. doi:10.5114/wo.2023.133505

Analiza IHC wykazała zróżnicowaną immunoekspresję POLD1 w jądrach komórkowych komórek CRC i komórek nabłonka błony śluzowej niezmienionego morfologicznie jelita grubego. Średni poziom immunoekspresji w komórkach raka był istotnie niższy niż w komórkach prawidłowego nabłonka. Poziom ekspresji POLD1 w komórkach raka nie korelował z danymi klinicznymi i patomorfologicznymi (lokalizacja guza, wielkość guza, obecność przerzutów w węzłach chłonnych, obecność przerzutów odległych czy stopień zaawansowania klinicznego TNM, a także z czasem przeżycia odległego). Wyniki powyższe potwierdzono analizując dane ekspresji mRNA *POLD1* w kohorcie 599 pacjentów z CRC

udostępnionych w projekcie TCGA. Podobnie, jak w przypadku białka, nie wykazano istotnych korelacji pomiędzy transkrypcji *POLD1*, a parametrami kliniczno-patologicznymi i przeżyciem pacjentów z CRC. Dodatkowo, w badanej grupie pacjentów przeżycie korelowało negatywnie z wyższym statusem T guza, obecnością przerzutów do regionalnych węzłów chłonnych i przerzutów odległych oraz wyższym stopniem zaawansowania TNM. Nie stwierdzono związku przeżycia w odniesieniu do płci, wieku i anatomicznej lokalizacji guza nowotworowego. Badania nie potwierdziły przydatności ekspresji POLD1 jako potencjalnego czynnika prognostycznego w CRC.

Omówienie pracy przeglądowej nr 1

Gola M, **Stefaniak P,** Godlewski J, Jereczek-Fossa B A, Starzyńska A. Prospects of POLD1 in Human Cancers: A Review. *Cancers (Basel)*. 2023;15(6):1905. doi: 10.3390/cancers15061905

Dane zebrane z aktualnego piśmiennictwa a przedstawione w tej pracy wskazują że rola POLD1 (tak na poziomie genu jak i białka) w odniesieniu do rozwoju i przebiegu nowotworów jest zróżnicowana w zależności od rodzaju nowotworu. Dotychczas stwierdzono zwiększoną ekspresję genu *POLD1* w przebiegu raka wątrobowo-komórkowego, raka piersi, niedrobnokomórkowego raka płuca co przekłada się na większe zaawansowanie oraz gorszą prognozę przebiegu choroby (Sanefuji i in. 2010; Qin i in. 2018; Zhang i in. 2021; Tang i in. 2022). Natomiast w raku brodawkowatym tarczycy obniżenie ekspresji *POLD1* związane było z gorszą charakterystyką morfologiczną guza (Siraj i in. 2020a). Podobnie wyniki badań materiału tkankowego CRC u chorych obszaru Półwyspu Arabskiego wskazują, że niska ekspresji białka POLD ma swoje przełożenie na większy rozmiar guza nowotworowego i większe zaawansowanie choroby (Siraj i in. 2020b), natomiast w raku endometrium jest to skorelowane z niskim zaawansowaniem choroby (Siraj i in. 2019). Na podstawie dokonanego przeglądu literatury, można wyciągnąć sformułować wnioski, że wiedza w zakresie ekspresji genu i białka POLD1 w przebiegu różnych typów nowotworów jest fragmentaryczna, a dotychczas prowadzone analizy są badaniami jednoośrodkowymi. Dalsze poszukiwania w tym zakresie mogą służyć do ustalenia przydatności POLD1 jako markera zaawansowania choroby i rokowania, a także jako potencjalnego czynnika predykcyjnego, przydatnego przy opracowywaniu, wdrażaniu i personalizacji nowych metod terapeutycznych.

Omówienie pracy przeglądowej nr 2

Stefaniak P, Majewska H, Zadrozny D, Godlewski J. Cancer in polyp of the large intestine – an interdisciplinary decision problem. *Gastroenterology Rev* 2021; 16 (4): 306-310. doi:10.5114/pg.2021.111481

Do problematyki oceny rozrostu nowotworowego w obrębie pojedynczego polipa w obrębie błony śluzowej jelita grubego odnosi się czwarta praca zawarta w tym cyklu. Inspirację do powstania pracy stanowiły trudności pojawiające się w procesie decyzyjnym po wykryciu ogniska raka gruczołowego w wyciętym kolonoskopowo polipie. Podczas kolonoskopii wykonanej jako badanie profilaktyczne (screeningowe) albo diagnostyczne (związane z objawami) często, w około 19,4% stwierdzana jest polip jelita grubego (Pox i in. 2012). Przeważnie pojedyncze uszypułowane polipy są wycinane w technice kolonoskopii zabiegowej a pozyskany polip jest wysyłany do badania histopatologicznego, które określa jego budowę morfologiczną. Problem kliniczny pojawia się w momencie stwierdzenia rozplemu raka gruczołowego w obrębie polipa. W niniejszej pracy przedstawiono aktualny przegląd literaturowy odnoszący się do tego zagadnienia. Ponadto wykonano obrazy mikroskopowe przekroju podłużnego polipa z naciekiem raka (materiał własny), które pokazuje niszczenie blaszki mięśniowej błony śluzowej – barwienie IHC na obecność desminy). Ta ilustracja obrazuje sposób oceny jakie stosują patomorfolodzy (T1 vs Tis) i przedstawienie zachowanej szypuły polipa, której ocena powinna być także zawarta w protokole patomorfologicznym. W tej pracy przedstawiono stosowane klasyfikacje Haggitt i Kikuchi oraz rekomendacje Polskiego Towarzystwa Gastroenterologicznego odnoszące się do nadzoru endoskopowego. Celem tej pracy było przedstawić podstawy naukowe i ocenę kliniczną prowadzącą do wydania racjonalnej decyzji terapeutycznej: obserwacja kolonoskopowa (z ryzykiem wznowy miejscowej) czy przekierowanie do operacji chirurgicznej – odcinkowa resekcja jelita grubego (z ryzykiem powikłań okołooperacyjnych) z uwzględnieniem stanu ogólnego pacjenta i jego zgody na proponowany sposób postepowania.

Podsumowanie

Przedmiotem przeprowadzonych badań była analiza immunoekspresji POLD1 w raku jasnokomórkowym nerki i raku jelita grubego oraz odpowiadającym im fragmentom niezmienionej struktury nerki lub jelita grubego oraz określenie korelacji ekspresji POLD1 z danymi kliniczno-patologicznymi i przeżyciem pacjentów. Przeprowadzone badania potwierdziły zróżnicowaną immunoreaktywność POLD1 w analizowanych skrawkach tkankowych i zmiany poziomu ekspresji tego czynnika w tkankach patologicznych, w porównaniu do niezmienionych nowotworowo tkanek.

Wnioski

1. Stwierdzono podwyższony poziom POLD1 w komórkach ccRCC w porównaniu do niezmienionych morfologicznie komórek nabłonka PCTs, który korelował z dobrym rokowaniem u pacjentów z ccRCC, wskazując na potencjalne zastosowanie POLD1 jako markera prognostycznego w tym nowotworze.

2. W skrawkach CRC poziom immunoekspresji POLD1 był niższy niż w enterocytach niezmienionej błony śluzowej jelita grubego, jednak nie potwierdzono prognostycznego znaczenia poziomu ekspresji POLD1 w tym nowotworze.

3. Wyniki przeprowadzonych prac eksperymentalnych oraz zgromadzone dane literaturowe wskazuje, że zmiany poziomu ekspresji POLD1 i ich przydatność POLD1 jako markera prognostycznego zależy od typu nowotworu.

4. Dane literaturowe dotyczące POLD1 wskazują na jego potencjalne znaczenie jako markera prognostycznego i predykcyjnego w różnych nowotworach złośliwych człowieka, brakuje jednak badań wieloośrodkowych, których celem byłaby walidacja przydatności POLD1 w poszczególnych typach nowotworów.

5. Stwierdzeniu nacieku „rak w polipie” jelita grubego wymaga skrupulatnej analizy otrzymanego opisu patomorfologicznego i interpretacji tego wyniku z zastosowaniem uznanych skali klasyfikacji w odniesieniu do stanu klinicznego danego pacjenta.

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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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Key Words: DNA polymerase, POLD1, clear cell renal cell carcinoma, ccRCC, patient's survival, prognostic factor.

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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).

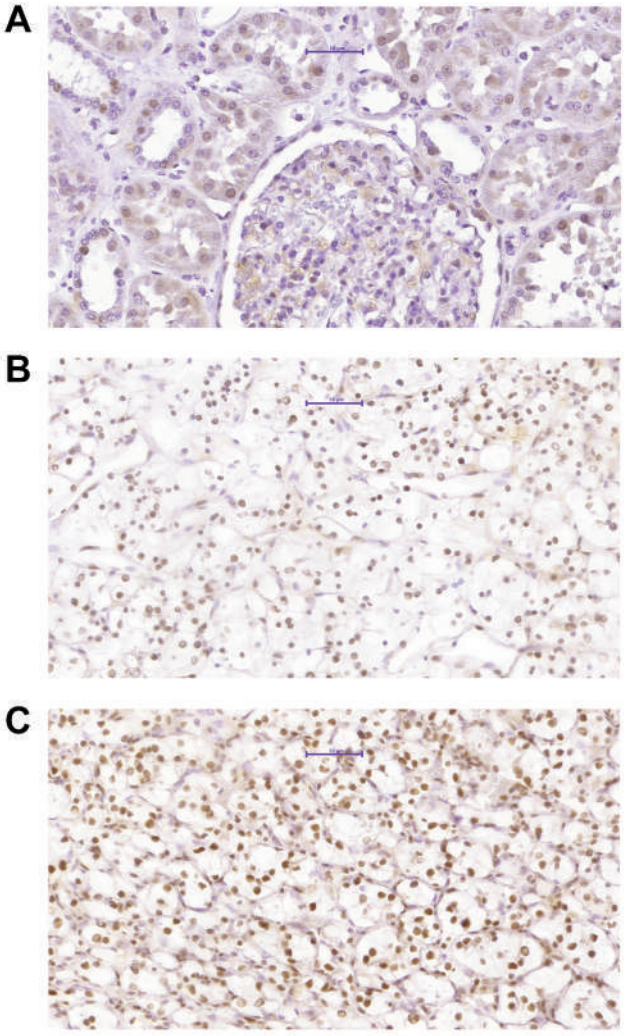


Figure 1. *Immunoreactivity of DNA polymerase delta 1 catalytic subunit (POLD1) in sections of unaltered kidney tissue and clear cell renal cell carcinoma (ccRCC). (A) The normal structure of the kidney's cortex, nuclear staining is visible in the tubular epithelium. Week POLD1 immunoreactivity (1 point) 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.*

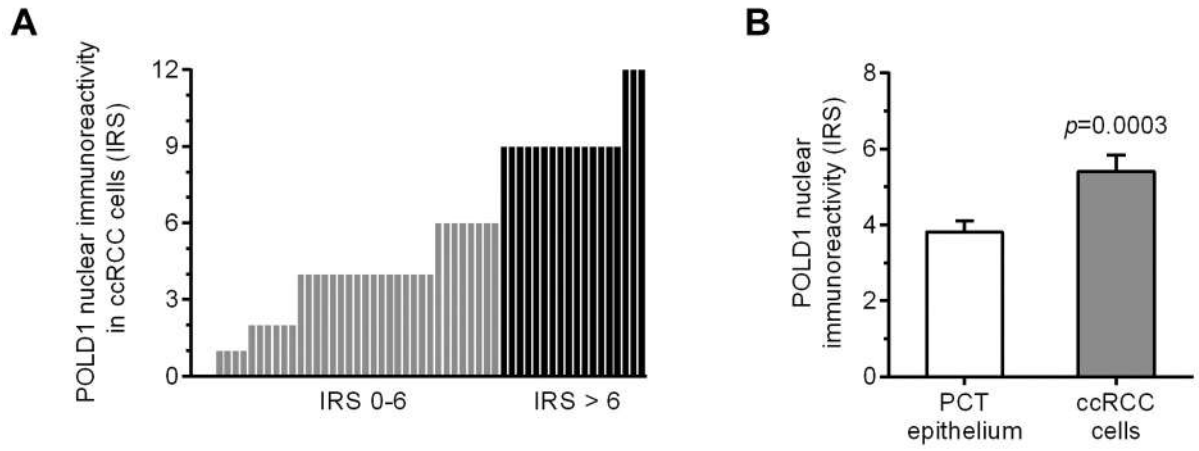


Figure 2. *Evaluation of DNA polymerase delta 1 catalytic subunit (POLD1) immunoexpression in the clear cell renal cell carcinoma (ccRCC) and unaltered renal tissues by immunohistochemistry. (A) POLD1 nuclear immunoreactivity in the tumor sections of individual patients with ccRCC is shown. Grey bars 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.

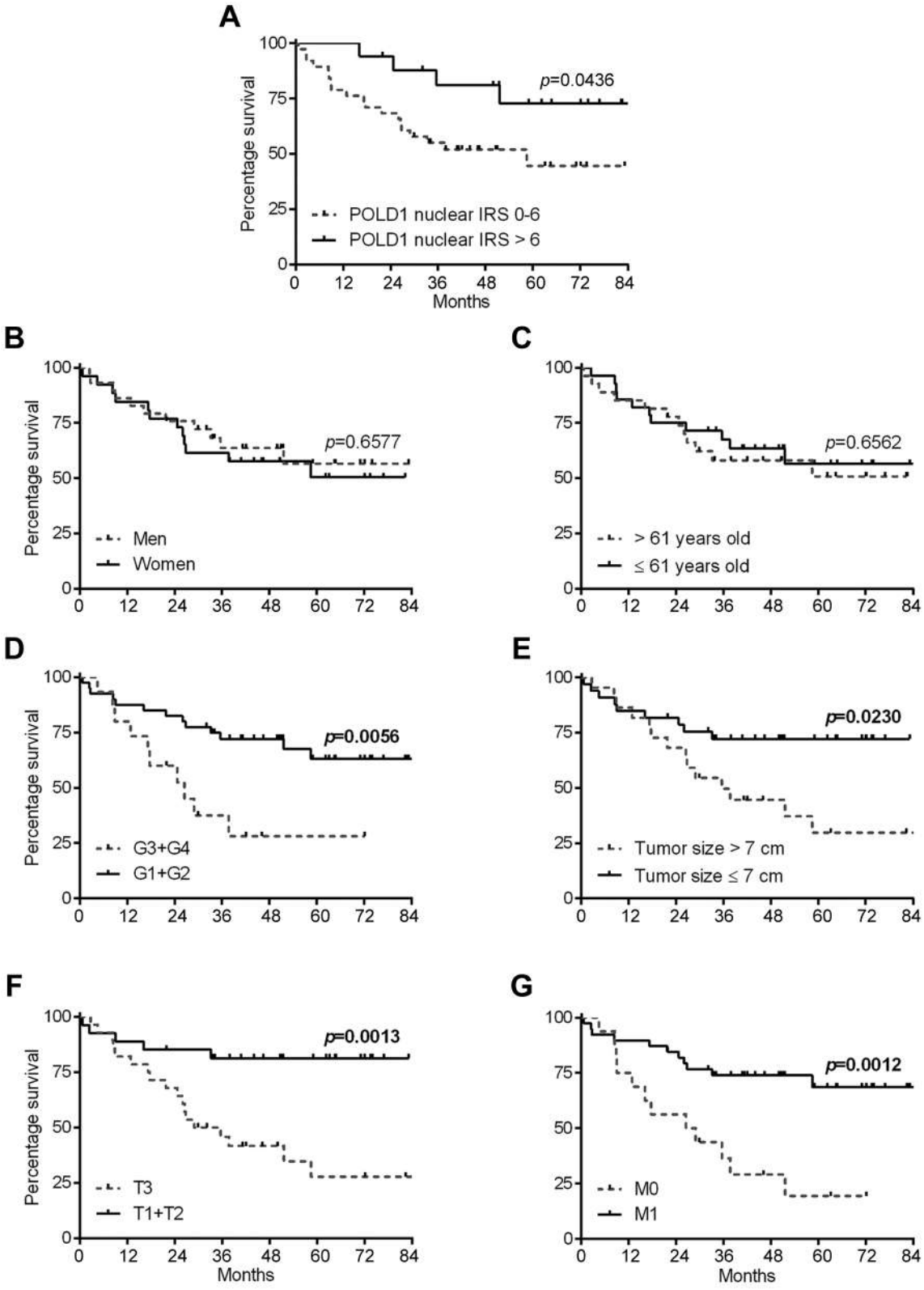


Figure 3. *Kaplan-Meier diagrams of overall survival of 56 patients with clear cell renal cell carcinoma (ccRCC) in correlation with (A) immunoexpression levels of DNA polymerase delta 1 catalytic subunit (POLD1), (B-C) demographic and (D-G) clinicopathological characteristics. Significant p-values (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

Altered immunoexpression of DNA polymerase delta 1 catalytic subunit (POLD1) in colorectal cancer

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Introduction: The study aimed to determine the immunoexpression levels of polymerase delta 1 catalytic subunit (POLD1), a catalytic and proofreading subunit of DNA polymerase delta, in the sections of colorectal cancer (CRC), and to evaluate the significance of POLD1 as a potential prognostic factor in CRC.

Material and methods: Paired, tumour and non-cancerous tissue samples of the large intestine distant to the neoplasm were collected from the postoperative material of 78 patients who underwent surgical resection of CRC tumours. Polymerase delta 1 catalytic subunit protein levels were determined using immunohistochemistry. Clinical, pathomorphological, and survival data of the patients were pooled. In addition, POLD1 mRNA expression levels of 599 CRC patients were extracted from The Cancer Genome Atlas (TCGA) datasets and subjected to statistical and survival analysis including the Kaplan-Meier method followed by the log-rank test.

Results: Immunoexpression of POLD1 was found in the nuclei of the tumour cells and epithelial cells of unchanged intestinal mucosa. Polymerase delta 1 catalytic subunit immunoreactivity in the tumour was heterogenous, and the average immunoreactivity score was decreased in cancer cells when compared to the mucosa of matched sections of unchanged large intestine ($p = 0.0259$). However, POLD1 expression at the protein and mRNA levels did not associate with clinicopathological characteristics of the patients and their survival.

Conclusions: Despite previous studies suggesting that POLD1 genetic alterations could be promising molecular biomarkers in CRC, our results do not support any prognostic significance of POLD1 expression in CRC.

Key words: colorectal cancer, prognosis, survival, POLD1, DNA polymerase delta 1.

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Introduction

Colorectal cancer (CRC) is the third most common and the second most deadly cancer worldwide, accounting for nearly 2 million new cancer cases and almost one million deaths worldwide in 2020 [1, 2]. Pathomorphological evaluation of postoperative material is routinely used in clinical practice, allowing for determination of CRC's features related to its invasion within the intestinal wall (T) as well as nodal (N) and distant metastasis (M) [3]. These classical morphological parameters are still the major basis for risk stratification and selection between different postoperative treatment options. Contemporary CRC treatment is realized as a personalized therapy and is based on the selection of neo- and/or adjuvant therapy in reference to the individual molecular cancer subtype, stage of disease, and general condition of the patient [2, 4, 5]. Therefore, it is necessary to identify and validate new proteins useful as prognostic and/or predictive markers that can be easily adopted for a routine immunohistochemical evaluation of postoperative CRC tissues.

DNA polymerase delta (Polδ) is an enzyme complex responsible for complementary synthesis of the lagging strand during DNA replication, and it participates, alongside DNA polymerase epsilon (Polε), in the replication of the leading DNA strand [6]. Polδ and Polε are heterotetrametric complexes, and their catalytic subunits are DNA polymerase delta 1 catalytic subunit (POLD1) and DNA polymerase epsilon, catalytic subunit (POLE), respectively [7]. Polymerase delta 1 catalytic subunit and POLE possess DNA polymerase and 3'–5' exonuclease activities, and they serve in the mechanisms of DNA repair [8]. Genetic alterations of exonuclease domains of *POLD1* and *POLE* may affect their DNA proofreading ability and are considered factors in the development of several human neoplastic diseases including hereditary and sporadic colorectal tumours [8–10]. Pathologic variants of *POLD1* and *POLE* can result in deficient DNA replication and repair predisposing to colorectal polyposis and subsequent neoplastic transformation [11, 12]. The studies based on colon cancer cell lines HCT116 and SW48 as well as sporadic CRCs indicate that *POLD1* gene mutation within its exonuclease domain impairs Polδ function resulting in accumulated mismatches of DNA base paring [13]. Colorectal tumours that develop on the basis of polymerase proofreading deficiency or impaired mismatch repair system are characterized by higher mutation burden and higher infiltration by neoantigen-specific, tumour-infiltrating T-cells, predisposing those tumours for immunotherapy [14]. This suggests that *POLD1* and *POLE* mutations can be predictive markers useful in identifying gastrointestinal tumours that are sensitive for treatment with immune checkpoint inhibitors [15, 16]. However, POLD1 mutations in

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sporadic CRC are rarer than those in POLE, and no individual mutational signature has been associated with POLD1 deficiency in microsatellite stable CRC [17], questioning the predictive significance of POLD1 genetic alterations in most molecular subtypes of sporadic CRCs.

While the genetic alterations of *POLD1* in CRC get most of the attention, the level of *POLD1* expression in postoperative material of CRC patients, and its association with the tumour progression and patients' outcomes have not been extensively studied, and the usefulness of POLD1 protein or mRNA level as a prognostic marker in CRC remains unclear. Therefore, the purpose of this study was to determine the expression levels of POLD1 protein in the tumour and matched normal tissues collected from CRC patients, subjecting tissue sections to a standard, cheap, and easy to adopt in any pathological laboratory method such as immunohistochemistry (IHC). In addition, *POLD1* mRNA expression data were extracted from The Cancer Genome Atlas (TCGA) CRC datasets and analysed.

Material and methods

Ethical statements

The study was approved by the Bioethical Commission of the University of Warmia and Mazury, Olsztyn, Poland (no. 40/2010 and 21/2017) and was conducted according to the guidelines of the Declaration of Helsinki (1975, as revised in 2000). Written informed consent was obtained from each patient included in this study.

Patients and collection of tissue samples

The colorectal cancer study group consisted of 78 patients (44 males and 34 females; mean age 67.5 years old, ranging 33–90). The tissue samples were collected from patients who underwent open surgery resection large interstine due to the CRC tumour. Only patients with histologically confirmed CRC (colonoscopic biopsy) and complete clinicopathological data (including computed tomography examination of abdominal and pelvis cavity) were qualified to the study. Patients with neoadjuvant chemotherapy and/or radiotherapy as well as patients with polyps in the colorectal region associated with the cancer were excluded from the analysis. The demographic and clinicopathological data of CRC patients are presented in Table 1. Shortly after resection 2 tissue specimens (whole wall of intestine, each 1–2 cm in size) were collected: one directly from the tumour and one from a distant, morphologically unchanged part of the large intestine. The samples collected from postoperative material were fixed in 4% buffered (pH 7.4) solution of paraformaldehyde. For clinical use, the cancer characteristic and staging according to the 8th edition of American Joint Committee on Cancer [5] were evaluated (pathologist H.M). Patients were followed-up, and the average time of observation was 52.2 months.

Immunohistochemistry and evaluation of immunohistochemical reactions

All sections of CRC lesions and matched normal, morphologically unchanged large intestine were subjected to immunohistochemical staining with the use of antibodies directed against POLD1 protein (HPA046524; Merck KGaA, Darmstadt, Germany) using a previously described protocol [18]. The polymerase delta 1 catalytic subunit nuclear immunoexpression levels were evaluated in CRC tumours and epithelial cells (enterocytes) of the matching large intestine according to the immunoreactive score system of Remmele and Stegner (IRS) [7] as previously described by Godlewski *et al*. [18]. The colorectal cancer patients were divided based on the median immunoexpression score in CRC cells (median IRS = 6) into 2 groups described as 'low', and 'high' POLD1 immunoexpression.

Bioinformatic analysis of The Cancer Genome Atlas datasets

Polymerase delta 1 catalytic subunit RNA-Seq gene expression data of 599 samples derived from primary CRC sites were extracted from the TCGA-COAD and TCGA-READ datasets [19] (accession date: 13 May 2023) and subjected to further bioinformatical analysis. Patients without complete demographic (sex, age) or staging (tumour-node-metastasis – TNM) data were excluded from the analysis. Follow-up data for overall survival (OS) and disease-free survival (DFS) of the patients were collected for 599 CRC cases from cBioPortal (accession date: 17 March 2023) [20]. Differential gene expression analysis was performed using DESeq2 [8] with absolute value of $\log_2$FoldChange ≥ 0.5 and p-value < 0.05 as the cut-off. Based on the median of normalized gene expression values of the *POLD1* gene the entire dataset was divided into 2 groups classified as 'low', and 'high' *POLD1* mRNA expression.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software, version 6.07 (GraphPad, La Jolla, CA, USA) and Statistica version 13 (TIBCO Software Inc., Palo Alto, CA, USA) software. The significance of differences between expression levels of POLD1 proteins in CRC cells and normal intestine epithelial cells were tested by the Wilcoxon matched-pairs signed-rank test. The ratios of POLD1 scores of paired cancer cells and epithelial cells of normal intestine sections were calculated. Correlations of *POLD1* mRNA and protein expression levels with demographic and clinicopathological data were evaluated using Fisher's exact test. The univariate associations of *POLD1* mRNA and POLD1 protein levels or demographic and clinicopathological data with patients' OS or DFS were plotted using the Kaplan-Meier method, and the survival differences between the patient cohorts were assessed by the log-rank test. Differences were considered statistically significant for a p-value lower than 0.05

Results

Polymerase delta 1 catalytic subunit protein was found in the nuclei of CRC cells (Figs. 1 A, B) and epithelial cells of unchanged mucosa of the large intestine (Figs. 1 C, D). In addition to CRC or epithelial cells, POLD1 immunoreactivity was noticeable in the nuclei of some stromal cells. However, when compared to the tumour cells

Table 1. No associations between clinicopathological features of colorectal cancer patients and DNA polymerase delta 1 catalytic subunit (POLD1) nuclear immunoreactivity (as determined by immunohistochemistry; *n* = 78) or POLD1 mRNA expression levels (as extracted from The Cancer Genome Atlas [TCGA] datasets TCGA-COAD and TCGA-READ; *n* = 599)

Parameters	Number of cases, *n*	POLD1 protein nuclear immunoreactivity in CRC cells			Number of cases, *n*	POLD1 mRNA expression level (TCGA-COAD and TCGA-READ)		
		IRS 0–6, *n* (%)	IRS > 6, *n* (%)	*p*-value		≤ median, *n* (%)	> median, *n* (%)	*p*-value
Total	78	47 (60.3)	31 (39.7)		599	300 (50.1)	299 (49.9)	
Gender								
Men	44	29 (65.9)	15 (34.1)	0.3509	318	161 (50.6)	157 (49.4)	0.8062
Women	34	18 (52.9)	16 (47.1)		281	139 (49.5)	142 (50.5)	
Age								
≤ 67 years old	37	21 (56.8)	16 (43.2)	0.6449	295	146 (49.5)	149 (50.5)	0.8065
> 67 years old	41	26 (63.4)	15 (36.6)		304	154 (50.7)	150 (49.3)	
Localization								
Colon and cecum	54	35 (64.8)	19 (35.2)	0.3161	443	225 (50.8)	218 (49.2)	0.5774
Rectum	24	12 (50.0)	12 (50.0)		156	75 (48.1)	81 (51.9)	
Primary tumour status								
T1–T3	63	37 (58.7)	26 (41.3)	0.7703	532#	262 (49.2)	270 (50.8)	0.2997
T4	15	10 (66.7)	5 (33.3)		67	38 (56.7)	29 (43.3)	
Regional lymph node status								
N0	45	29 (64.4)	16 (35.6)	0.4832	342	162 (47.4)	180 (52.6)	0.1375
N1 + N2	33	18 (54.5)	15 (45.5)		257	138 (53.7)	119 (46.3)	
Distant metastasis								
M0	56	31 (55.4)	25 (44.6)	0.2026	513$	260 (50.7)	253 (49.3)	0.4868
M1	22	16 (72.7)	6 (27.3)		86	40 (46.5)	46 (53.5)	
TNM stage								
I + II	43	27 (62.8)	16 (37.2)	0.6478	332	160 (48.2)	172 (51.8)	0.3242
III + IV	35	20 (57.1)	15 (42.9)		267	140 (52.4)	127 (47.6)	

IRS – immunoreactive score of Remmele and Stegner, TCG – The Cancer Genome Atlas, TNM – tumour-node-metastasis
p-values were calculated using Fisher's exact test
 # Includes one Tis patient
$ includes MX patients

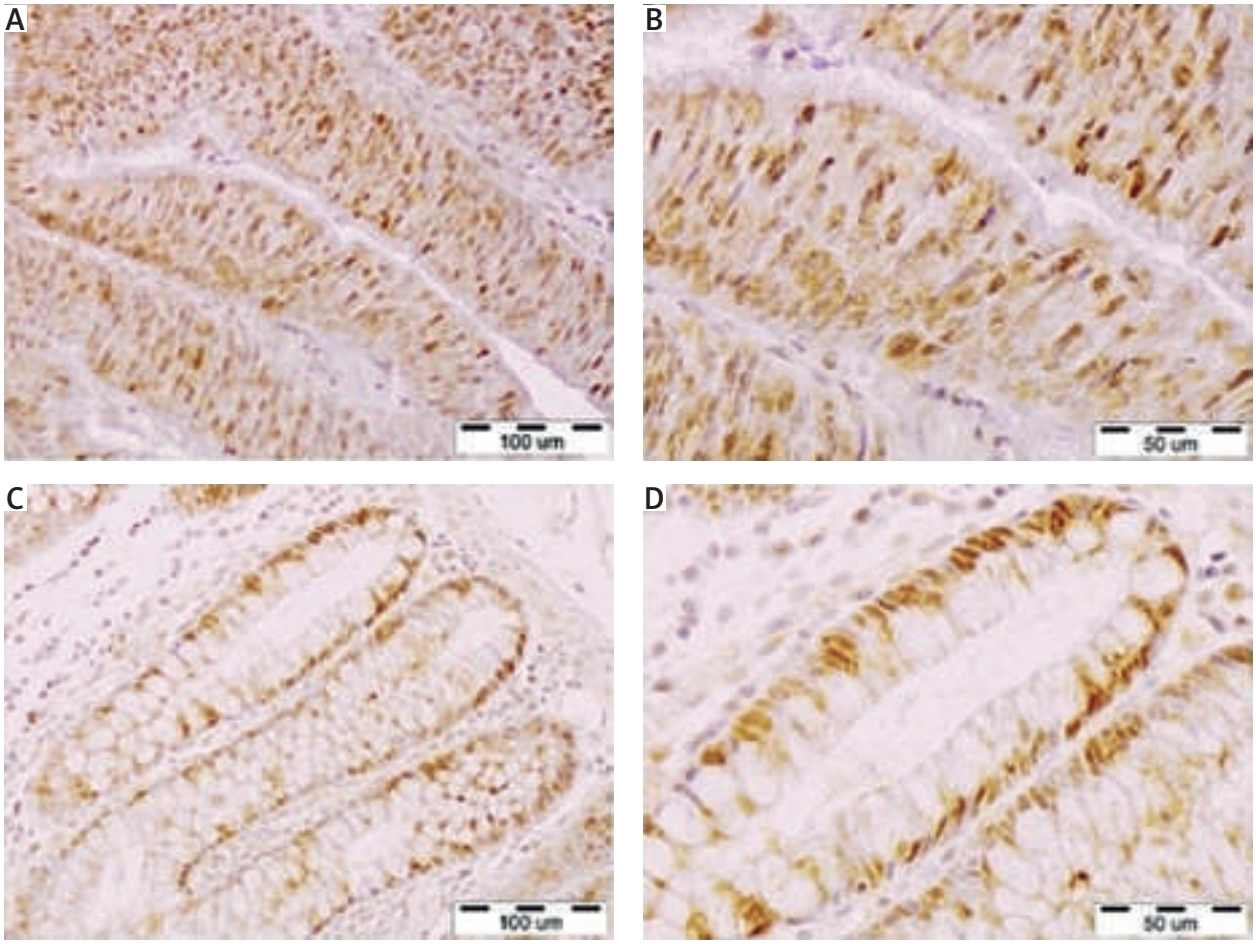


Fig. 1. Immunohistochemical staining of DNA polymerase delta 1 catalytic subunit (POLD1) protein in representative sections of the tumour and large intestine tissues of a colorectal cancer (CRC) patient. Nuclear immunoreactivity of POLD1 in CRC cells (**A, B**), nuclear immunoreactivity of POLD1 in epithelial cells of the matched, unchanged mucosa of the large intestine (**C, D**). Magnification ×200 (**A, C**), ×400 (**B, D**)

(Figs. 1 A, B) or epithelial cells of intestinal mucosa (Figs. 1 C, D), the density and staining intensity of POLD1-positive cells in the stroma were low. Polymerase delta 1 catalytic subunit immunoexpression in levels CRC sections were heterogenous, and their score ranged from IRS = 2 to IRS = 12. Polymerase delta 1 catalytic subunit 1 immunoreactivity levels were equal to or lower than the median value (IRS = 6) in 47/78 (60.3%) and higher than the median IRS in 31/78 (39.7%) tumour sections (Fig. 2 A). Polymerase delta 1 catalytic subunit immunoexpression was decreased in 33 (42.3%) and increased in 20 (25.6%) tumours when compared to the POLD1 immunostaining in the epithelial cells of the matched section of noncancerous large intestine. In the sections derived from 25 CRC patients (32.1%), the CRC cells and matched epithelial cells did not differ in regard to the level of POLD1 protein expression. The average immunoreactivity level of POLD 1 was significantly decreased in the nuclei of CRC cells as compared to the epithelial cells of unchanged large intestine mucosa (IRS 6.40 ±0.33 and IRS 7.06 ±0.33, respectively; $p = 0.0259$) (Fig. 2 B).

Polymerase delta 1 catalytic subunit expression levels in the tumour cells did not correlate with demographic (sex, age) and clinicopathological (tumour localization, primary tumour status, regional lymph nodes status, presence of distant metastasis, and TNM stage) data of CRC patients (Table 1, left panel).

Similarly, *POLD1* mRNA expression data extracted from TCGA-COAD and TCGA-READ datasets did not correlate with the demographic characteristics of the patients and the progression of CRC (Table 1, right panel) and did not reveal significant associations between the levels of *POLD1* mRNA and OS or DFS of the CRC patients (Figs. 3 A, B).

Consequently, immunoexpression of POLD1 in CRC cells did not correlate with CRC patients' outcomes ($p = 0.1856$) (Fig. 3 C). Among the demographic and clinicopathological data subjected to survival analysis, the higher status of primary tumour (HR = 2.14, $p = 0.0477$), N1 or N2 status of nodal metastasis (HR =2.07; $p = 0.0371$), presence of distant metastases (HR = 3.12; $p = 0.0007$), and higher TNM stage (HR = 2.51; $p = 0.0089$) correlated with worse prognosis in CRC patients (Figs. 3 G–J) while the other parameters such as sex, age, tumour localization did not reveal prognostic significance (Figs. 3 D–F).

Discussion

Most of the previous studies explored the prevalence of pathogenic *POLD1* variants and the association of genetic alterations with CRC patients' survival and therapy effectiveness. Our studies aimed to investigate POLD1 immunoexpression in postoperative CRC material, and to test the association between protein levels in reference

to the clinicopathological data and patients' outcomes. The immunoexpression levels of POLD1 in our analysed CRC specimens were heterogenous, allowing the cohort to be divided into the 2 subgroups that were then analysed for correlations with disease progression and patients' outcomes. However, we did not find any significant association between the POLD1 immunoreactivity and clinicopathological data of the patients. Moreover, the survival analysis carried out in the cohort of 78 patients and 2 TCGA-derived datasets of 599 patients did not reveal any prognostic significance of POLD1 expression in CRC.

A previous study investigating POLD1 immunoexpression in a cohort of 1069 CRC patients from the Middle East revealed significant correlations between low POLD1 levels and mucinous histological type of the tumour, higher primary tumour status, and higher tumour stage, suggesting a suppressive role of POLD1 protein in the progression of CRC [21]. However, the latter study also failed to disclose significant associations between the POLD1 immunoexpression and patients' outcomes [21]. Interestingly, in a recent study by Kim *et al*. [22] using a mouse model of colon dysplasia and HCT116 and SW480 cell lines, it was shown that the expression of POLD1 in CRC can be controlled via the β-catenin/c-MYC/E2F1 axis [22]. Moreover, inhibition of the β-catenin/c-MYC/E2F1 axis resulted in decreased POLD1 expression followed by increased DNA replication stress, DNA damage response, apoptosis, and overall repression of the tumour growth. Correlations between POLD1 and c-MYC as well as observed results of POLD1 reduction showed that this enzyme was essential for CRC tumourigenesis. Indeed, POLD1 activity decrease both tumour mutational burden and immunogenicity, maintaining genomic integrity of cancer cells above the critical level [23]. Therefore, both low POLD1 immunoexpression level and/or harbouring of *POLD1* exonuclease domain mutations shall be considered as part of the signature that potentially correlates with longer survival and benefits from treatment with immune checkpoint inhibitors such as those targeting programmed death-1 (PD-1) and its ligand (PD-L1) [5, 16, 24].

Polymerase delta 1 catalytic subunit and POLE are the catalytic subunits of Polδ and Polε, with DNA polymerase and proof-reading exonuclease activities that are essential for DNA replication and maintenance of cellular genetic homeostasis [17]. Germline and somatic pathogenic variants of *POLD1* and *POLE* with altered proof-reading capabilities have been demonstrated as driver mutations predisposing to the development of hypermutated but microsatellite stable CRC [12, 25]. Interestingly, the genetic alterations of *POLE* exonuclease domain have been reported in both familial and sporadic CRC, while there is little evidence of equivalent *POLD1* somatic mutations [11, 17]. Despite much reported evidence for somatic *POLD1* mutations predisposing to develop hypermutated sporadic and colitis-associated CRC, most of the studied *POLD1* alterations were associated with hereditary colorectal syndromes [25, 26]. Mutation status of the *POLD1* gene has recently been proposed as a promising predictive marker in CRC [15]. Analysis of genomic data from TCGA dataset including 2674 CRC patients revealed that *POLD1/POLE* mutations were present in 7.37% of tumours and

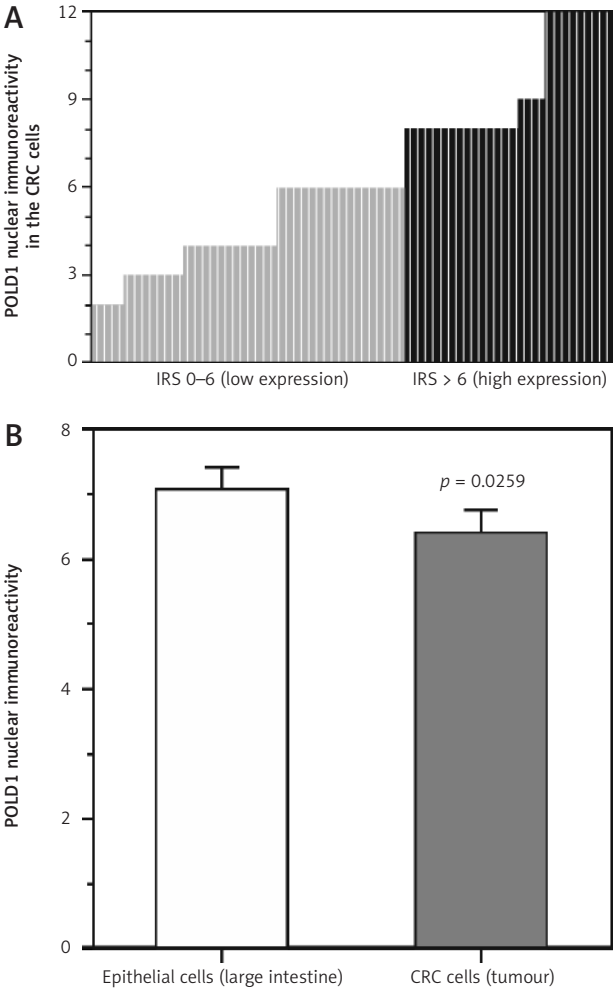


Fig. 2. Evaluation of DNA polymerase delta 1, catalytic subunit (POLD1) immunoexpression in the colorectal cancer (CRC) cells and epithelial cells of morphologically unchanged large intestine by immunohistochemistry. Polymerase delta 1, catalytic subunit nuclear immunoreactivity in the tumor sections of individual patients with CRC are shown. Grey bars represent patients revealing low POLD1 immunoreactivity, black bars represent patients with high levels of POLD1 immunoreactivity (**A**), the average nuclear immunoreactivity of POLD1 in the tumor cells are shown in relation to POLD1 levels in the epithelial cells of morphologically unchanged large intestine (**B**). Data are presented as the means ±SEMs (n = 78)

CRC – colorectal cancer, IRS – immunoreactive score of Remmele and Stegner, POLD1 – polymerase delta 1 catalytic subunit

that the presence of alterations can have predictive significance for positive immune checkpoint inhibitor therapy outcomes [16]. However, the results of our study suggest that the level of POLD1 expression, unlike its genetic status, has no prognostic significance in CRC.

The expression levels of POLD1 and its prognostic or predictive significance have been studied only in several human solid cancers. The study carried out on 432 endometrial carcinoma specimens revealed an association between elevated POLD1 immunoexpression and higher tumour grade [27]. Increased POLD1 expression was shown in breast cancer tissues and breast cancer cell line MCF-7, and this alteration could be at least partially attributed to decreased p53 activity in this tumour [28]. Another study on this tumour revealed that elevated expression of POLD1

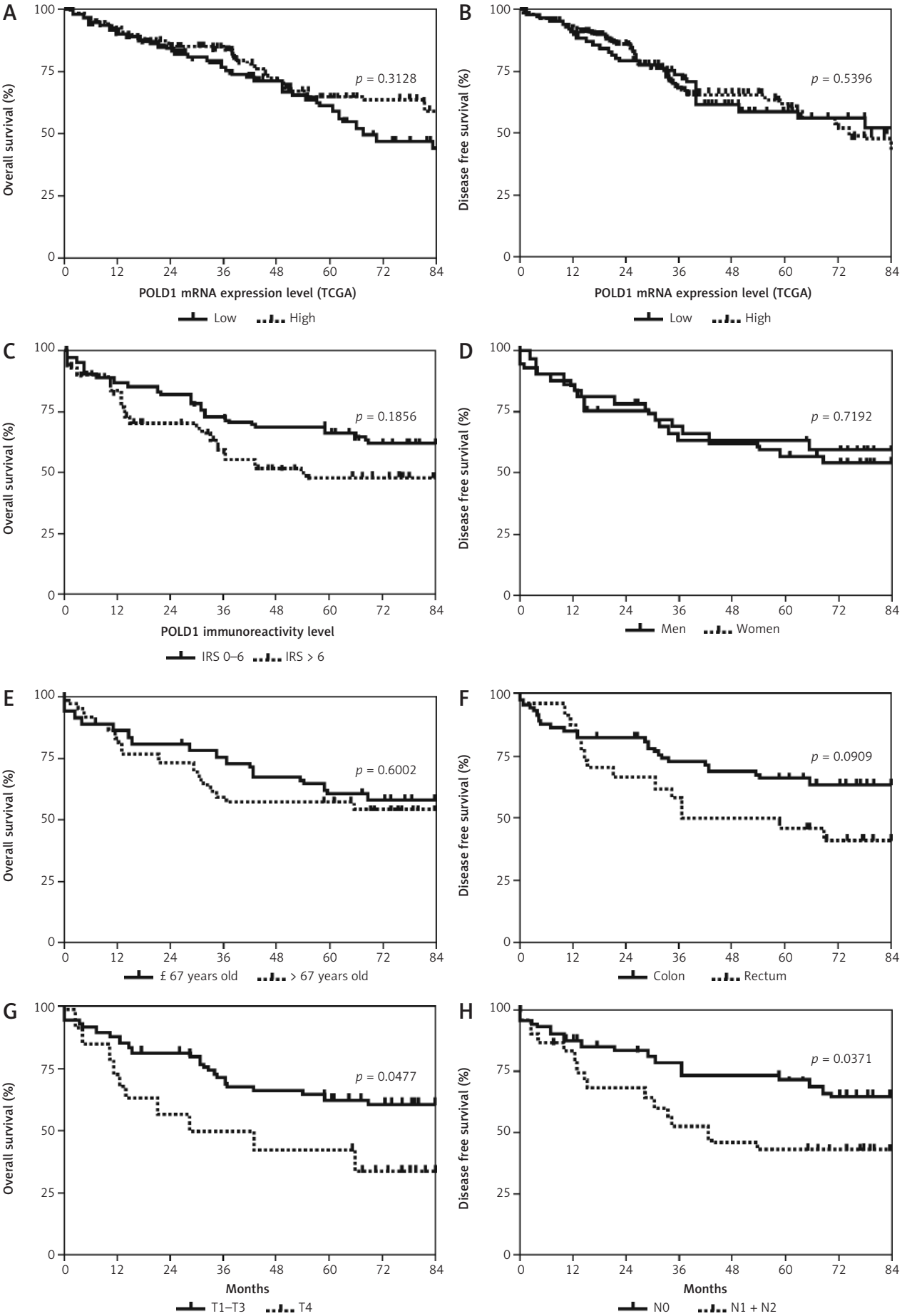


Fig. 3. Kaplan-Meier diagrams presenting the survival of patients with colorectal cancer (CRC) regarding the expression levels of the polymerase delta 1 catalytic subunit (POLD1) (**A–C**) and demographic or clinicopathological characteristics of the patients (**D–J**): overall survival (**A**) and disease-free survival (**B**) of CRC patients regarding *POLD1* mRNA expression levels (The Cancer Genome Atlas datasets; *n* = 599). Overall survival of CRC patients (*n* = 78) regarding the nuclear POLD1 immunoreactivity in cancer cells (**C**), sex (**D**), age (**E**), tumour localization (**F**), primary tumour status (**G**), regional lymph node status (**H**)

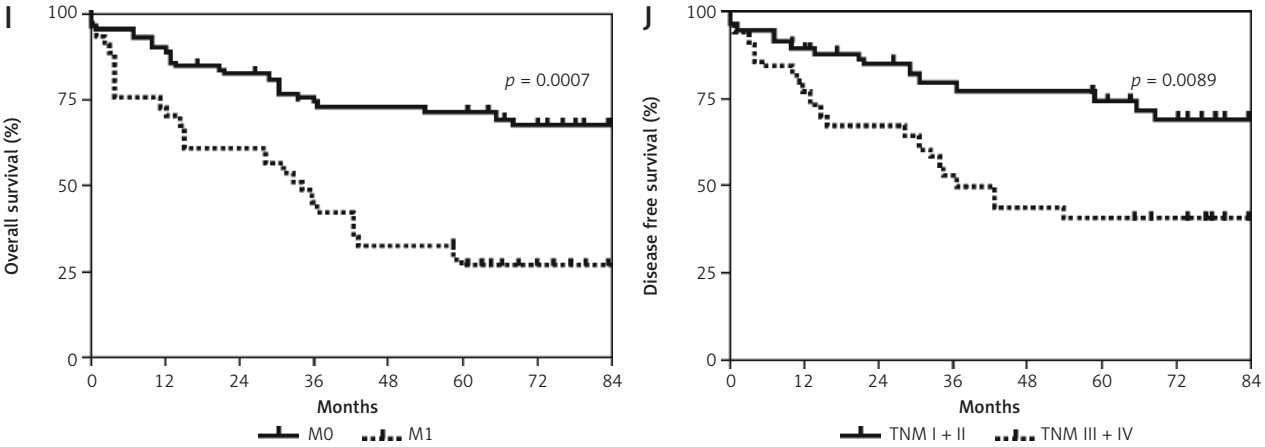


Fig. 3. Cont. Distant metastases (**I**), and tumour-node-metastasis stage (**J**). Significant *p*-values (< 0.05) for corresponding log-rank tests are given in bold

correlates with metastasis to the lymph node, higher tumour grade, negative p53 immunostaining, and higher Ki-67 index [29]. The same study revealed prognostic significance of POLD1 in breast cancer while its levels correlated with worse DFS and incidence of triple-negative tumours [29]. In addition, experiments carried out in triple-negative breast cancer cell line MDA-MB-231 demonstrated antiapoptotic properties of POLD1 overexpression in these cells, suggesting that POLD1 could be considered as a potential therapeutic target in breast cancer [30]. Microarray profiling of non-small cell lung cancer specimens revealed that altered *POLD1* expression can be a part of the signature associated with the development of lung cancer from chronic obstructive pulmonary disease. Elevated expression of POLD1 was shown to correlate with poor prognosis of lung adenocarcinoma [31]. Zhao *et al*. [32] applied bioinformatical analysis and IHC to investigate the role of Polδ family members in hepatocellular carcinoma, showing that elevated expression of POLD1 is associated with worse prognosis and development of an immunosuppressive tumour microenvironment. These findings were in line with another studies on hepatocellular carcinoma [33, 34], which documented significant associations between the expression levels of Polδ subunits, in particular POLD1, and patients' outcomes, tumour infiltration by immune cells, and genomic alterations of the tumour. While most studies suggest an oncogenic character of POLD1 in human malignancies, our previous study on clear cell renal cell carcinoma showed that higher levels of POLD1 protein were associated with longer OS of the patients. The association between the disease progression and decreased POLD1 immunoexpression was also found in a group of 300 papillary thyroid carcinoma patients [35]. The results of the above studies suggest that the potential oncogenic or tumour suppressive role of *POLD*1 and its prognostic or predictive significance can be a tumour-specific feature, rationalizing the present and future studies on POLD1 immunoexpression in various types of human cancers.

Conclusions

The present study was limited to immunohistochemical assay only due to routine application of this method in postoperative evaluation of patients with various neoplasms. Our analysis revealed that the immunoexpression of POLD1 is altered in CRC cells, but the protein levels did not correlate with the progression of the disease and patients' outcomes. This observation was supported by the analysis of *POLD1* mRNA expression in CRC that used TCGA datasets. While the genetic status of the POLD1 gene is being investigated for its prognostic or predictive significance in CRC, the results of our study do not support the usefulness of POLD1 expression levels as a prognostic marker in CRC.

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The authors declare no conflict of interest.

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cancers

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Review

Prospects of POLD1 in Human Cancers: A Review

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Simple Summary: Polymerase delta 1 (POLD1), a catalytic and proofreading subunit of the human DNA polymerase delta (Polδ), plays a vital role in maintaining the stability of the genome. In recent years, *POLD1* germline and somatic mutations, as well as gene-expression patterns, have been extensively studied in cancers. Nevertheless, the complex regulatory mechanisms of *POLD1* expression and their clinical relevance are yet to be elucidated. This review aims to summarize the current research status regarding the role of POLD1 in neoplastic processes.

Abstract: Cancer is the second leading cause of death globally, exceeded only by cardiovascular disease. Despite the introduction of several survival-prolonging treatment modalities, including targeted therapy and immunotherapy, the overall prognosis for the metastatic disease remains challenging. Therefore, the identification of new molecular biomarkers and therapeutic targets related to cancer diagnosis and prognosis is of paramount importance. DNA polymerase delta 1 (POLD1), a catalytic and proofreading subunit of the DNA polymerase δ complex, performs a crucial role in DNA replication and repair processes. Recently, germline and somatic mutations of the *POLD1* gene have been acknowledged in several malignancies. Moreover, diversified *POLD1* expression profiles have been reported in association with clinicopathological features in a variety of tumor types. With this review, we aim to summarize the current knowledge on the role of POLD1 in cancers. In addition, we discuss the future prospects and clinical applications of the assessment of *POLD1* mutation and expression patterns in tumors.

Keywords: DNA repair; polymerase delta; germline and somatic mutations; DNA replication

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1. Introduction

Within the next twenty years the number of new cancer cases is expected to nearly double globally [1]. In addition, the observed increase in incidence will presumably be paralleled by a rise in cancer-related deaths [1]. The projected increase is expected to be driven by both aging and the growth of the human population worldwide [1]. Despite the rapid progress in cancer diagnosis and treatment, when it is diagnosed at a metastatic stage the prognosis remains poor [2]. Therefore, the identification of novel molecular diagnostic, prognostic, and predictive biomarkers may be highly warranted.

The polymerase delta 1 (*POLD1*) gene encodes the major catalytic and proofreading subunit of the DNA polymerase δ (Polδ) holoenzyme [3]. It is crucial that *POLD1* expression is precisely regulated during the cell cycle [4]. With regard to the Polδ complex, it plays an essential role in DNA replication as it synthesizes the DNA at the lagging strand.

Furthermore, the important error-correcting ability provided by the exonuclease activity of this enzyme contributes significantly to replication fidelity [5]. In addition, Polδ is involved in DNA repair upon exposure to mutagens [6].

Both germline and somatic alterations in the proofreading domain of *POLD1* have been implicated in various diseases, including cancers [7]. Moreover, *POLD1* mutation is thought to be strongly associated with genomic instability, mutator phenotype, and tumorigenesis [7–9].

Although *POLD1* germline mutation was primarily linked to colorectal and endometrial cancers, more recent data have also found a connection between this mutation and an increased risk of many other tumor types [7,10]. Notably, two clinical conditions regarding the cancer-predisposing nature of the *POLD1* mutation have been mentioned in the literature: the first, defined as polymerase proofreading-associated polyposis [11] and the second, the recently described POLE/POLD1-associated tumor syndrome, which have expanded the spectrum of possible tumors affecting people with inherited DNA polymerase-related mutations [12]. Nonetheless, somatic mutations of the *POLD1* gene are inadequately studied in cancer [7]. Thus, they warrant further investigation to be appropriately addressed clinically and practically.

As for immunohistochemical POLD1 protein expression levels in malignancies, their correlation with clinicopathological characteristics has lately been confirmed [13–25]. Notably, the downregulation of *POLD1* expression also occurs under physiological conditions, such as replicative senescence to mediate aging [26]. Advancing our understanding of the *POLD1* expression patterns in various cancer types in relation to meaningful clinical and pathological parameters may facilitate the translation of research knowledge into clinical practice.

Past articles have thoroughly reviewed the role of POLD1 in tumors in 2016 [7,9]. Therefore, our aim is to summarize and update the current understanding of the *POLD1* mutations and expression changes in human cancers. Moreover, we address possible therapeutic implications and future challenges in *POLD1*-mutated cancers.

2. *POLD1* Gene and a Polymerase Delta Holoenzyme in Health

The DNA polymerase delta 1 catalytic subunit (*POLD1*) gene is located on chromosome 19 at q13.3-q13.4 [27,28]. The human *POLD1* gene is composed of 27 exons and 26 introns. It encodes a large 125-kDa protein known as the POLD1/p125 subunit, a catalytic and proofreading subunit of the human DNA polymerase delta (Polδ). The fluctuations of the *POLD1* promoter activity imply that the transcriptional mRNA and protein levels are regulated during the cell cycle [4]. The *POLD1* gene promoter is activated by Sp1, Sp3 transcription factors, cell cycle-related E2F1, and CCCTC-binding factor (CTCF) [29,30]. On the other hand, the p53 protein, the "genome guardian", and cyclin-dependent kinase inhibitor p21 are able to inhibit the Sp1-stimulated *POLD1* promoter activity, thus repressing *POLD1* gene expression [31,32].

Song et al. discovered that a cell cycle-dependent element (CDE)/cell cycle gene homology region (CHR) element—is an active functional element in the *POLD1* promoter, crucial for the cell cycle regulation of the *POLD1* gene [32]. They also showed that E2F1 and p21 regulate the *POLD1* promoter via binding to the CDE/CHR element [32]. Interestingly, p53-mediated inhibition of *POLD1* transcription seems to be indirect [33]. p53 does not directly bind to the CDE–CHR motifs. Instead, p53 triggers indirect p21-dependent *POLD1* downregulation via the p53–p21–DREAM–CDE/CHR pathway [33]. In addition, CTCF mainly binds to the *POLD1* promoter in site 3 and 4 regions [30]. It has been shown that these two regions play crucial roles in CTCF-mediated *POLD1* regulation [30]. Additionally, microRNA-155-mediated suppression of the forkhead transcription factor FOXO3a subsequently contributes to the decreased expression of *POLD1* at the mRNA and protein levels [34].

Moreover, *POLD1* expression has been causally linked to age-related cellular senescence and two POLD1-related mechanisms of aging have been proposed [26,30]. Gao et al.

showed that the attenuation of the binding affinity of E2F1 for the *POLD1* promoter, via age-related decline in E2F1 expression and increased CpG island 3 methylation in the *POLD1* promoter region, causes a reduction in *POLD1* expression in replicative senescence [26]. Hou et al. proposed, however, that an age-related decline in CTCF levels affects the binding affinity of CTFC to the *POLD1* promoter, thus driving down *POLD1* expression [30]. It is, therefore, well established that down-regulation of *POLD1* is a characteristic age-related change occurring in humans [26,30,35].

Human Polδ holoenzyme has been believed to be a heterotetramer in unstressed, non-dividing cells composed of a catalytic subunit (POLD1) and three accessory subunits, POLD2 (p50), POLD3 (p66/p68), and POLD4 (p12) [36]. However, it has recently been suggested that the POLD4 subunit may occur as a dimer in the human Polδ complex [37]. Therefore, human Polδ potentially exists under a pentameric structure [37]. Through the catalytic POLD1 subunit, in association with both replication factor C (RFC) and proliferating cell nuclear antigen (PCNA), Polδ plays a pivotal role in discontinuous DNA synthesis at the lagging strand during genome replication. Moreover, its 3′–5′ exonuclease domain provides an error-correcting activity during DNA synthesis [38]. It has also been shown that Polδ is required for break-induced telomere synthesis [39].

A pivotal role for POLD1 in maintaining the enzymatic function of the Polδ complex suggests that adequate cellular *POLD1* expression levels should be sustained for the smooth functioning of genomic DNA replication [40]. Novel data suggest that POLD1 is protected from degradation while being interconnected with the three different subunits in the Polδ complex, whereas excessive non-associated POLD1 is degraded to intercept the formation of incomplete and inactive Polδ units [40]. Moreover, results obtained in an experimental study using a murine model revealed the overall low levels of POLD1-3 subunits in POLD3-deficient cells [41].

Surprisingly, it has recently been shown that POLD1 plays a unique role in both the nucleus and the cytoplasm [42]. Subcellular fractionation has revealed that more than 60% of the POLD1 content occurs in the cytoplasm [42]. Regarding its cytoplasmic function, the POLD1 protein localized at the Golgi complex controls microtubule growth [42]. Shen et al. demonstrated that POLD1 undergoes bidirectional nucleocytoplasmic transport [43]. In addition, they also substantially unraveled the mechanisms underlying both the nuclear import and export of the POLD1 protein [43]. Figure 1 summarizes the mechanisms of *POLD1* gene regulation. Moreover, it outlines the physiological role of the POLD1 protein within the cytoplasm and nucleus.

Interestingly, during normal cell cycle progression, conversion of heterotetrameric Polδ (Polδ4) to the heterotrimer (Polδ3) takes place via POLD4/p12 subunit degradation [40,41]. It has been shown that POLD4 undergoes proteasomal degradation initiated by its polyubiquitination, in synchrony with the S phase [44,45]. Contrarily, no alteration in POLD1, POLD2, and POLD3 levels with the progression of the cell cycle was observed in Zhang et al.'s studies [45]. Although both Polδ4 and Polδ3 are active polymerases, Polδ3 seems to be the predominating form of human Polδ during the S phase of the cell cycle [6,45]. Moreover, a direct comparison of Polδ3 with Polδ4 has shown that the heterotrimeric complex displays a higher ratio of exonuclease to polymerase activity in vitro than four-subunit Polδ4 [6]. In addition, POLD4 degradation in response to DNA damage after the application of ultraviolet, methyl methanesulfonate, hydroxyurea, and aphidicolin, causes the concomitant conversion of Polδ4 to a heterotrimeric form [46]. Therefore, exposure to the agents causing DNA damage following Polδ4 to Polδ3 conversion suppresses DNA synthesis, while at the same time favoring proofreading activity under conditions of DNA replication stress.

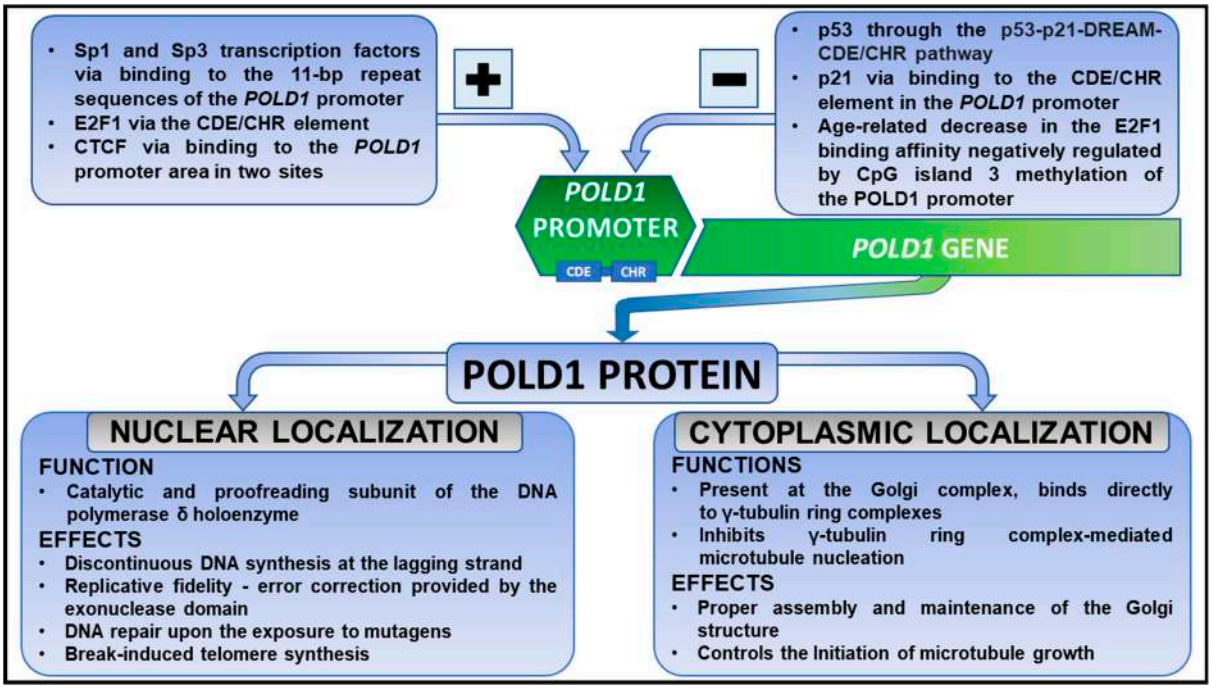


Figure 1. Regulation of *POLD1* gene expression within the cell and physiological roles of POLD1 depending on its cellular localization. The figure highlights positive and negative regulators of the *POLD1* promoter. Apart from well-known functions of POLD1 exhibited in the nucleus, recent studies have also noticed its role within the cytoplasm. bp, base pair; CDE/CHR, cycle-dependent element/cell cycle gene homology region; CTCF, CCCTC-binding factor; DREAM, dimerization partner, RB-like, E2F, and MuvB core complex.

3. *POLD1* Gene and a Polymerase Delta Holoenzyme in Non-Oncogenic Processes

Numerous studies have documented the physiological role of both Polδ holoenzyme and POLD1 protein. As they are involved in multiple biological processes, including DNA replication and DNA repair, a growing body of studies in recent years has highlighted the role of germline and somatic mutations in *POLD1–POLD4* genes in the genesis of human pathologies. Mandibular hypoplasia, deafness, progeroid features, and progressive lipodystrophy (MDPL) syndrome is a rare autosomal dominant disorder caused by heterozygous de novo mutations in the *POLD1* gene [47,48]. Notably, exome sequencing revealed that mutations in different POLD1 domains may lead to a major phenotypic variability in MDPL syndrome [48]. Recent data have also revealed that *POLD1* mutations causing reduced Polδ polymerase activity with a maintained exonuclease activity can lead to a non-syndromic sensorineural hearing loss in an autosomal recessive manner [49]. In addition, several cases of autosomal recessive *POLD1*-linked combined immunodeficiency have been reported [50–52]. Additionally, Nichols-Vinueza et al. described a potential role for POLD1 in B-cell maturation [52].

4. POLD1 in Cancers

Knowing that Polδ is crucial in genome maintenance and is involved in the DNA proofreading processes, it is not surprising that mutations in the *POLD1* gene have been linked to genomic instability, mutator phenotype, and carcinogenesis [7–9]. In this paragraph, we aim to discuss the influence of both germline and somatic *POLD1* mutations on different malignancies. Moreover, the changes in the *POLD1* expression level in relation to clinicopathological parameters will be addressed.

4.1. Colorectal Cancer

Colorectal cancer (CRC) is the third most frequently diagnosed cancer worldwide but is second in terms of mortality [1]. Almost a third of all CRC cases have a familial component, however, only about 5% of these develop as a consequence of well-defined inherited syndromes, whereas the majority of them do not possess a known genetic background [53,54]. To date, colorectal cancer (CRC) seems to be the most extensively studied malignancy concerning *POLD1* mutations.

Among patients with a family history of CRC and multiple or large colorectal polyps, a minor fraction of carriers of *POLD1* heterozygous germline mutations can be found [55,56]. Regarding somatic pathogenic variants affecting *POLD1* in CRC, they are thought to be extremely rare findings [57]. Unfortunately, the overall incidence of *POLD1* mutation in CRC patients remains unknown. Since the first description in 2013 of a correlation between the *POLD1* mutation and dominantly inherited intestinal adenomas and carcinomas [55], two terms regarding this phenomenon have been coined. Originally, this syndrome was described as "polymerase proofreading-associated polyposis" [11], however, the term "POLE/POLD1-associated tumor syndrome" was recently proposed instead [12], as among some patients with a germline mutation in DNA polymerase subunits *POLE* and/or *POLD1*, expanded extracolonic tumor spectrum and absence of colonic polyposis have been noted. Interestingly, tumors with *POLE*/*POLD1* mutations were found to be hypermutated, chromosomally unstable, and, surprisingly, microsatellite stable [55]. Moreover, recent data suggested the occurrence of an abundance of tumor-infiltrating lymphocytes (TILs) in *POLE*/*POLD1*-mutant CRCs [58]. In general, lymphocytes located in the tumor tissue region have emerged as the activators of a patient's pre-existing intratumoral immunity after exposure to ICI treatment [58].

Unfortunately, data regarding *POLE*/*POLD1* status to predict the efficacy of immunotherapy in CRC treatment are scarce, thus conducting research in this field would be reasonable.

Recently, Siraj et al. investigated the correlation of immunohistochemical POLD1 protein expression with clinicopathological features in Middle Eastern CRCs [13]. They observed that low *POLD1* expression, found in more than half of CRC cases, was correlated with larger tumor size, adenocarcinoma histology, and stage III tumors [13]. Moreover, in our study focused on patients with CRC using IHC analysis, lower *POLD1* nuclear expression in CRC cells compared with normal epithelial colon cells was observed. *POLD1* expression levels in the tumor cells did not correlate with clinicopathological factors and the prognosis of CRC patients (data not published yet).

In Figure 2, we present representative immunohistochemical staining patterns of POLD1 within healthy, benign polypoid, and malignant tissues.

4.2. Endometrial Cancer

With its incidence continuing to rise, endometrial cancer (EC) is the most common gynecological malignancy in developed countries [1,59]. Although the *POLD1* mutations in general are very scarce in EC [14,60], it is now well established that *POLD1* germline mutations predispose people to EC [11,14]. Presumably, the frequency of *POLD1* mutations in EC may be population dependent [60,61]. Among 47 South East Asian women with grade 3 (according to FIGO grades) endometrioid ECs, a presence of two pathogenic germline *POLD1* mutations was detected [61]. Whereas Church et al.'s data revealed only one germline *POLD1*-mutated endometrioid EC among 154 patients with different FIGO grades enrolled in a British study [60].

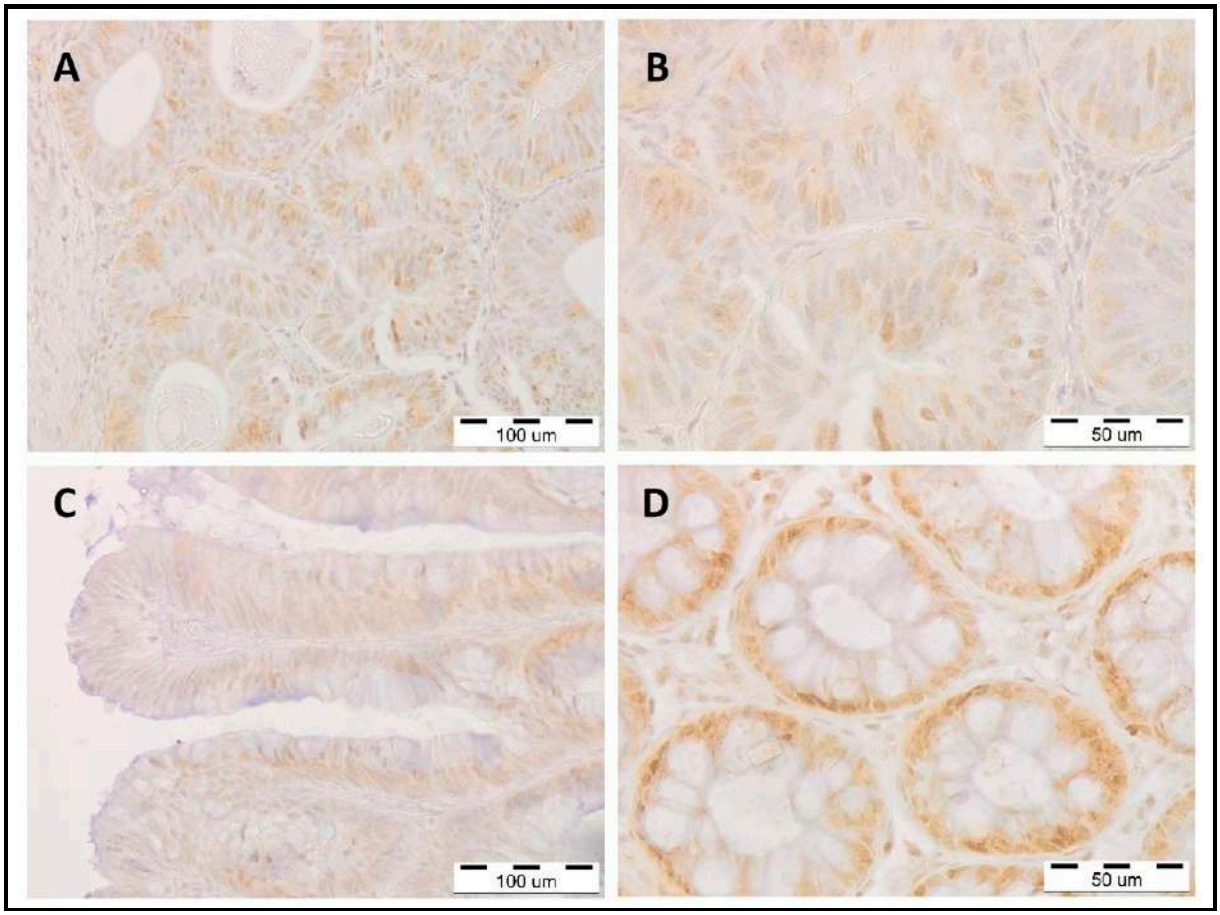


Figure 2. Immunohistochemical staining against POLD1 within tissues of the human large intestine. (**A**,**B**) Represent *POLD1* expression in the colon cancer tissue; (**C**) *POLD1* expression in villous adenomas; (**D**) *POLD1* expression in epithelial cells of normal mucosa of the large intestine, view of the transverse section of intestinal crypts. All sections were stained using immunohistochemical methods: primary antibody—rabbit anti-human antibody against POLD1 (HPA046524; Sigma-Aldrich, St. Louis, MO, USA), next—secondary antibody HRP-conjugated (ready-to-use dilution; ImmPRESS Universal reagent Anti-Mouse/Rabbit Ig, Vector Laboratories). Finally, the sections were stained in diaminobenzidine (DAB; Dako, Glostrup, Denmark) and counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO, USA). The magnification was (**A**,**C**) 200× and (**B**,**D**) 400×; particular bars are labeled in each photo.

Moreover, EC was found to be the most common malignancy among female *POLD1* germline mutation carriers [62]. Palles et al.'s studies revealed that EC is the most frequent extraintestinal cancer in polymerase proofreading-associated polyposis syndrome [62]. Surprisingly, one recent study on Chinese genomic cancer population data suggests that EC may be the most common malignancy with *POLD1* mutations among all cancers [63].

Even though *POLD1*-mutated cancers are believed to be primarily microsatellite stable [55], some ECs and CRCs possess microsatellite instability (MSI) [63–67]. Notably, Haraldsdottir et al. have suggested that the deficient DNA mismatch repair system in endometrial and colorectal cancers resulting in MSI phenotype, may occur mainly in tumors with somatic rather than germline *POLD1*/*POLE* mutations [64]. In addition, one study that assessed an endometrial dedifferentiated carcinoma molecular profile suggested that *POLD1* and *MLH1* (a gene involved in DNA mismatch repair), as well as high microsatellite instability (MSI-H) and increased tumor mutation burden (TMB), may be peculiar to this rare distinct subtype of EC [68].

As it is widely known that patients with MSI-H tumors can benefit from immunotherapy [69], routine clinical genetic testing for both *POLD1*/*POLE* and MSI-related mutations may help identify patients with endometrial and colorectal cancers who could respond well to immunotherapy [70]. To date, only one study evaluated POLD1 protein immunohistochemical expression in EC and its association with the clinicopathological profile [14]. Low expression of *POLD1* was noted in almost 60% of Saudi EC cases and significantly correlated with grade 1 tumors [14].

Nevertheless, conducting research to establish both prognostic and predictive implications of *POLD1* mutation and/or POLD1 protein expression for EC patients is justified.

4.3. Renal Cancer

Clear cell renal cell carcinoma (ccRCC) is the most common histopathological subtype of renal cancer, accounting for up to 85% of all kidney cancers [71]. Current studies have highlighted the role of DNA damage repair pathways in ccRCC. Two research groups have recently analyzed DNA polymerase epsilon and delta and their possible prognostic significance for patients with ccRCC [15,72]. Our team was the first to report that, unexpectedly, POLD1 nuclear protein expression levels correlated with better prognosis in patients with ccRCC [15]. Moreover, we have also demonstrated that the level of POLD1 immunoexpression in tumor cells does not correlate with the demographic and clinicopathological characteristics of ccRCC patients [15]. This unanticipated data demonstrating the positive correlation of POLD1 nuclear immunoreactivity with longer overall survival among ccRCC patients, in contrast to several reports regarding other malignancies, may suggest that the potential role of POLD1 can be considered as cancer specific [14,17].

Eventually, subsequent molecular studies will be crucial to clarify the role of POLD1 in ccRCC tumorigenesis.

4.4. Liver Cancer

Hepatocellular carcinoma (HCC), accounting for 75–85% of primary liver cancers [1], is the second most lethal malignancy after pancreatic cancer, with a 5-year relative sur-vival of 18% [59]. The role of POLD1 in HCC was first presented in 2010 [16]. Sanefuji et al. demonstrated that *POLD1* overexpression induced by tp53 mutation correlates with a high degree of vascular invasion, poor cellular differentiation, and a poorer prognosis in HCC [16]. They have also suggested that POLD1 may contribute to cancer cell dedifferentiation in human HCC [16]. Furthermore, apoptosis of HCC cells was shown to be related to the inhibition of *POLD1* gene expression [73]. Similarly, apoptosis of triple-negative breast cancer (BC) cells was driven by the reduction in *POLD1* expression [74].

Recently, Tang et al. conducted a thorough analysis of the predictive and prognostic value of POLD1 in HCC [17]. It was shown that *POLD1* is significantly upregulated in HCC in comparison with adjacent healthy liver tissue [17]. Additionally, *POLD1* overexpression correlated with increased levels of alpha-fetoprotein, high TNM stage (III and IV), and predicted worse prognosis in HCC [17]. In addition, aberrantly high expression of *POLD1* in HCC tissue was demonstrated to be caused by DNA copy number gain, low *POLD1* methylation, and downregulation of small noncoding miR-139-3p [17]. Lastly, *POLD1* expression positively correlated with the immune infiltration levels of B cells, macrophages, dendritic cells, and CD4+ T cells in the HCC tumor microenvironment [17]. Moreover, an in vivo orthotopic liver injection model revealed that POLD1 knockdown in HCC cells diminished tumor incidence, size, and lung metastases in mice [75].

Taken together, an in-depth exploration of HCC tumorigenesis can aid in the development of novel clinical treatment modalities.

4.5. Breast Cancer

With an estimated 2.3 million new cases in 2020, breast cancer is the current leading cause of global cancer incidence, constituting 11.7% of all cancers diagnosed worldwide [1]. Patients harboring germline *POLD1* mutations are at higher risk of developing BC com-

pared with the general population [76,77]. Somatic mutations, although insufficiently documented, have also been reported in BC [78,79]. Furthermore, even though BC cases are infrequently associated with MSI [80], this particular group of tumors may be *POLD1*-mutated as well [81].

Research conducted by Zhang et al. highlighted the mechanisms underlying changes in *POLD1* expression in BC [18]. They have shown that *POLD1* expression and the methylation level of the *POLD1* promoter are both increased in BC tissues and cell lines [18]. Moreover, *POLD1* expression and the methylation of the *POLD1* gene promoter were found to be inhibited by the p53 protein through the suppression of DNA methyltransferase 1 and Sp1 activities in BC [18]. In the following studies, they observed that sirtuin 1, a member of the sirtuin family, enhances proliferative, migratory, and invasive properties of BC cells in vitro by inhibiting the p53 protein expression and, therefore, promoting the *POLD1* upregulation [82].

To date, data regarding the expression patterns and clinical value of *POLD1* among patients with BC are scarce. However, novel studies conducted by Qin et al. revealed that increased *POLD1* expression is linked to poor prognosis in BC [19]. Elevated POLD1 levels were related to lymph node metastasis, histological grade, p53 status, and the ki-67 index [19]. Moreover, survival analysis showed that BC patients with high POLD1 levels had poorer disease-free survival in comparison with patients with low *POLD1* expression [19]. In addition, *POLD1* status likely affects the treatment response, especially in triple-negative breast cancer [83].

Thus, these data suggest that the POLD1 protein can be considered as a potential prognostic biomarker in BC [19].

4.6. Other Cancers

Although *POLD1* mutations in cancers seem to be understudied, they may serve as promising prognostic/predictive biomarkers in the future and, importantly, future targets for novel POLD1-oriented therapies. Nevertheless, apart from malignancies mentioned in the previous paragraphs, several different cancers have been reported to harbor *POLD1* mutation as well. Wang et al.'s studies that aimed to assess the clinical benefits of immune-checkpoint inhibitor (ICI) treatment have estimated that across almost 50,000 patients with different cancer types, *POLD1* mutation occurred in 1.37% of cases [10].

For instance, the overall frequency of *POLD1* mutations was estimated to be 2.5% among Chinese patients with lung cancer [84]. Furthermore, a recent analysis revealed that POLD1 is associated with the development of non-small cell lung cancer (NSCLC) from chronic obstructive pulmonary disease [20]. Interestingly, high expression levels of *POLD1* indicated poor prognosis in lung adenocarcinoma but not in squamous cell carcinoma [20]. Additionally, in both mesothelioma, a rare and deadly cancer, and esophageal squamous cell carcinoma, POLD1 has been found to play a significant role in resistance to platinum-based chemotherapy [85,86].

In papillary thyroid cancer, low *POLD1* expression implies poor clinicopathological characteristics [21]. Recently, POLD1 protein level was shown to be negatively correlated with the expression of cadherin-16 in this cancer [87].

In gastric adenocarcinoma, *POLD1* mutation occurred in 2.77% of 613 patients [88]. Zhu et al. have also shown that using immunotherapy in *POLE*/*POLD1*-mutated tumors may have potentially positive implications [88].

Comprehensive molecular profiling has identified the presence of *POLD1* mutations in brain tumors [62,89–91]. Additionally, *POLD1* expression in this heterogeneous group of neoplasms was only described in 1p19q co-deleted lower-grade gliomas [24]. Yet, the clinical significance of *POLD1* mutations and changes in the POLD1 protein level in central nervous system tumors is not fully understood.

Studies concerning uveal melanoma, the most common primary intraocular malignancy in adults, have discussed the changes in *POLD1* expression patterns within patients with this cancer [22,23].

Much research has been conducted to investigate the possible clinical significance of POLD1 in gynecologic malignancies. Besides endometrial carcinoma, cervical and ovarian cancers were also assessed for the importance of *POLD1* mutations and expression changes [92,93]. Interestingly, the *POLD1* gene was more frequently altered among Chinese women with locally advanced cervical cancer than in Caucasian patients [92]. Furthermore, using cisplatin-resistant and cisplatin-sensitive ovarian cancer cell lines, Xing et al. demonstrated that *POLD1* was highly expressed in the platinum-sensitive group, whereas chemoresistant cell lines possessed a reduced expression of *POLD1* [93].

Intriguingly, in acute lymphoblastic leukemia (ALL), *POLD1* upregulation may promote the relapse of this disease [25]. Thus, its expression could be proposed as the potential diagnostic marker and therapeutic target for the treatment of relapsed ALL [25].

A summary of studies discussing the relationship of *POLD1* expression level with clinicopathological characteristics was provided in Table 1.

Table 1. Changes in *POLD1* expression levels and their implications in different types of cancer.

Type of Cancer	Number of Patients	*POLD1* Status	Stage/ Grade	Detection Method	Clinicopathological Relevance	Ref.
Colorectal cancer	1069	Low	I–IV	IHC	• Low protein expression had a significant association with adenocarcinoma histology, larger tumor size, and stage III tumors	[13]
Endometrial cancer	419	Low	I–IV; mainly type I EC (88.2%)	IHC	• Low protein expression had a significant association with grade 1 tumors and a trend toward type I EC	[14]
Clear cell renal cell carcinoma	56	High	I–III; mainly lower grades (G1–G2 in 73.2%)	IHC	• POLD1 protein expression levels in the tumor cells did not correlate with clinicopathological data • Strong POLD1 protein nuclear immunoexpression in cancer cells correlated with better prognosis	[15]
Hepatocellular carcinoma	339	High	I–IV; mainly stage I and II tumors (50.1% and 24.8%, respectively)	mRNA data retrieved from the GEPIA2 database IHC	• High *POLD1* expression was significantly correlated with increased levels of alpha-fetoprotein and advanced TNM stage • *POLD1* upregulation was an independent indicator of poor OS • DNA copy gain, low *POLD1* methylation, and downregulation of miR-139-3p may cause *POLD1* high expression • *POLD1* expression correlated with the immune infiltration levels of B cells, macrophages, dendritic cells, and CD4+T cells	[17]
Breast cancer	84	High	G1–G3; invasive breast cancer treated with radical mastectomy	RT-qPCR Western blot	• Increased *POLD1* gene expression was significantly associated with lymph node metastasis, histological grade, p53 status, and ki-67 index • High *POLD1* gene expression was associated with poor DFS	[19]
Lung cancer	–	High	I–IV	RT-qPCR	• Gradually higher *POLD1* gene expression was associated with the gradually worse prognosis of lung adenocarcinoma	[20]

Table 1. *Cont.*

Type of Cancer	Number of Patients	*POLD1* Status	Stage/ Grade	Detection Method	Clinicopathological Relevance	Ref.
Papillary thyroid cancer	286 (females in 77.7%)	Low	I–IV; mainly stage I tumors (68.3%)	IHC	• Decreased POLD1 protein expression was significantly associated with the follicular variant of PTC, distant metastasis, and stage IV tumors	[21]
Lower-grade gliomas with 1p19q codeletion	280	Low	WHO grade II–III	RT-qPCR Western blot	• Lower expression of the *POLD1* gene was associated with longer PFS and OS in patients who received chemotherapy	[24]
Childhood Acute lymphoblastic leukemia	90	High	–	–	• In 90 matched diagnosis and relapse pairs of ALL bone marrow samples, *POLD1* expression was significantly upregulated in relapsed ALL in comparison to the newly diagnosed ALL • *POLD1* upregulation was miR-520H mediated	[25]

5. Conclusions and Future Directions

With cancer incidence rates continuing to rise globally, there is a strong need for the development of new biomarkers and treatment strategies [1]. The genetic mutations and expression profiles in *POLD1*-mutated cancers have recently gained remarkable attention. Nevertheless, the complex regulatory mechanisms of *POLD1* expression in cancers remain to be fully characterized.

Both *POLD1* germline and somatic mutations within the proofreading domain are considered rare in a variety of malignancies [9,10,63]. Additionally, the presence of pathogenic *POLD1* germline alterations has been linked with some hereditary tumors, such as colorectal and endometrial cancers [55].

Based on the primary role of Polδ in genome maintenance, mutations in the catalytic *POLD1* subunit lead to genomic instability, mutator phenotype, and malignant transformation [7,9]. Missense mutations are believed to be the most common mutations in *POLD1*-associated tumors, and no mutation hotspots have been identified so far [70,84].

It should be noted that *POLD1* has been considered haploinsufficient in the literature [9,47,55]. Contrarily, a *POLD1* haplosufficiency has been recently revealed in the yeast model [94] and in germline monoallelic *POLD1* alteration carriers [95,96]. It has been speculated that a solitary heterozygous *POLD1* mutation in the exonuclease domain without concurrent mutations in other repair systems causes only a modest increase in the mutation rate [95]. In addition, Schamschula et al. suggested that *POLD1*-mutated cancers acquire the ultra-high TMB only with concurrent mismatch repair deficiency [66]. It is hypothesized that there are no cancers with an exclusive heterozygous *POLD1* mutation and only a complete loss of the two *POLD1* alleles can lead to cancer [95]. Therefore, both germline and somatic *POLD1* mutations triggering tumor formation may turn out to be biallelic [95]. Nevertheless, an attempt to organize the current knowledge and deepen understanding of POLD1 biology in carcinogenesis would be of paramount importance and could pave the way for novel anti-cancer drugs.

Whereas an overwhelming number of studies in the last decades have focused on *POLD1* pathogenic variants in a wide range of neoplasms, only a few studies have validated the prognostic and predictive utility of POLD1 mRNA and protein expression in human cancers to date. So far, *POLD1* expression and its clinical significance have been investigated in colorectal, endometrial, renal, liver, breast, lung, and thyroid cancers [13–15,17,19–21]. Nevertheless, these data are mainly limited to single-institution experiences.

In recent years, various immunotherapy-based cancer treatments have been approved for clinical use and many additional drugs are still being developed, thus opening a new

era in cancer treatment [97,98]. Moreover, ICI treatment is considered a new standard of care across many cancer indications [97]. There are several biomarkers for predicting the outcome of ICI treatment, among which expression of programmed death-ligand 1 (PD-L1), MSI status, and TMB are the most commonly used [63,97]. Moreover, high levels of TILs (especially the CD8+ T cells subpopulation [99]), common in MSI cancers, may predict the response to ICI treatment in various malignancies [100]. As some ICI recipients fail to respond to the treatment, it is therefore crucial to identify accessory molecular biomarkers for predicting the ICI response to prevent immunotherapy overtreatment [98–102].

Some reports have suggested that *POLE*/*POLD1* mutations and/or *POLE*/*POLD1* expression patterns may be useful to predict immunotherapy effectiveness [10,70]. Previous studies investigating the association of *POLE*/*POLD1* mutation with MSI status in various cancers have shown ambiguous results. Although cancers harboring *POLD1* mutation are believed to be primarily microsatellite stable, some of them may display an MSI-H phenotype [55,63–68]. Wang et al.'s study on a cohort of almost 50,000 patients with different solid cancer types assessed the impact of *POLE*/*POLD1* mutations on immunotherapy outcomes [10]. Their analysis demonstrated that among ICI-treated cancer individuals, patients with either *POLE* or *POLD1* mutations have significantly longer overall survival in comparison with those without [10]. Notably, the ICI treatment outcomes were similar, regardless of whether these mutations were located within the exonuclease domain or outside of it [10]. Regrettably, *POLE*/*POLD1* alterations cannot be independent factors for predicting the benefit from immunotherapy [101]. In addition, a study conducted by He et al. suggested that *POLE*/*POLD1* mutations in the proofreading domain may result in DNA repair defects and an exceptionally high TMB, thereby generating high neoantigen load levels that, in turn, positively correlate with the sensitivity to ICI treatment [63]. Moreover, tumors expressing higher numbers of neoantigens are associated with a better response to immunotherapy in patients with CRC, melanoma, and NSCLC [63]. However, little is known about the influence of *POLD1* expression level on the efficacy of ICI in cancer treatment.

In the last decade, whole-genome sequencing identified specific mutational signatures that describe the patterns of mutations that arise during tumorigenesis [103,104]. Several single-base substitution (SBS) signatures have been described concerning *POLD1*-mutated tumors; SBS10c and SBS10d are linked to defective POLD1 proofreading [105], whereas SBS20 is associated with concurrent *POLD1* mutations and mismatch repair deficiency [65]. Groundbreaking studies have shown that the presence of specific SBS signatures may have prognostic and therapeutic implications [106]. Notably, Ma et al. reported that *POLD1* functional mutations generating SBS signatures associated with POLD1 activity lead to augmented immunogenicity [107]. Moreover, patients with cancers generating *POLD1*-mutation-related SBS signatures demonstrated an enhanced response to ICI treatment [107].

Given the variety of immuno-oncology treatments currently in use for therapies in patients with different cancer types, including melanoma, NSCLC, CRC, and HCC [97,108], novel research examining the relationship between the expression of the POLD1 protein and the clinical outcomes in patients receiving immunotherapy would be of great importance. Several clinical trials are ongoing to evaluate the impact of *POLD1* mutation on treatment outcomes (NCT05103969, NCT03810339, NCT03428802, NCT03491345, NCT04969029).

With the advancement of knowledge regarding cancer biology, substantial progress in the development of new diagnostic and prognostic molecular biomarkers as well as targeted therapeutic strategies against oncological diseases can be expected within the next few years.

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Review paper

Cancer in a polyp of the large intestine – an interdisciplinary decision problem

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Abstract

Large intestine polyps are commonly found during colonoscopies. Pedunculated colon polyps can be totally removed using an endoscopic invasive technique. A problem arises when the pendulated polyp contains cancerous infiltration. The aim of the article was a presentation of the clinical decision process concerned with the presence of cancer invasion tissue within colorectal polyps. Review of literature source and presentation of histological sample photography. A correct interpretation of the pathomorphological protocol is crucial for the therapeutic decision, which should be consistent with the actual recommendations of gastroenterological societies. Local treatment is considered as complete when the adenocarcinoma is well or moderately differentiated without any microinvasion of blood and lymphatic vessels and the resection margin is more than 1 mm from the cancer tissue infiltration. In the contemporary clinical practice patients with a colon polyp require rational clinical decisions, which are based on the actual recommendations.

Introduction

The current secondary prevention of colorectal cancer involves colonoscopy examinations. During this examination, the mucosa of large intestinal wall is controlled, and their condition is assessed, especially the potential presence of polypoid or neoplastic lesions. Endoscopic biopsy is performed when the mucosa is affected by inflammation, or when polypoid lesions, adenomas, or tumours are present. Pendulated polyps of the colon or rectum can be removed totally using an endoscopic invasive technique. Then, the histopathological examination evaluates potential dysplastic or neoplastic tissue presence in such endoscopically resected polyps. The infiltration of pathological tissue affects parts of the polyp's structure, and the clinician must correctly interpret the pathomorphology report to assess the stage of the tumour and prepare the final treatment decision. These decisions vary from referral patient observations by endoscopy monitoring to classifying the patient for a surgical procedure – segmental resection of the large intestines. This implies that the decision should be rational, consistent with the current treatment recommendations and the principles of *evidence-based medicine*.

Discussion

The normal epithelium of the large intestines is the simple columnar epithelium. It consists mainly of absorptive cells and Goblet cells. The columnar absorptive cells have distinct differentiation of the surface part. The apical surface of the cell is multiplied by the microvilli and adapted to absorb water and the other substances while the lateral surfaces ensure further transport of water and the other substances to the intercellular space; moreover, via the intercellular junctions, they form a dense and regular arrangement of the epithelial cells. The Goblet cells are unicellular glands that produce and secrete the mucus, which is a protective and lubricant layer covering the mucosal surface.

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The mucosa lining the internal lumen of the intestine is arranged in the intestinal crypts, which are deep indentations of the epithelium, forming simple tubular glands. The epithelium lines on the lamina propria which is composed of loose connective tissue with blood and lymphatic vessels and a thin layer of smooth muscles- the muscularis mucosae [1].

The structural distortion of the mucosa that is seen in an endoscopic examination has a tissue mass shape called a polyp. Depending on the shape and presence of the connecting pedicle, the polyp is called pedunculated, semi-pedunculated, sessile, or flat, respectively. Proliferations of the intestinal epithelium, in which produce cancer development, originate from adenomas or serrated lesions [2].

Initially, because of the adenomatous polyposis coli (*APC*) gene mutation and the occurrence of subsequent mutations (of the suppressor genes and protooncogenes), uncontrolled proliferation of epithelial cells takes place. Within the mucosa, proliferating cells form adenomatous lesions. A different degree of distortions of cellular morphology is found, including variations in the shape and size of the nuclei. The regular arrangement and normal structure of the glandular crypts are disturbed by lesions, and the sum of these morphological changes is called as dysplasia or intraepithelial hyperplasia. The severity of mucosal distortions is referred to as low-grade or high-grade dysplasia. Depending on the surface shape of the polypoid structures as development of the glandular tubules or visible villi, they are divided into 3 subtypes: tubular adenomas, villous adenomas, or tubulovillous adenomas, depending on the predominant component. These polypoid lesions have a potential for neoplastic transformation in the adenoma-cancer sequence [3].

The mutations in the mutator genes group, mainly *MSH2* and *MLH1,* result in the development of serrated lesions. This name originates from a microscopic image – the epithelium has different heights of cells which form the glands, and these lumen resemble the teeth of a saw [3].

The probability of a malignant neoplasm significantly increases with larger polyps and the presence of a villous component. The presence of adenoma with the following criteria: size ≥ 1 cm and/or high-grade dysplasia and/or the villous formation ≥ 20%, is considered as high risk of cancer development [4].

The polyps can be single, but in genetically determined alternations they can be multiple or even uncountable when the internal surface of the large intestine is entirely lined by them.

Today, colonoscopy is carried out as a diagnostic investigation in patients with specific symptoms: pain in the abdominal cavity, bleeding from the lower gastrointestinal tract or faecal occult blood, hypochromic anaemia, changes in the consistency of faeces, changes in the frequency of stools, and disturbances of the gastrointestinal transit time.

Furthermore, since 2000 in Poland, colonoscopy has been a part of colorectal cancer prevention to facilitate the early diagnosis of this neoplasm. This is carried out within the screening program, under the invitation (for people aged 55–64 years) and without invitation (upon patient's request) [5].

If any abnormalities in the large intestine mucosa (inflammation, polyp, and neoplasm) are detected, tissue samples are collected due to the histopathological evaluation. The polyp should be collected whole for a further histopathological verification if it is not too large for the endoscopic loop.

Colorectal polyps detected during colonoscopies performed under the screening programs are a common finding and the incidence is 19.4%, including adenomas which were detected in 6.4% as well as colorectal cancers – 0.9%. Cancers were diagnosed mostly in early-stage, I° – 47.3% [6].

The incidence of colorectal polyps as well as the risk of cancer increase with the age of the persons recruited to the endoscopy screening [6]; therefore, the correct evaluation of a polyp and potential malignant colorectal polyp is very important. The rules of the protocol in the case of colon polyps were developed by the European Commission and published in a monography in 2020. They were later adopted as recommendations of the National Societies for Gastroenterology [4, 7].

Depending on the number of polyps, their size (less than 1 cm, 1–2 cm, and bigger than 2 cm), confirmation of adenoma and/or high-grade dysplasia and/or villous arrangement, the patient is qualified to the following groups: high, average, and low risk of neoplastic transformation and the potential development of colorectal cancer. The appropriate colonoscopy follow-up examinations are recommended, respectively, at 1-, 3-, and 10-year intervals. In the case of large polyps that can be resected in parts by using the "bite-by-bite" endoscopic polypectomy technique, the resection site should be tattooed, and the surgical scar should be examined after 2–3 months [7] (Table I).

In clinical practice, the problem with the decision occurs when the pendulated polyps has a cancerous infiltration. A proper interpretation of such a finding is critical for the course of the patient's case.

According to the pathological assessment, the stage of a neoplasm is evaluated using the 7th edition of TNM AJCC/UICC. The infiltration limited to the mucosa (intraepithelial) is assigned as pTis stage, whereas the in-

filtration of cancer into the lamina propria (including the muscularis mucosae) and deep penetration into the submucosa is considered as pT1, the first stage of invasive cancer. The head and stalk of the polyp are composed from the mucosa and the submucosa, and due to this the infiltration which is limited only to the polyp structure, without penetration into the wall of the large intestine, could be estimated as pTis or pT1 stage, respectively.

In pathomorphology analysis, the depth of cancerous infiltration within the pedunculated polyp is assessed using the Haggitt classification [8] (Figure 1). Haggitt level 4 is reserved for the infiltration of cancer below the polyp stalk, or it refers to squat polyps (Table II).

The submucosal infiltration of the intestinal wall is a starting point of the Kikuchi classification, which is used to assess squat, sessile, and semi-sessile polyps [9] (Table III).

After resection of the polyp, the local cancer treatment is considered as completed when all the following conditions are met: the presence of well or moderately differentiated tubular adenoma (malignancy stage G1 or G2), without vascular micro-invasion, and resection margin > 1 mm from the cancer infiltration. When the above-mentioned criteria are met, the patient should be examined after 3 and 6 months and then redirected for further colonoscopy monitoring at 1-year intervals [4, 7].

The resection site of a malignant polyp (scar) should be tattooed directly during colonoscopy or 7–14 days after the first resection date. A tattoo is extremely helpful in finding the site during a possible surgical procedure or further endoscopy follow-up [10].

The strongest parameters indicating that the neoplastic tissue has been left after polypectomy and subsequent risk of local recurrence is high, at > 20%, (each of the points 1–3) and 8–15% (point 4) are: 1) resection

Table I. Eligibility of the patients for the risk groups (points 1-3) and respective recommendations for the endoscopic follow-up, according to the recommendations of the Polish Society of Gastroenterology [7]

Risk group	Number of polyps	Polyp size	Next colonoscopy
1. Low risk	1–2 tubular adenomas; low-grade dysplasia	Smaller than 10 mm	Without supervision, the next prophylactic colonoscopy in 10 years
2. Average risk	3–4 tubular adenomas	Smaller than 10 mm	Examination in 3 years
	1 adenoma or more with high-grade dysplasia and/or with villous component		
	1 adenoma or more	10–20 mm	
3. High risk	5 adenomas or more	Smaller than 10 mm	After 1 year [7]
	1 adenoma or more	Over 20 mm	
"Bite-by-bite" polypectomy of sessile and flat polyps			After 2–3 months
Malignant colorectal polyp (when the criteria for oncological endoscopic resection are met)			First examination after 3 months, the next after 6 months, and the following examinations after 12–24 months [10]

Table II. The Haggitt classification for the assessment of cancerous infiltration within the pedunculated polyp [8]

1	Carcinoma invading through the muscularis mucosae into the submucosa, but limited to the head of the polyp (above the junction between the adenoma and its stalk)
2	Carcinoma invading to the level of the neck of the adenoma (junction between adenoma and its stalk)
3	Carcinoma invading any part of the stalk
4	Carcinoma invading into the submucosa of the bowel wall below the stalk of the polyp but above the muscularis propria

Table III. The modified Kikuchi classification, used for sessile polyps to stage cancer infiltration in the submucosa of the large intestine wall [9, 10]

sm1	Slight submucosal invasion from muscularis mucosa to the depth of 200 to 300 µm	
	Sm1a	Less than a quarter of the width of the tumour invading the submucosa
	Sm1b	Between a quarter and a half of the width of the tumour invading the submucosa
	Sm 1c	More than a half of the width of the tumour invading the submucosa
sm2	Intermediate invasion	
sm3	Carcinoma invasion near the inner surface of the muscularis propria	

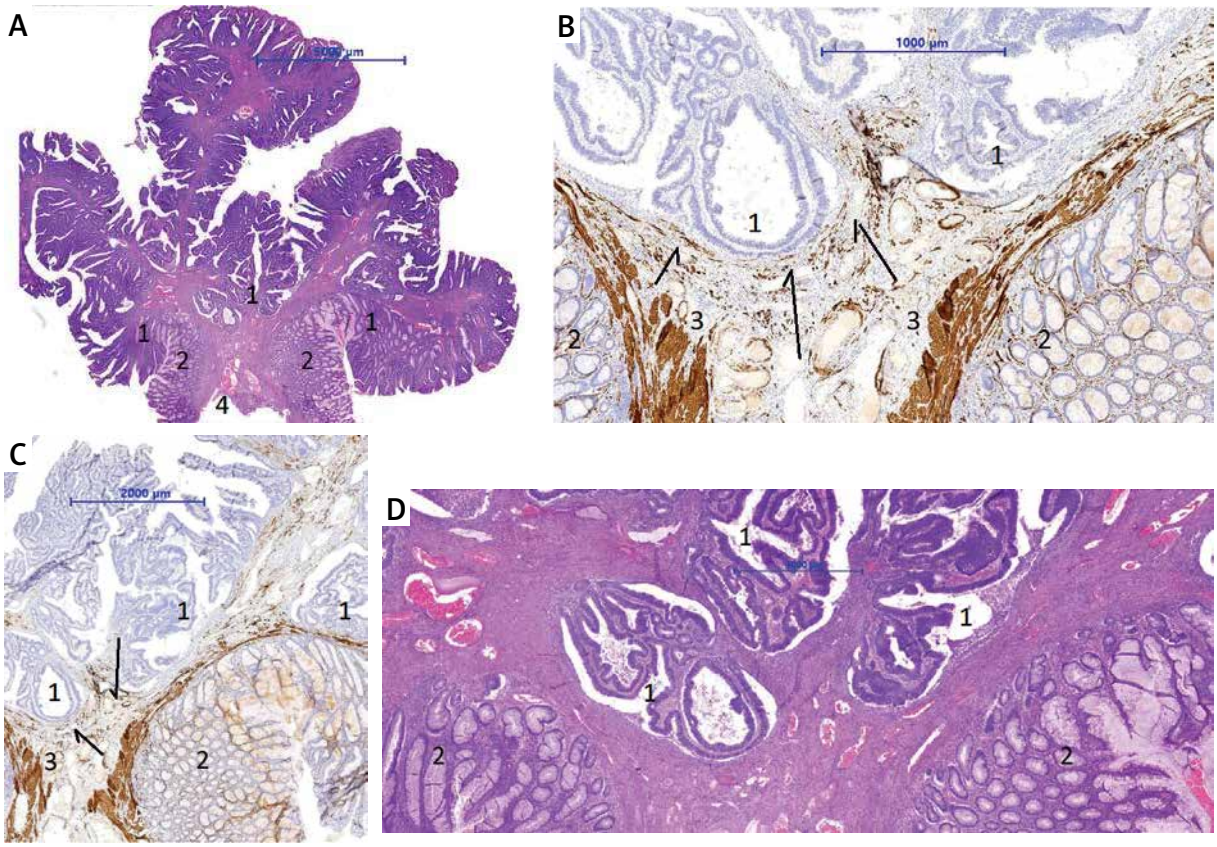


Figure 1. Pedunculated colon polyp, assessed with the Haggitt classification as level 3. **A**, **D** – H + E staining; the subsequent magnifications show infiltration of the polyp stalk by cancerous tissue (numbers 1); A, at the stalk level, there is regular intestinal epithelium (numbers 2), and the margin of the polyp resected with the endoscopic loop is tagged with the number 4. **B**, **C** – Immunohistochemical staining against desmin; to demonstrate smooth muscle tissue of the muscularis mucosae (numbers 3), the arrows indicate destruction of the muscularis mucosae from the cancer infiltration. The magnification bar: **A** – 5000 µm, **B** – 1000 µm, **C** – 2000 µm, and **D** – 1000 µm is labelled in each photo, respectively

margin < 1 mm, 2) pedunculated polyp, Haggitt level 4, 3) sessile polyp, Kikuchi 3, 4) poor differentiation.

The above-mentioned factors imply that segmental large intestine resection should be performed if the general patient's condition allows for this procedure.

The other factors with lesser significance include the following: the sessile type of polyp – Kikuchi, sm2, lymphovascular invasion, mucinous tumour, and tumour budding [10].

Most patients with the diagnosis "cancer within colon polyp" are proceed using the special regulations of the "fast track for oncological diagnosis and treatment". Based on the performed examinations: computed tomography (CT) scan of the thorax, abdomen cavity, and pelvis plus carcinoembryonic antigen (CEA) level, which preclude or confirm cancer dissemination, the decision concerning further treatment is made. If the lesion is located at the rectum level, endorectal ultrasound (EUS) or magnetic resonance imaging (MRI) should be carried out as a part of the procedure. The decision is taken during the oncology consultation, in which the patient and oncological team (surgical oncologist, clinical oncologist, and radiotherapist) take part. In the "cancer within colon polyp" case, a correct interpretation of colonoscopy and histopathological reports is crucial, and the decisions should be consistent with the recommendations of the societies for gastroenterology as well as colorectal surgery. This decision also includes the risk analysis of the potential surgery and the perioperative complications with regard to the patient's age and general condition. At the same time, the factors indicating persistent cancer tissue in the margin of excision and/or a substantial risk for local cancer recurrence and, consequently, the strong need for surgical intervention with segmental intestinal resection and regional lymph draining cannot be ignored. The final decision about management is taken by a well-informed patient who has full knowledge of their clinical situation.

Conclusions

In the contemporary clinical practice, the management of the patient with a colon polyp, both diagnosis and subsequent follow-ups, require cooperation between an endoscopist physician and a pathologist. The presence of cancerous tissue in the polyps warrants the participation of a radiologist and a surgical oncologist. This is an example of the multidisciplinary management that allows rational clinical decisions to be taken based on current recommendations.

Acknowledgments

The authors declare no conflict of interest.

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w sprawie nadania stopnia doktora

Olsztyn 16.02.2024
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OŚWIADCZENIE
o samodzielnym napisaniu rozprawy doktorskiej

Świadoma(-y) odpowiedzialności prawnej oświadczam, że rozprawa doktorska pod tytułem Białko POLD1 jako czynnik prognostyczny w raku jelita grubego i raku odbytnicy. została napisana przeze mnie samodzielnie i nie zawiera treści uzyskanych w sposób niezgodny z obowiązującymi przepisami.

Oświadczam również, że przedstawiona rozprawa nie jest i nie była wcześniej podstawą do ubiegania się o nadanie stopnia naukowego doktora.

Ponadto oświadczam, że treść rozprawy doktorskiej przedstawionej przez mnie do obrony, zawarta na przekazywanym nośniku elektronicznym, jest identyczna z jej wersją drukowaną.

W związku z powyższym oświadczam, że wyrażam zgodę na poddanie rozprawy doktorskiej procedurze antyplagiatowej.

Przemysław Stefaniak
(podpis kandydata)

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Jednocześnie wyrażam zgodę na przedłożenie tej pracy przez Przemysława Stefaniaka jako część rozprawy doktorskiej w formie zbioru spójnych tematycznie publikacji naukowych.

Jednocześnie oświadczam że samodzielna i możliwa do wyodrębnienia część tej pracy wykazuje indywidualny wkład Przemysława Stefaniaka polegający na opracowaniu koncepcji, wykonywania pracy eksperymentalnej, opracowania i interpretacji wyników, pisania tekstu tej pracy.

Jacek Kieżun

2. Niniejszym składam oświadczenie że w publikacji naukowej: ***Stefaniak P,*** *Krazinski B E, Majewska H, Kiezun J, Godlewski J. Altered immunoexpression of DNA polymerase delta1 catalytic subunit (POLD1) in colorectal cancer. Contemp Oncol (Pozn) 2023; 27(3):147-154,* mój wkład merytoryczny polegał na przygotowaniu metodologii eksperymentu, udział w wykonaniu preparatów histologicznych oraz przygotowaniu dokumentacji zdjęciowej zawartej w tej pracy.

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Jacek Kieżun

Olsztyn 30.01.24

Dr n.med Hanna Majewska

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Przewodniczący Rady Naukowej Dyscypliny Nauki Medyczne

Uniwersytetu Warmińsko-Mazurskiego w Olsztynie

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Hanna Majewska

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Hanna Majewska

Olsztyn 30.01.24

Dr hab.n.med Dariusz Zadrożny, Prof. UW-M

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Przewodniczący Rady Naukowej Dyscypliny Nauki Medyczne

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Dariusz Zadrożny

Olsztyn 30.01.24

Lek. Michał Gola

Katedra Histologii i Embriologii Człowieka

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Przewodniczący Rady Naukowej Dyscypliny Nauki Medyczne

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Michał Gola

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Prof. Barbara Alicja Jereczek-Fossa, M.D. Ph.D.
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Olsztyn 30.01.24

Prof. dr hab. Anna Starzyńska
Zakład Chirurgii Stomatologicznej,
Wydział Lekarski, Gdański Uniwersytet Medyczny

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Anna Starzyńska