UNIWERSYTET MIKOŁAJA KOPERNIKA W TORUNIU

Collegium Medicum
im. Ludwika Rydygiera w Bydgoszczy

Bydgoszcz 2023 r.

UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Lekarski
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Kinaza CDK9 jako nowy cel terapeutyczny oraz czynnik prognostyczny w nowotworach złośliwych

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu

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Bydgoszcz 2023r.

Spis treści

Wykaz stosowanych skrótów

BCLC (ang. *Barcelona Clinic Liver Cancer staging system*) - klasyfikacja barcelońska oceniająca kliniczne zaawansowanie raka wątrobowokomórkowego

BLCA (ang. *bladder cancer*) - rak pęcherza moczowego

CDK (ang. *cyclin-dependent kinase*) - kinaza cyklino-zależna, kinaza zależna od cyklin

EMA (ang. *European Medicines Agency*) - Europejska Agencja Leków

FDA (ang. *Food and Drug Administration*) - Urząd ds. Żywności i Leków

HCC (ang. *hepatocellular carcinoma*) - rak wątrobowokomórkowy

iASPP (ang. *inhibitor of apoptosis-stimulating protein of p53*) - inhibitor białka p53 stymulującego apoptozę

MM (ang. *multiple myeloma*) - szpiczak mnogi

OS (ang. *overall survival*) - przeżycie całkowite

PFS (ang. *progression-free survival*) - przeżycie do progresji

PROTAC (ang. *Proteolysis Targeting Chimera*) - chimera ukierunkowana na proteolizę

TCGA (ang. *The Cancer Genome Atlas*) - Atlas Genomowy Nowotworów

P-TEFb (ang. *Positive-Transcription Elongation Factor b*) - pozytywny czynnik elongacji transkrypcji

TMA (ang. *tissue microarray*) - mikromacierze tkankowe

TP53 (ang. *Tumor Protein 53*) - białko p53

wt-p53 (ang. *wild-type tumor protein 53*) - “dzikie”, niezmutowane białko p53

Rozdział 1. Wykaz publikacji stanowiących podstawę rozprawy doktorskiej

Rozprawę doktorską stanowi cykl czterech artykułów:

1. **Borowczak, J.**, Szczerbowski, K., Maniewski, M., Zdrenka, M., Słupski, P., Antosik, P., Kołodziejska, S., Sekielska-Domanowska, M., Dubiel, M., Bodnar, M. & Szylberg, Ł. The Prognostic Role of CDK9 in Bladder Cancer. Cancers 14, (2022). doi:10.3390/cancers14061492

   IF: 6.575 *MNISW*: 140

2. **Borowczak, J.**, Szczerbowski, K., Maniewski, M., Zdrenka, M., Słupski, P., Andrusewicz, H., Łysik-Miśkurka, J., Rutkiewicz, P., Bodnar, M. & Szylberg, Ł. The prognostic role of p53 and its correlation with CDK9 in urothelial carcinoma. Clin. Transl. Oncol. (2022). doi:10.1007/s12094-022-02994-6

   IF: 3.340 *MNISW*: 70

3. **Borowczak, J.**, Szczerbowski, K., Stec, E., Grzanka, D. & Szylberg, Ł. CDK9: Therapeutic Perspective in HCC Therapy. Curr. Cancer Drug Targets 20, 318–324 (2020). doi: 10.2174/1568009620666200212124357

   IF: 3.428 *MNISW*: 70

4. **Borowczak, J.**, Szczerbowski, K., Ahmadi, N. & Szylberg, Ł. CDK9 inhibitors in multiple myeloma: a review of progress and perspectives. Med. Oncol. 39, 39 (2022). doi:10.1007/s12032-021-01636-1

   IF: 3.738 *MNISW*: 70

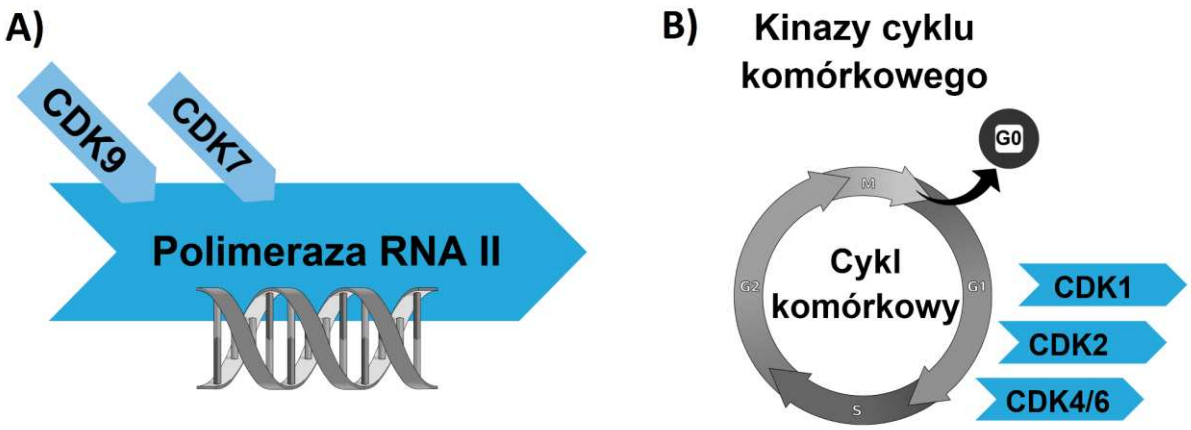
Sumaryczny IF: 17.081 Sumaryczna punktacja *MNiSW*: 350

Rozdział 2. Wprowadzenie

W ostatnich dekadach nastąpił znaczący postęp w terapii nowotworów i opiece onkologicznej. Nowoczesne badania profilaktyczne oraz rozwój metod diagnostycznych zaowocowały zwiększeniem się odsetka wczesnych rozpoznań nowotworów oraz przedłużeniem czasu przeżycia chorych. Dzięki rozwojowi nowych metod terapeutycznych, takich jak immunoterapia czy terapia celowana, nawet u chorych w zaawansowanym stadium choroby znacząco wydłużył się oczekiwany czas przeżycia [1,2].

Niestety, obecnie dostępne terapie systemowe mają liczne ograniczenia, które wynikają z ograniczonej biodostępności substancji aktywnych, ich niezadowalającej penetracji w głąb guza oraz powikłań związanych z toksycznością [3]. Ponadto parakrynna aktywność mikrośrodowiska guza, dysregulacja cyklu komórkowego i akumulacja zmian genetycznych mogą znacznie ograniczyć efektywność terapii [4]. Pomimo częstych remisji uzyskanych po zastosowaniu pierwszej linii leczenia systemowego, rozwijająca się lekooporność i rychły nawrót choroby prowadzą do nieuchronnego pogorszenia się stanu zdrowia pacjenta [3,5]. W związku z tym, poszukiwanie nowych markerów prognostycznych i celów terapeutycznych jest kluczowe dla dalszego postępu w terapii nowotworów.

Zdolność do unikania apoptozy jest jednym z podstawowych znamion choroby nowotworowej [6]. Naturalną konsekwencją rozpowszechnienia tej tezy poprzez Hanahana i Weinberga stało się rosnące zainteresowanie białkami kontrolującymi cykl komórkowy i ich wykorzystaniem w terapii nowotworów. Grupą, która w ostatnim czasie cieszy się szczególnym zainteresowaniem, są kinazy zależne od cyklin (CDK). Obecnie znanych jest 20 kinaz cyklinozależnych, kodowanych przez 21 genów [7]. Spośród nich CDK od 1 do 6, 14 do 18 oraz CDK 20 kontrolują cykl komórkowy, natomiast CDK od 7 do 13 oraz CDK 19 regulują transkrypcję [8,9]. CDK funkcjonują w kompleksach z cyklinami, poprzez które kontrolują przeżycie i proliferację komórki (Rycina 1) [10,11].

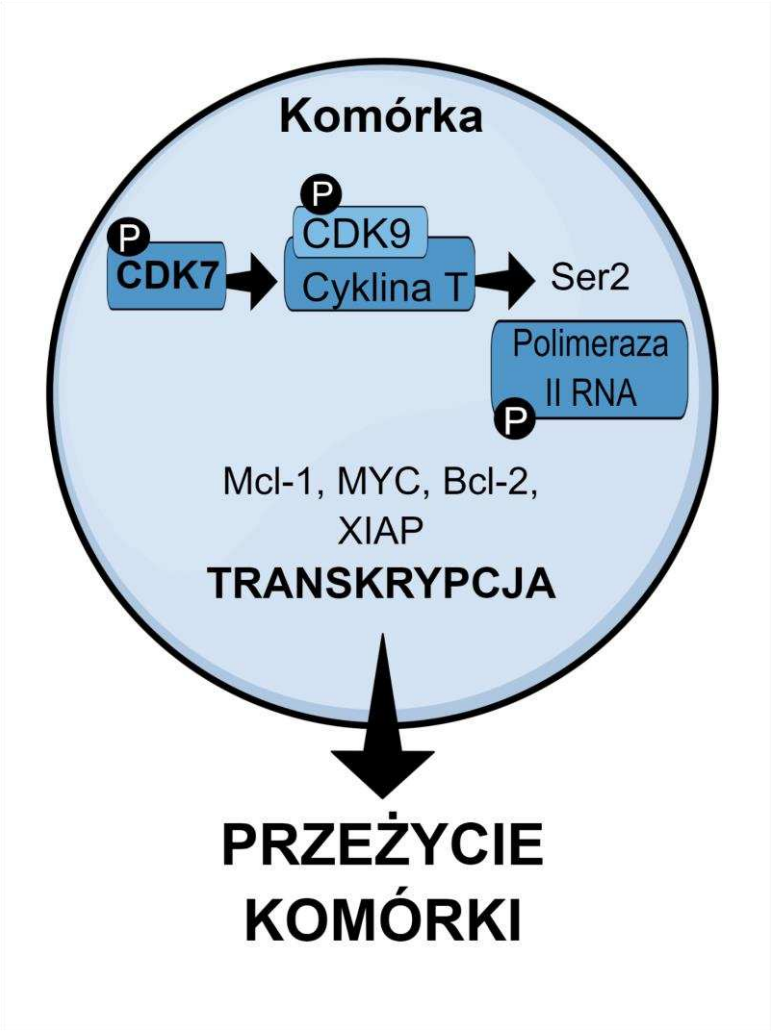


Rycina 1. Mechanizm działania głównych kinaz cyklinozależnych [12]. Kinazy transkrypcyjne, zwłaszcza CDK7 i 9, regulują aktywność polimerazy II RNA i transkrypcję (Rycina 1A). Kinazy cyklu komórkowego, takie jak CDK1, 2 i 4/6, są regulatorami punktów kontrolnych, a ich aktywność umożliwia przejście cyklu komórkowego do interfazy (G1 i G2), fazy syntezy (S) i fazy mitozy (M) (Rycina 1B).

Inhibitory CDK4/6, między innymi abemaciclib, palbociclib i ribociclib, zostały niedawno zaaprobowane przez Urząd ds. Żywności i Leków (FDA) oraz Europejską Agencję Leków (EMA) jako leki stosowane w terapii zaawansowanego i rozsianego HR+/HER2- raka piersi [13]. Inhibitory CDK7 są obecnie testowane w leczeniu pacjentów z drobnokomórkowym rakiem płuca, rakiem trzustki i rakiem piersi (NCT05394103 i NCT04247126). Niemniej jednak, za jeden z centralnych ośrodków regulacji transkrypcji i obiecujący cel terapeutyczny uważana jest dzisiaj CDK9 [14].

CDK9 jest białkiem regulującym transkrypcję i występującym w dwóch znanych izoformach, $CDK9_{42}$ i $CDK9_{55}$ [15]. Łącząc się z cyklinami T1 i T2a, tworzą one pozytywny czynnik elongacji transkrypcji (P-TEFb), który fosforylując polimerazę II RNA wydłuża transkrypcję (Rycina 2) [8,14]. Niemniej jednak, obie izoformy wykazują pewną funkcjonalną odmienność. Zwiększona ekspresja $CDK9_{42}$, ale nie $CDK9_{55}$, jest związana z nasiloną proliferacją komórek [16]. Natomiast $CDK9_{55}$ współtworzy mechanizmy naprawy DNA poprzez szlak sygnałowy związany z białkiem Ku70 [17]. Podobną różnorodność funkcyjną zaobserwowano w stosunku do cyklin wiążących się z CDK9. Cyklina T jest niezbędna do prawidłowego różnicowania się limfocytów, monocytów i adipocytów, podczas gdy nadekspresja cykliny K, wynikająca z aktywacji p53, sugeruje jej rolę w mechanizmach

odpowiedzi na stres komórkowy [18]. Aktywacja ścieżki sygnałowej CDK9 sprzyja powstawaniu białek antyapoptotycznych, takich jak Bcl-2, XIAP, MYC i Mcl-1. Dysregulacja tego szlaku zaburza homeostazę komórki, zapobiega jej programowanej śmierci i prowadzi do niekontrolowanej mitogenezy, będącej zaczątkiem choroby nowotworowej [8,10,19,20].



Rycina 2. Mechanizm działania CDK9. CDK7 fosforyluje CDK9, powodując jej związanie z cykliną T i powstanie dodatniego kompleksu elongacji transkrypcji. Kompleks ten, poprzez fosforylację domeny C-końcowej polimerazy II RNA na serynie 2, warunkuje transkrypcję białek antyapoptotycznych i przyczynia się do przeżycia komórki nowotworowej.

Większość autorów jest zgodna co do prognostycznej roli CDK9 w nowotworach złośliwych człowieka. Jej nadekspresja koreluje z nasileniem cech histologicznej złośliwości i krótszym czasem przeżycia pacjentów z rakiem trzustki, rakiem endometrium i kostniakomięsakiem, a także jest czynnikiem predykcyjnym nawrotu raka surowiczego jajnika

[21–24]. Jednakże Schlafstein i wsp. wykazali, że wysoka ekspresja CDK9 jest związana z dłuższym czasem przeżycia pacjentów z rakiem piersi, którzy nie osiągnęli kompletnej remisji po neoadjuwantowej chemioterapii [25]. Fenomen ten nie został dotychczas w pełni wyjaśniony. Berthet i Kaldis w swojej publikacji zasugerowali, że dobrze zróżnicowane komórki są bardziej wrażliwe na zaburzenia cyklu komórkowego [26]. Badania na modelach zwierzęcych wykazały istotne różnice w mechanizmach kontroli cyklu komórkowego dojrzałych komórek eukariotycznych i embrionalnych komórek macierzystych [27]. W czasie podziału, komórki nowotworowe zaczynają zachowywać się podobnie do komórek macierzystych. Hamowanie aktywności kinaz zależnych od cyklin pozwala odwrócić ten trend i sprawia, że komórki nowotworowe nabierają cech komórek dobrze zróżnicowanych. Wydaje się zatem, że nadekspresja CDK ma szczególne znaczenie na wczesnych etapach rozwoju choroby, kiedy relatywnie dojrzałe komórki nowotworowe są wciąż zależne od czynników pobudzających wzrost [24,28].

Schlafstein i współpracownicy wyciągnęli podobne wnioski, argumentując, że niska ekspresja CDK9 może zaburzać naprawę DNA i prowadzić do niestabilności genetycznej, a w związku z tym agresywnego przebiegu choroby [25]. Stała aktywność CDK9 w czasie cyklu komórkowego jest niezbędna do zachowania równowagi pomiędzy białkami proapoptotycznymi i antyapoptotycznymi [29]. Ze względu na swoją rolę w apoptozie, CDK9 wchodzi w interakcje z czynnikami transkrypcyjnymi i białkami supresorowymi, takimi jak p53, a ich wzajemna regulacja warunkuje przeżycie lub śmierć komórki [30]. Zmiany aktywności tych białek mają zatem bezpośrednie przełożenie na aktywność i funkcjonowanie CDK9 [31,32]. Przykładowo, jednym z najczęstszych zdarzeń w ewolucji nowotworu jest mutacja p53. Prowadzi ona do stopniowego narastania niestabilności genetycznej. Jej wystąpienie jest także czynnikiem ryzyka progresji nieinwazyjnego raka urotelialnego pęcherza moczowego do raka inwazyjnego [33,34]. Z tego powodu, rak urotelialny pęcherza moczowego (BLCA) staje się naturalnym przedmiotem dalszych badań.

W literaturze istnieją liczne wątpliwości dotyczące terapeutycznego stosowania inhibitorów CDK9, wynikające z ich niskiej skuteczności w monoterapii. Przyczyną niepowodzeń jest najprawdopodobniej niska selektywność pierwszej generacji inhibitorów CDK9, takich jak flavopiridol, powodujący wysoką toksycznością terapii [35]. Chociaż w 2021 roku Anshabo i wsp. oraz Mandal i wsp. scharakteryzowali CDK9 jako potencjalny cel

terapeutyczny, brak jest w literaturze światowej prac podsumowujących postępy w użyciu nowej generacji inhibitorów CDK9 w terapii poszczególnych nowotworów [8,19].

Blokada CDK9, ze względu na swój potencjał do przełamywania oporności komórek na apoptozę, cieszy się dużym zainteresowaniem w nowotworach hematologicznych. W dotychczasowych badaniach pacjenci z przewlekłą białaczką limfocytową leczeni dinaciclibem, inhibitorem CDK9 pierwszej generacji, osiągnęli dłuższy czas przeżycia wolnego od progresji (13.7 vs. 5.9 miesięcy), dłuższy całkowity czas przeżycia (21.2 vs. 16.7 miesięcy) oraz wyższy odsetek odpowiedzi na leczenie (40% vs. 8.3%) niż pacjenci leczeni zaaprobowanym przez FDA przeciwciałem monoklonalnym skierowanym przeciwko limfocytom B (ofatumumabem). Co istotne, zdecydowane większe korzyści z terapii dinaciclibem zaobserwowano wśród pacjentów z delecją p53 (czas przeżycia 21.2 vs. 5.4 miesiące), wskazując na skuteczność inhibitorów CDK9 w tej grupie pacjentów [36]. Praca Ghia i współautorów otworzyła również drogę dla oceny skuteczności CDK9 w terapii guzów litych. Chemioterapuetyk paclitaxel może indukować apoptozę poprzez ścieżki sygnałowe związane z p53/p21. Z tego powodu ocena korelacji między ekspresją CDK9 i p53 może pozwolić na wstępną ocenę potencjalnego zastosowania inhibitorów CDK9 w terapii zaawansowanych nowotworów złośliwych [37,38].

Wyniki badań dotyczących innych nowotworów hematologicznych są jednak niejednoznaczne i nie zostały wcześniej uporządkowane. Druga generacja inhibitorów CDK9 charakteryzuje się większą selektywnością, rzadszymi działaniami niepożądanymi i może poprawić tolerancję terapii [8]. Rozwój technologii wytwarzania leków umożliwił zastosowanie metod alternatywnych do inhibicji kinazy CDK9. Chimery ukierunkowane na proteolizę (PROTAC) umożliwiają selektywną degradację CDK9 oraz sprzężenie inhibitorów CDK9 z innymi substancjami aktywnymi [39]. Niestety, zalety i wady różnych metod terapii ukierunkowanej na CDK9 nie zostały jeszcze w pełni poznane.

W ostatnich latach przeprowadzono liczne badania przedkliniczne oceniające skuteczność inhibitorów CDK9 w raku wątrobowokomórkowym (HCC) [40,41]. Wczesne próby kliniczne w HCC stają się coraz bardziej prawdopodobne, dlatego pojawiła się potrzeba usystematyzowania dotychczasowych doniesień, określenia potencjalnej grupy badanej oraz momentu włączenia terapii. Inhibitory CDK9 zdają się zmniejszać oporność na inne leki, nawet u pacjentów o niekorzystnymi profilu cytogenetycznym, dlatego mogą stanowić uzupełnienie

podstawowych schematów terapeutycznych. Biorąc pod uwagę synergistyczne działanie flavopiridolu i sorafenibu, koncepcja zastosowania blokady CDK9 w terapii raka wątrobowokomórkowego wymaga pogłębionej analizy [42,43].

Rozdział 3. Cel pracy z uzasadnieniem podjętej tematyki badawczej

Główne cele pracy:

I. Ocena wartości predykcyjnej CDK9 w przewidywaniu rokowania pacjentów z nowotworami złośliwymi na przykładzie raka urotelialnego pęcherza moczowego.
II. Ocena korelacji między ekspresją CDK9 a występowaniem histopatologicznych cech złośliwości raka urotelialnego pęcherza moczowego.
III. Ocena korelacji pomiędzy ekspresją białek regulatorowych cyklu komórkowego i CDK9 oraz ich wpływu na rokowanie pacjentów z rakiem urotelialnym pęcherza moczowego.
IV. Analiza możliwości zastosowania inhibitorów CDK9 w terapii celowanej oraz zaproponowanie sposobu ich włączenia do obecnych schematów terapeutycznych.
V. Wskazanie, które grupy pacjentów mogą osiągnąć największe korzyści kliniczne w związku z hamowaniem aktywności CDK9.

Szczegółowe cele realizowane w poszczególnych publikacjach:

1. Uwzględniając najnowsze doniesienia o niejednoznacznej roli CDK9 w prognostyce rokowania w nowotworach złośliwych, zdecydowaliśmy o jej sprawdzeniu w niezbadanym dotychczas raku. Praca oryginalna “The Prognostic Role of CDK9 in Bladder Cancer” miała zweryfikować, czy ekspresja CDK9 umożliwia oceną rokowania pacjentów z rakiem urotelialnym pęcherza moczowego. Zakładaliśmy, że wysoka ekspresja CDK9 związana będzie ze złym rokowaniem, a wynik badania stanowić będzie podstawę do terapeutycznej blokady tej kinazy.

2. Kontynuując oceną wartości prognostycznej CDK9 w raku urotelialnym pęcherza moczowego, zdecydowaliśmy o ocenie korelacji pomiędzy CDK9 a innymi białkami regulującymi cykl komórkowy. Praca oryginalna "The prognostic role of p53 and its correlation with CDK9 in urothelial carcinoma" miała sprawdzić czy znaczenie prognostycznej CDK9 zmienia się w zależności od wysokości ekspresji i obecności mutacji białka p53, jednej z najczęstszych zmian genowych w raku urotelialnym pęcherza moczowego.

3. Artykuł przeglądowy "CDK9: Therapeutic Perspective in HCC Therapy" miał na celu usystematyzowanie dotychczasowej wiedzy na temat zastosowania inhibitorów kinazy CDK9 w raku wątrobowokomórkowym. W związku z perspektywą nieodległych badań klinicznych, szczególne znaczenie miało podsumowanie dotychczas przeprowadzonych prób oraz analiza możliwości włączenia inhibitorów CDK9 do obecnie stosowanych schematów terapeutycznych. Biorąc pod uwagę, że wybór metody leczenia pacjentów z rakiem wątrobowokomórkowym jest bezpośrednio związany ze stopniem jego klinicznego zaawansowania, praca ta miał zidentyfikować grupę pacjentów mogących odnieść największą korzyść z leczenia.

4. Z powodu braku badań klinicznych z zastosowaniem inhibitorów CDK9 w raku wątrobowokomórkowym zdecydowaliśmy się na pogłębienie analizy o doniesienia z badań klinicznych w nowotworów hematologicznych. Publikacja "CDK9 inhibitors in multiple myeloma: a review of progress and perspectives" miała za zadanie wyjaśnić wczesne niepowodzenia w użyciu terapii ukierunkowanej na CDK9, uwzględniając różnice w mechanizmie działania poszczególnych inhibitorów, potencjalne skutki uboczne i skuteczność kliniczną. Zakładając ich niewielką skuteczność w monoterapii, skupiliśmy się na analizie interakcji pomiędzy blokerami CDK9 a lekami stosowanymi obecnie w praktyce klinicznej, a także znalezieniu alternatywy dla hamowania aktywności CDK9.

Rozdział 4. Publikacje

The Prognostic Role of CDK9 in Bladder Cancer

Borowczak J, Szczerbowski K, Maniewski M, Zdrenka M, Słupski P, Antosik P, Kołodziejska S, Sekielska-Domanowska M, Dubiel M, Bodnar M, Szylberg Ł.

Cancers (Basel). 2022 Mar 15;14(6):1492.

doi: 10.3390/cancers14061492.

IF: 6.575 MNISW: 140

cancers

MDPI

Article

The Prognostic Role of CDK9 in Bladder Cancer

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Simple Summary: In this article, we investigated the prognostic role of cyclin-dependent kinase 9 expression in urothelial carcinoma. High CDK9 expression has recently been associated with shorter patient survival time, but its role in urothelial carcinoma has not yet been explored. The expression of CDK9 was higher in cancer than in normal urothelial tissue and correlated with tumor grade, stage, and invasiveness. To our surprise, patients with high CDK9 expression lived longer than patients with low CDK9 expression. In The Cancer Genome Atlas database cohort, high CDK9 RNA concentration correlates with longer survival of patients and CDK9 status remained a statistically significant prognostic factor in multivariate analysis. It seems that CDK9 not only regulates the expression of anti-apoptotic genes, leading to longer survival of cancer cells, it also facilitates DNA repair, preventing the build-up of genomic instability, crucial in the initiation and progression of bladder cancer. The results suggest that CDK9 overexpression is not always associated with a worse prognosis, while cell maturity and disease stage may influence the efficacy of potential targeted therapy.

Abstract: Introduction: Most patients with urothelial carcinoma are diagnosed with non-invasive tumors, but the prognosis worsens with the progression of the disease. Overexpression of cyclin-dependent kinase 9 has been recently linked to increased cancer proliferation, faster progression, and worse prognosis. However, some cancers seem to contradict this rule. In this work, we explored the prognostic role of CDK9 expression in urothelial carcinoma. Materials and Methods: We performed immunohistochemical analysis on 72 bladder cancer samples. To assess a larger group of patients, the Cancer Genome Atlas (TCGA) database containing 406 cases and transcriptomics information through the Human Pathology Atlas were analyzed. Results: CDK9 is overexpressed in urothelial cancer tissues when compared to normal urothelial tissues ($p < 0.05$). High CDK9 expression was observed in low-stage, low-grade, and non-muscle-invasive tumors ($p < 0.05$). The patients with high CDK9 expression had a significantly higher 5-year overall survival rate than those with low CDK9 expression (77.54% vs. 53.6% in the TMA group and 57.75% vs. 35.44% in the TCGA group, respectively) ($p < 0.05$). The results were consistent in both cohorts. Multivariate Cox regression analysis indicated that low CDK9 status was an independent predictor for poor prognosis in the TCGA cohort (HR 1.60, CL95% 1.1–2.33, $p = 0.014$). Conclusions: High CDK9 expression predicts a favorable prognosis in urothelial carcinoma and is associated with clinicopathological features characteristic for early-stage disease. The decrease in CDK9 expression can be associated with the build-up of genetic instability and may indicate a key role for CDK9 in the early stages of urothelial carcinoma.

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Citation: Borowczak, J.; Szczerbowski, K.; Maniewski, M.; Zdrenka, M.; Słupski, P.; Antosik, P.; Kołodziejska, S.; Sekielska-Domanowska, M.; Dubiel, M.; Bodnar, M.; et al. The Prognostic Role of CDK9 in Bladder Cancer. *Cancers* **2022**, *14*, 1492. https://doi.org/10.3390/cancers14061492

Academic Editor: Francesco Massari

Received: 20 February 2022
Accepted: 11 March 2022
Published: 15 March 2022

Keywords: CDK9; bladder cancer; expression; prognosis; survival

1. Introduction

Bladder cancer is the ninth most frequent malignancy worldwide, with approximately 430,000 cases a year. It ranks 13th in terms of mortality with almost 200,000 deaths per year [1–3]. The most common histological subtype is urothelial cancer, which accounts for approximately 90% of the cases. Over half of the patients are diagnosed in the early stage of the disease with non-invasive tumors and are successfully treated radically. This results in a high 5-year survival rate of up to 77.1%. However, when the disease is more advanced, these numbers drop dramatically, reaching a 4.7% survival rate in metastatic cancer [2]. Genetic heterogeneity, reactive increase in DNA repair, and mechanisms modifying the intracellular drug concentration may limit the response to therapy [4]. Therefore, there is a need for novel treatment options as well as novel prognostic markers.

Cyclin-Dependent Kinase 9 (CDK 9)

Cyclin-dependent kinase 9 (CDK9) is a transcription regulating protein [5]. Together with cyclin T, CDK9 forms positive transcription elongation factor-B (P-TEFb), which activates RNA polymerase II (RNA POL II), and through this mechanism, stimulates transcription [5,6]. The following translation results in the formation of anti-apoptotic proteins, such as MYC or Mcl-1 [7]. This disrupts cellular homeostasis, shifting the apoptotic balance towards the survival of cells [8]. At the same time, the recruitment of P-TEFb is required for the differentiation of muscles [9], neurons [10], or adipocytes [11]. Furthermore, CDK9 promotes tumor growth via the p53 related pathway [12,13]. Its overexpression is associated with poor prognosis in various neoplasms, such as pancreatic cancer and osteosarcoma [14,15]. CDK9 inhibitors are being tested for the treatment of multiple malignancies, including multiple myeloma, acute myeloid leukemia, prostate cancer, and hepatocellular carcinoma, making CDK9 a valid potential therapeutic target and a novel prognostic marker [16–19]. In this work, we aimed to investigate whether there is a connection between the CDK 9 expression and individual clinical features of bladder cancer, such as stage, grade, presence of metastasis, and survival time. We assessed the prognostic value of CDK9 expression in urothelial cancer and validated the findings in The Cancer Genome Atlas Program database.

2. Materials and Methods

2.1. Patients and Tissue Samples

All tissue specimens were collected from patients diagnosed with urothelial carcinoma and treated in the Department of Urology between November 2009 and July 2018. Our study includes 72 cases of bladder cancer (study group) and 32 cases of normal urothelial mucosa (control group), collected immediately during either transurethral resection of bladder tumor (TURBT) or radical cystectomy (RC). Clinical data, including age, sex, overall survival, tumor differentiation (grade), stage T, lymph nodes invasion, metastasis, tumor size, cancer invasiveness, progression, and recurrence were obtained (Table 1). The study was conducted following the Declaration of Helsinki, and the protocol was approved by the Bioethics Committee (KB881/2019).

Table 1. Clinicopathological characteristics of the study group.

Variables		*n* (%)
Age	Mean	71.5 years (range 45–88 years)
Sex	Female	11 (15.28%)
	Male	61 (84.72%)

Table 1. *Cont.*

Variables		*n* (%)
Grade	low	34/72 (47.22%)
	high	38/72 (52.78%)
Stage	T1	39/72 (54.17%)
	T2	20/72 (27.78%)
	T3	9/72 (12.5%)
	T4	4/72 (5.56%)
Tumor size	≥3 cm	39/72 (54.17%)
	<3 cm	33/72 (45.83%)
Lymph node metastases	N0	61/72 (84.72%)
	N1–3	9/72 (12.5%)
	Unknown	2/72 (2.78%)
Distant metastasis	No	62/72 (86.11%)
	Yes	7/72 (9.72%)
	Unknown	3/72 (4.17%)
Invasiveness	NMIBC	36/72 (50%)
	MIBC	35/72 (48.61%)
	Unknown	1/72 (1.39%)
Progression	Yes	17 (23.61%)
	No	34 (47.22%)
	Unknown	21 (29.17%)
Recurrence	Yes	26 (36.11%)
	No	8 (11.11%)
	Unknown	38 (52.78%)
Mean recurrence time		21.07 months
Type of procedure	TURBT	35 (48.61%)
	PC	31 (43.06%)
	Unknown	6 (8.33%
Disease course	Alive	29/72 (40.28%)
	Dead	43/72 (59.72%)
Median follow-up time		60 months (range 5–60 months)

2.2. Sample Staining

The expression of CDK9 was determined using IHC assays according to the protocol described in Buchholz et al.'s study [20]. In the beginning, standardization and optimization of the IHC method were performed on a recommended tissue, based on the antibody datasheet and reference sources (The Human Protein Atlas: https://www.proteinatlas.org (accessed on 11 November 2021); Uhlen et al., 2010 [21]). In brief, 3 µm thick sections of the tissue arrays were baked for 1 h at 60 °C before xylene deparaffinization and subsequent rehydration through graded ethanol (99.8%; 96%; 90% and 80%). Tissue sections were incubated with a primary rabbit monoclonal anti-CDK9 antibody (1:200; 40 min; cat. no: ab76320, Abcam, Cambridge, MA, USA). Primary antibodies were visualized using the UltraView Universal DAB Detection Kit (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA) followed by color development using 3,3-diaminobenzidine. The slides were counterstained with hematoxylin II for 12 min and blue reagent for 4 min. Finally, tissue sections were dehydrated in increasing ethanol concentrations (80, 90, 96, and 99.8%), cleared in xylenes (I–IV), mounted using a mounting medium, and examined.

2.3. Image Acquisition and IHC Analysis

Initially, the clinical data were blinded and the images were captured using an optical microscope at ×10 magnification with a color video camera attached to a computer system. For each sample, two experienced pathologists selected the most representative regions

and acquired images. The analysis was performed using the ImageJ 1.53j version (NIH, Bethesda, MD, USA) (Java 1.8.0_172) and the IHC profiler plugin. Nuclear CDK9 expression was obtained by calculating the H-score. To determine CDK9 expression in cancer cells, the standard protocol designed by Verghese et al. was followed [22]. The highly positive zone was found to be ranging from 1 to 60; 61 to 120 for the positive zone; 121 to 170 for the low positive zone; and 181 to 220 for the negative zone, respectively. The intensity values ranging from 221–255 predominantly represent fatty tissues, stroma, or background artifacts that do not contribute to pathological scoring and were therefore excluded from the score determination zones. H-score was assigned using the formula (1 × (%cells low positive) + 2 × (%cells positive) + 3 × (%cells high positive)), obtaining a value from 0 to 300.

2.4. Statistical Analysis

All statistical analyses were performed using Statistica version 13.3 (Statsoft) and Microsoft Excel 2019. The value of $p < 0.05$ was considered statistically significant. Continuous variables were tested for normality by the Kolmogorov–Smirnov test. The relations between compared groups, due to the categorical character of variables, were analyzed using the Mann–Whitney U Test. More than two independent groups were compared using the ANOVA Kruskal–Wallis test. Correlation between clinicopathological characteristics and CDK9 expression was evaluated using Spearman's rank correlation coefficient. Univariate and multivariate analyses of potential predictors for overall survival were performed using Cox proportional hazard regression. Results were expressed as hazard ratio (HR) and 95% confidence interval (CI). The two-sided p-value of <0.05 was considered to indicate statistical significance. The relation between CDK9 expression with overall survival was evaluated with a log-rank test and presented using Kaplan–Meier analysis.

3. Results

3.1. Patients Characteristics

We explored the relevance of CDK9 expression in human urothelial carcinoma by comparing normal urothelial mucosa and urothelial carcinoma of bladder cancer patients. Table 1 summarizes the characteristics of the TMA cohort. The research group consisted of 11 females and 61 males. The mean age of patients was 71.5 years (range 45–88 years) and the median follow-up time was 5 years. Among 72 patients, 34 (47.22%) were diagnosed with low-grade tumors and 38 (52.78%) were diagnosed with high-grade tumors. 39 (54.17%) tumors were classified as T1, 20 (27.78%) as T2, 9 (12.5%) as T3, and 4 (5.56%) as T4. The samples were categorized as low stage (T1) or high stage (T2-4). Nine (12.5%) patients were diagnosed with lymph node metastases and seven (9.72%) patients had distant metastases at the time of diagnosis. The mean 5-year overall survival time was 45.3 months, ranging from 5.0 to 60.0 months.

3.2. CDK9 Is Overexpressed in Bladder Cancer

To explore the characteristics of CDK9 staining patterns in urothelial cancer and control samples, we performed immunohistochemical staining using a monoclonal CDK9 antibody (1:200; 40 min; cat. no: ab76320, Abcam, Cambridge, MA, USA). CDK9 expression was present in all examined samples in both study and control groups. Strong immunoreactivity was observed in bladder cancer samples and was significantly higher than in the control group (median H-SCORE = 204 vs. 170.5 respectively, $p = 0.0022$) (Figure 1). CDK9 is overexpressed in urothelial carcinoma.

3.3. CDK9 Expression Correlates with Disease Course in Bladder Cancer TMA Cohort

According to the Mann–Whitney U test, CDK9 expression was significantly higher in the lower stage (pT1 vs. pT2–4; $p = 0.0172$), lower grade (low vs. high; $p = 0.04$), and non-invasive tumors (NMIBC vs. MIBC; $p = 0.0075$) (Figure 2). The detailed description of CDK9 expression in selected groups is assembled in Table 2. CDK9 expression in T1 tumors was significantly higher than in the T2–T4 group and in the control. However,

we found no significant difference between CDK9 expression in the T2–T4 group and the control. Spearman's correlation coefficient showed a weak to moderate negative correlation between CDK9 expression and tumor stage, grade, size, and invasiveness ($p < 0.05$). CDK9 expression did not correlate with metastasis, lymph node invasion, recurrence, or progression of the disease ($p > 0.05$) (Table 2).

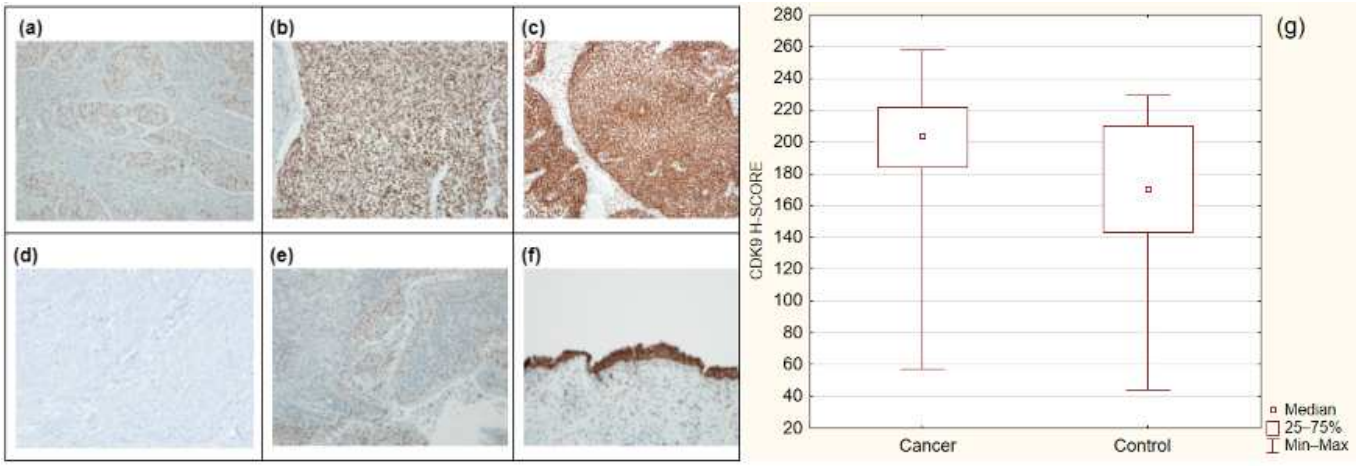

Figure 1. Representative cross-sectional staining patterns at x10 magnitude; (**a**) invasive bladder cancer with low CDK9 expression; (**b**) non-invasive bladder carcinoma with medium CDK9 expression; (**c**) non-invasive bladder cancer with high CDK9 expression; (**d**) CDK9 negative control; (**e**) normal mucosa with low CDK9 expression; (**f**) normal mucosa with high CDK9 expression and positive reaction in the cells of the stromal inflammatory infiltration; (**g**) CDK9 expression in cancer and control groups ($p = 0.0022$).

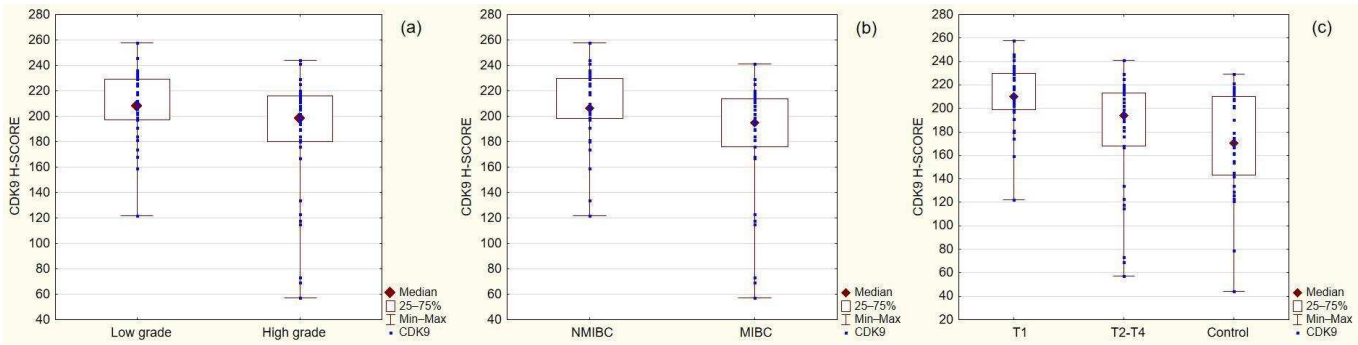

Figure 2. CDK9 expression depending on: (**a**) tumor grade ($p = 0.04$); (**b**) tumor invasiveness ($p = 0.0075$); (**c**) tumor stage ($p = 0.0001$). NMIBC—non-muscle-invasive bladder cancer; MIBC—muscle-invasive bladder cancer.

To determine the prognostic value of CDK9 expression in patients with urothelial carcinoma, we dichotomized the samples into low and high CDK9 expression groups, with the cutoff point being 219 H-score. Patients with high CDK9 expression had a significantly higher 5-year overall survival (OS) rate than patients with low CDK9 expression (77.54% vs. 53.6%, respectively; $p = 0.04$) (Figure 3). The Kaplan–Meier analysis of OS by quartiles showed significant differences in OS between patients in the lower and upper quartiles of CDK9 expression ($p = 0.039$) (Figure 3).

Table 2. Correlation between CDK9 expression and clinical predictors for bladder cancer.

Clinical Data	Total *N*	Median CDK9 Expression (Min–Max)	Q1	Q3	Statistical Differences between Groups ($p < 0.05$)	CDK 9 Expression Correlation (Spearman's Correlation Coefficient)
Cancer group	72	204 (57–258)	184	222	-	
Low grade	34	208 (122–258)	197	229	$p = 0.04$	-0.283 ($p < 0.05$)
High grade	38	198.5 (57–244)	180	216		
T1	39	210 (122–258)	199	230	$p = 0.0001$	-0.35 ($p < 0.05$)
T2–T4	33	194 (57–241)	168	213		
NMIBC	36	206 (122–258)	198	229	$p = 0.0075$	-0.34 ($p < 0.05$)
MIBC	35	195 (57–241)	176	214		
N0	61	206 (57–258)	190	225	$p = 0.31$	0.05 ($p > 0.05$)
N1-3	9	184 (69–246)	167	218		
M0	62	204.5 (57–258)	181	224	$p = 0.91$	0.026 ($p > 0.05$)
M1	7	205 (69–244)	184	225		
Progression	17	195 (69–244)	197	226	$p = 0.19$	0.16 ($p > 0.05$)
Lack of progression	35	210 (115–258)	184	212		

NMIBC—non-muscle-invasive bladder cancer; MIBC—muscle-invasive bladder cancer; N0, N1—3-lymph node metastasis; M0, M1—distant metastasis; Q—quartile.

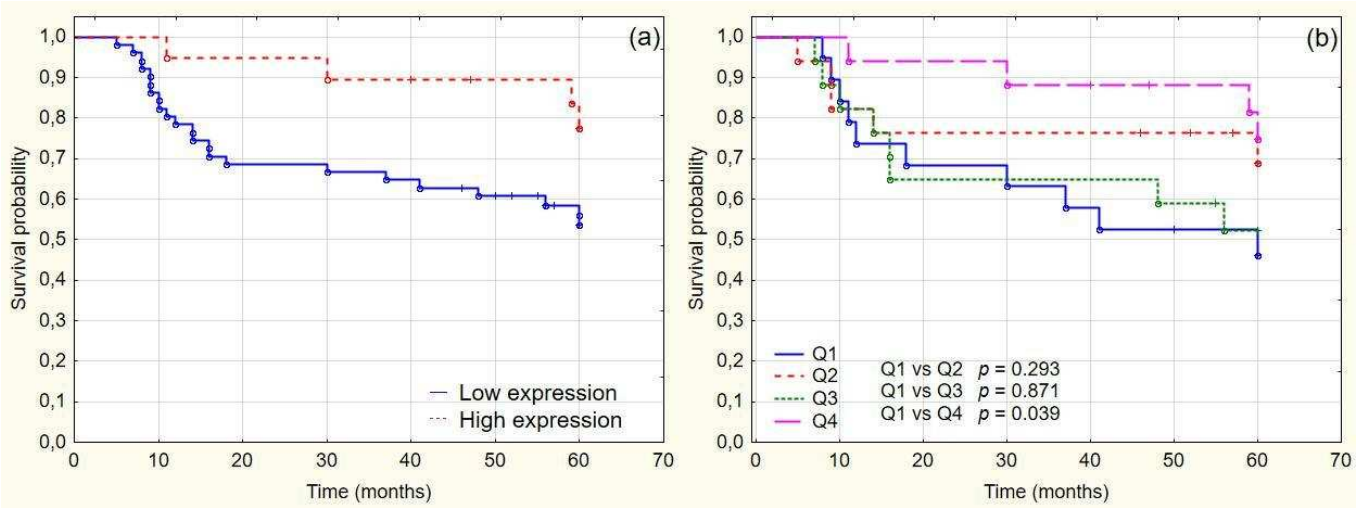

Figure 3. OS analysis of TMA cohort (**a**) overall survival in low CDK9 and high CDK9 groups (5-year OS 77,54% vs. 53.6%, respectively; $p = 0.04$); (**b**) overall survival by CDK9 expression quartiles.

Univariate Cox regression analysis showed that the type of procedure, stage, grade, invasiveness, tumor size, lymph node invasion, presence of distant metastases, and progression were significant prognostic factors. In multivariate analysis, only the occurrence of progression remained statistically significant (<0.05) (Table 3). CDK9 status was not statistically significant for the prognosis of overall survival (HR 2.7, CI95% 0.93–7.82, $p = 0.06$), but due to borderline statistical significance and small group size, we decided to explore the prognostic value of CDK9 in the TCGA urothelial cancer cohort.

Table 3. Univariate and multivariate analysis of overall survival.

Viable	Univariate Analysis			Multivariate Analysis		
	RR	95% CI	*p*-Value	RR	95% CI	*p*-Value
Age (<70 vs. >70)	0.45	0.17–1.2	0.112	-	-	-
Sex (M vs. F)	0.64	0.19–2.13	0.47	-	-	-
Stage (T1 vs. T2–T4)	0.16	0.06–0.4	0.0001	0.36	0.02–8.55	0.53
Grade (low vs. high)	0.17	0.06–0.45	0.0003	0.85	0.14–5.26	0.86
Invasiveness (NMIBC vs. MIBC)	0.13	0.05–0.34	0.00004	0.65	0.06–6.81	0.72
Lymph node metastasis (N0 vs. N1–3)	0.26	0.11–0.63	0.003	1.1	0.21–5.74	0.91
Distant metastasis (M0 vs. M1)	0.17	0.07–0.42	0.0001	0.35	0.08–1.56	0.17
Tumor size (<3 cm vs. >3 cm)	0.30	0.13–0.72	0.007	0.43	0.12–1.57	0.2
Recurrence (Y/N)	0.35	0.05–2.5	0.295	-	-	-
Progression (Y/N)	22	6.08–79.48	0.000002	7.96	1.48–42.5	0.015
CDK9 (low vs. high)	2.7	0.93–7.82	0.06	-	-	-

3.4. TCGA Urothelial Bladder Cancer Cohort

We found that CDK9 expression correlates with higher OS rate, lower stage, and grade (Figure 2, Table 2). However, due to a relatively small number of cases, with only 13 tumors being T3 or T4, as well as a lack of statistical significance in Cox regression analysis and contradictory reports from other researchers, we deemed it necessary to validate our findings. To assess a larger group of patients, we accessed The Cancer Genome Atlas (TCGA) database and obtained transcriptomics information through the Human Pathology Atlas. The TCGA cohort consisted of 406 cases with urothelial bladder cancer, out of which 273 samples were high stage (T3 or T4) (Table 4) [23]. The Ensembl gene id, available from TCGA, was used to map the TCGA RNA-seq data and the FPKMs (number of fragments per kilobase of exon per Million reads). Based on the FPKM value of CDK9, the samples were dichotomized into the low expression and high expression groups. Univariate Cox regression analysis showed that age, stage, and CDK9 status were statistically significant prognostic factors. All predictors, including CDK9 status (HR 1.60, CL95% 1.1–2.33, $p = 0.014$) remained significant in multivariate analysis (Table 5). The 5-year survival rate in patients with high CDK9 expression reached 57.75% and was significantly higher than the 35.44% 5-year survival rate in the low CDK9 expression group ($p < 0.005$) (Figure 4). We found no differences between patients' OS by quartiles in the Kaplan–Meier analysis (Figure 4). The results obtained from the TCGA cohort are consistent with the findings in the TMA cohort.

Table 4. Baseline characteristics of TGCA ($n = 406$) cohort.

Clinical Data		*n* (%)
Age (years)		68.1 (range 34–90)
Median follow-up time		1.44 years
Sex	male	299/406 (73.65%)
	female	107/406 (26.35%)
Stage	I	2/406 (0.49%)
	II	129/406 (31.77%)
	III	140/406 (34.48%)
	IV	133/406 (32.76%)
Disease course	Alive	227/406 (55.91%)
	Dead	179/406 (44.09%)

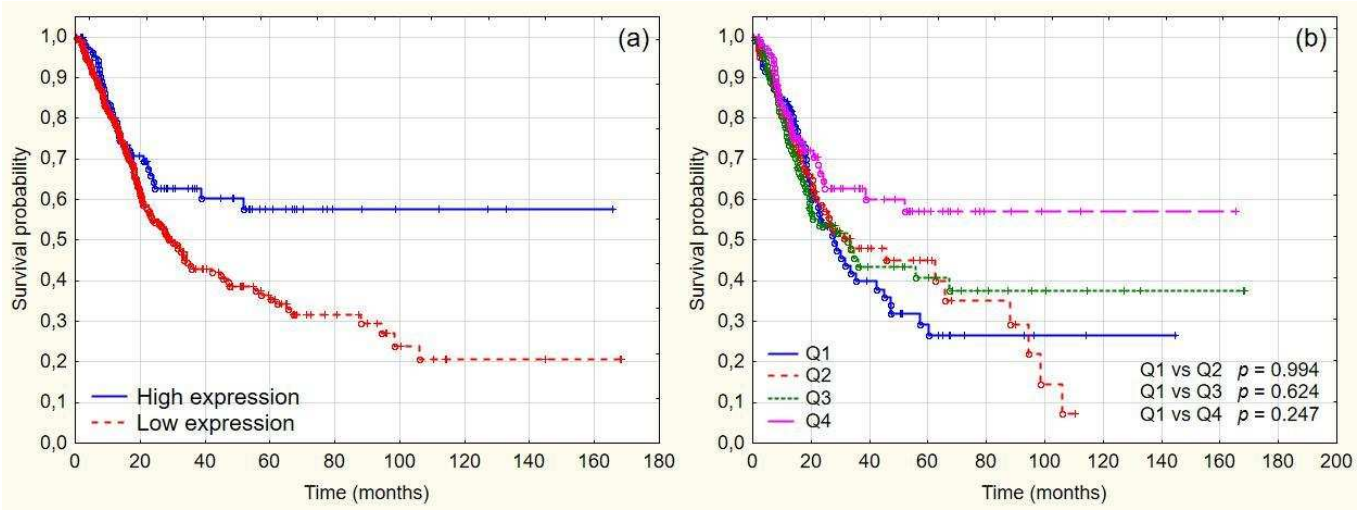

Figure 4. Overall survival analysis of TCGA cohort (**a**) overall survival in low CDK9 and high CDK9 groups (5-year OS 57.75% vs. 35.44%), respectively (p = 0.009); (**b**) overall survival by CDK9 expression quartiles.

Table 5. Univariate and multivariate analysis of overall survival in the TCGA cohort.

Variable	Univariate Analysis			Multivariate Analysis		
	RR	95% CI	*p*-Value	RR	95% CI	*p*-Value
Age (<70 vs. >70)	0.63	0.47–0.85	0.002	0.64	0.48–0.86	0.003
Sex (M vs. F)	1.16	0.83–1.6	0.38	-	-	-
Stage (T1 vs. T2–T4)	0.46	0.32–0.67	0.00004	0.48	0.33–0.69	0.00008
CDK9 (low vs. high)	1.61	1.11–2.33	0.01	1.60	1.1–2.33	0.014

CDK9 expression was prognostic in the TCGA cohort, and its high expression predicts longer overall survival in urothelial bladder cancer.

4. Discussion

4.1. The Prognostic Role of CDK9 in Cancers

The presented results show that although CDK9 is overexpressed in all stages and grades of bladder cancer when compared to normal bladder tissue, its expression decreases in line with higher grade and stage. With the Kaplan–Meier estimator, the results indicate that CDK9 may predict a good prognosis in patients with bladder cancer. However, available literature mentions an ambiguous prognostic role of CDK9, which differs in various types of cancers.

Kretz et al. [15] showed that CDK9 is overexpressed in pancreatic ductal adenocarcinoma and higher CDK9 expression correlates with shorter survival times in PDAC patients. In Ma et al.'s study, CDK9 expression is inversely correlated to the percent of tumor necrosis post-neoadjuvant chemotherapy, an important predictive factor for disease outcomes in osteosarcoma patients and correlates with worse prognosis [14]. Wang et al. reported that in ovarian cancer high-CDK9 expression correlated with significantly shorter overall survival time and disease-free survival. CDK9 expression was also significantly higher in the patient-paired metastatic and recurrent tissue when compared to primary ovarian cancer tissue [24]. Similarly, Parvathareddy et al. reported that high CDK9 expression is an indicator of poor prognosis, tumor recurrence, and high Ki-67 index in epithelial ovarian cancer (441 samples) [25]. On the other hand, Schlafstein et al.'s study revealed that high CDK9 expression was associated with longer overall survival starting in patients at 3 years after the initial surgery, who did not achieve complete response after neoadjuvant chemotherapy [26].

4.2. CDK9 in Cell Differentiation and Carcinogenesis

Berthet and Kaldis [27] suggested that well-differentiated cells are more sensitive to cell cycle dysregulation. In mouse models, cell cycle regulating mechanisms were different in embryonic stem cells and differentiated cells [28]. When tumor cells proliferate, they start to behave similarly to stem cells. However, inhibition of CDK can affect proliferation, and in this scenario, tumor cells behave more like differentiated cells. It seems plausible that in specific tumors, CDK overexpression may be pivotal especially in the early stages of disease when relatively well-differentiated cells are more dependent on Cdk/cyclin complexes and the genomic instability is still limited. Schlafstein et al. drew similar conclusions, arguing that if low CDK9 expression leads to increased DNA damage and genetic instability, then the disease in patients with low CDK9 expression may be more aggressive [26,29]. This thesis seems to be reinforced by De Falco et al.'s study, where higher expression of CDK9 was observed in PNET and neuroblastoma tumors with more differentiated cells [10].

In our study CDK9 expression was the highest in the low-stage tumors, suggesting that CDK9 overexpression may play an important role in cancer development, but its role decreases when the genomic instability increases (Figure 2). The main implications of CDK9 overexpression are summarized in Figure 5 [12,13,30]. Similarly, CDK9 expression was higher in the low-grade group than in the high-grade group, which can be attributed to the increasing independence from cell cycle regulators in higher-grade cancers and suggest that the role of CDK9 may be marginalized as the disease progresses [31] (Figure 2).

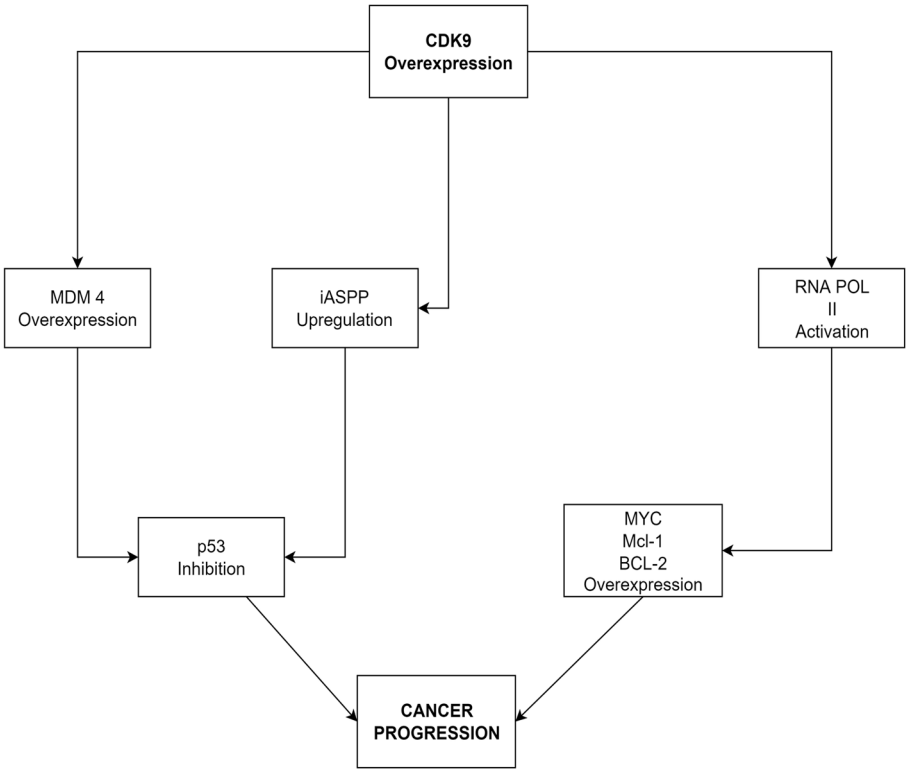

Figure 5. The role of CDK9 overexpression in cancer progression. CDK9 activates the mouse double minute 4 (MDM4) and the inhibitor of apoptosis-stimulating protein of p53 (iASPP) proteins, which inhibit the tumor-suppressing functions of p53 and disturb genomic integrity. Overexpression of CDK9 increases the activity of RNA polymerase II and causes an increase in anti-apoptotic proteins, such as c-Myc, Mcl-1, and Bcl-2, preventing the programmed death of cancer cells. Both pathways lead to the accumulation of genetic changes and the progression of the disease [12,13,30].

4.3. CDK9 and Genome Stability

Yu et al. suggested that CDK9 plays a key role in maintaining genome integrity in response to replication stress [32]. CDK9 silencing in U2OS cells resulted in delayed progres-

sion through S-phase. Similar results were observed in human telomerase-immortalized epithelial cells, suggesting that the effects are independent of cell type. The recovery defect was similar to that after treatment of aphidicolin, a DNA polymerase inhibitor. In the absence of exogenous damage, CDK9-silencing caused no changes in proliferation and apoptosis. The induction of DNA damage after CDK9 knockdown led to replication fork instability and breakdown, even in the absence of added genotoxic agents [32–34]. Those findings suggest that CDK9 is needed to complete DNA synthesis and contributes to maintaining genome integrity in a response to replication stress. Interestingly, only the deficit in cyclin K, but not cyclin T1 or cyclin T2, impaired the cell cycle recovery, suggesting that cyclin K is the regulatory subunit of CDK9, which mediates its activities in the RSR [32].

Low-grade bladder cancers are usually non-muscle-invasive, but due to the increasing genomic instability, they may progress to invasive tumors [35,36]. In Vaish et al. study cancer microsatellite instability was observed frequently in high-stage (40.6%) and high-grade (59.4%) tumors [36]. CDK9 accumulates in response to replication stress and lifts the burden of transcriptional stress by limiting the amount of single-stranded DNA in cells. CDK9 knockdown increases the spontaneous DNA damage signaling in replicating cells and impairs their ability to recover from a transient cell cycle arrest [32]. Relatively high CDK9 expression in the non-muscle-invasive and low-grade cancer groups (Figure 2) seems to be in line with those reports.

The p53 human suppressor gene plays a pivotal role in maintaining genomic stability by regulating the cell cycle, cell differentiation, DNA repair, and apoptosis [37]. The loss of p53 function is associated with lower overall survival of bladder cancer patients and is the most prevalent in high-grade, high-stage, and muscle-invasive cancers [38–41]. CDK9 and p53 form a feedback loop, in which CDK9 phosphorylates p53 and renders its ability to cause cell cycle arrest and apoptosis, while p53 increases CDK9 gene expression [42]. In response to DNA damage, p53 also activates the transcription of cyclin K, critical for genomic maintenance and replication shock response. As cyclin K and cyclin T differ structurally, the CDK9-cyclin K complex can act independently of cyclin T [29,43]. The reduction in CDK9 activity may be a direct consequence of p53 mutation, its subsequent loss of function, and dysregulation of the p53-CDK9 feedback loop. In that sense, CDK9 expression may be an indicator of p53 functionality. We hypothesize that in low-grade urothelial cancer, CDK9 overexpression may diminish p53 activity, facilitating progression. However, in high-grade tumors, p53 is usually mutated or inactive, therefore the relatively reduced expression of CDK9 would limit the stabilizing activity of cyclin K and further impair DNA repair mechanisms [44].

The pathways between CDK9, p53, and other tumor-suppressive proteins are well established, but it is still unclear whether the decrease in CDK9 activity arises from their interactions. The assumption that the relationship between CDK9 and other proteins differs in nature when compared to other malignancies seems doubtful. The relatively decreased CDK9 expression may be a direct manifestation of genomic instability. The most frequent genetic alteration in transitional cell carcinoma is the loss of chromosome 9, occurring in >50% of bladder tumors for all grades and stages [45]. Deletions of chromosome 9 more frequently affect 9q than 9p and are more prevalent in higher-grade tumors [46,47]. Tumors with deletions of the regions 9ptr-p22, 9q22.3, 9q33, and 9q34 recur more rapidly than those without deletion [47]. Loss of heterogeneity of 9q is considered a very early genomic alteration in bladder cancer pathogenesis and the most common event amongst a series of copy number changes, suggesting that loss of 9q leads to a rapid increase in genomic instability [45]. Since the CDK9 gene is located on chromosome 9q34, it is possible that the decrease in CDK9 expression is a result of CDK9 knockdown in genetically unstable cells and reflects the destabilization of the genome [48,49]. According to this hypothesis, in cancers with a relatively lower frequency of somatic mutations, the expression of CDK9 is not hindered by genomic instability and high CDK9 expression may predict a poor prognosis. However, in cancers where somatic mutations are more frequent, the genomic instability, including early deletions of 9q, may decrease the expression of CDK9. This

statement seems to be true for bladder cancer and lung cancer, which are characterized by a high frequency of somatic mutations and in which low CDK9 expression correlates with a shorter overall survival time [50,51]. In those tumors, low CDK9 expression may be an indicator of more aggressive disease.

Low CDK9 expression in urothelial cancer tissues correlates with more advanced, higher-grade, and muscle-invasive disease, therefore subjecting low-expression patients to more aggressive therapy may provide clinical benefits. DNA repair gene mutations are prevalent in this group; therefore, combined therapy of CDK9 inhibitors with other agents that impair DNA repair, such as PARP inhibitors, may be beneficial [52]. However, co-inhibition of CDK9 and PARP has yet to be proven in urothelial cancer cell lines [45]. Furthermore, CDK9 silencing resulted in no modification of DNA repair genes in SAS and FaDu cells, suggesting another mechanism of action [29,46,53]. On the other hand, in the early stage of urothelial cancer, where CDK9 expression is the highest, CDK9 inhibition can inhibit transcription of anti-apoptotic proteins, impair tumor growth, reactivate wild-type p53 and increase its concentration, thereby preventing disease progression [12,47,54]. As CDK9 has yet to be investigated as a therapeutic target in urothelial carcinoma, preclinical studies should be performed before attempting clinical trials.

5. Conclusions

Higher CDK9 expression correlates with a lower grade, lower stage, and non-muscle-invasive bladder cancer. Urothelial bladder cancer patients with higher CDK9 expression had a higher 5-year overall survival rate when compared to the low CDK9 expression group. Contrary to results from other malignancies, CDK9′s role in bladder cancer seems different. Its high expression seems to be more significant in low-stage tumors, where p-53 mutations are rare and the genome is stable. Along with the increase in genomic instability, CDK9 decreases due to a decrease in p53 functionality, deletions of chromosome 9q, or dedifferentiation of cancer cells. Although our findings suggest that the CDK9 influence on disease progression is not clearly negative, there are no proven mechanisms that would confirm CDK9′s duality in carcinogenesis. The ambiguous role of CDK9 needs further evaluation.

Author Contributions: Conceptualization: J.B., K.S. and M.M.; writing—original draft preparation: J.B., K.S., M.M. and Ł.S.; formal analysis: M.Z., P.S. and M.B.; visualization: J.B., K.S., P.A. and Ł.S.; staining evaluation: M.Z., S.K., M.M., K.S. and J.B.; investigation: P.S., M.B., M.Z. and P.A.; acquisition: P.A., S.K., P.S. and M.B.; writing—review and editing: J.B., M.S.-D. and M.D. All authors have read and agreed to the published version of the manuscript.

Funding: The authors did not receive support from any organization for the submitted work.

Institutional Review Board Statement: The study was conducted following the Declaration of Helsinki, and the protocol was approved by the Bioethics Committee (KB881/2019).

Informed Consent Statement: The requirement for patient consent was waived due to the retrospective nature of the study by the Ethics Committee.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions. Publicly available datasets were analyzed in this study. This data can be found here: https://v21.proteinatlas.org/ENSG00000136807-CDK9/pathology/urothelial+cancer/ (accessed on 10 November 2021) [24].

Conflicts of Interest: The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Rozdział 4. Publikacje

The prognostic role of p53 and its correlation with CDK9 in urothelial carcinoma

Borowczak J, Szczerbowski K, Maniewski M, Zdrenka M, Słupski P, Andrusewicz H, Łysik-Miśkurka J, Rutkiewicz P, Bodnar M, Szylberg Ł.

Clinical and Translational Oncology. 2023 Mar;25(3):830-840.

doi: 10.1007/s12094-022-02994-6.

IF: 3.340 MNISW: 70

Clinical and Translational Oncology
https://doi.org/10.1007/s12094-022-02994-6

RESEARCH ARTICLE

The prognostic role of p53 and its correlation with CDK9 in urothelial carcinoma

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Received: 23 September 2022 / Accepted: 25 October 2022

Abstract

Purpose The mutation of p53 is considered a pivotal step in bladder cancer pathogenesis. Recently, distinct interactions between p53 and CDK9, a transcription regulator, have been described. In this work, we explored the prognostic role of p53 expression and evaluated its associations with CDK9 in urothelial carcinoma.

Materials and methods The research group consisted of 67 bladder cancer samples and 32 normal urothelial mucosa samples. All specimens were analyzed using ImageJ and the IHC profiler plugin. To validate the results, 406 cases from The Cancer Genome Atlas database were analyzed.

Results P53 and CDK9 are overexpressed in urothelial cancer tissues when compared to normal urothelial tissues ($p < 0.05$). High p53 expression was observed in metastatic tumors and tumors with high CDK9 expression ($p < 0{,}05$). High p53 expression was predictive for shorter survival in patients with non-muscle-invasive bladder cancer (HR = 0.107 [0.012–0.96]; $p = 0.046$) but did not correlate with prognosis in the muscle-invasive group. In high CDK9 cancers, high p53 expression correlated with the occurrence of high-grade and muscle-invasive tumors ($p < 0.05$).

Conclusion High expression of p53 correlates with unfavorable clinical features of bladder cancer. CDK9 is associated with the expression of p53, possibly through interactions with p53 inhibitors. Since the blockade of CDK9 in other malignancies reactivates wild-p53 activity, confirming the crosstalk between p53 and CDK9 in bladder cancer may be another step to explain the mechanism of tumor progression in its early stages.

Keywords P53 · CDK9 · Bladder cancer · Expression · Prognosis

Introduction

Bladder cancer is one of the most common malignancies worldwide, with approximately 524,000 cases annually [1]. In 2019, its incidence and mortality rates increased and were estimated at 6.5 and 2.9 per 100 000, respectively, accounting for 229,000 deaths and 4.39 million disability-adjusted life years [1]. In many European countries, the prevalence of bladder cancer is still on a rise, presumably due to the popularity of smoking and an aging population [2]. The survival time and rate depend on early diagnosis; the 5-year survival reaches up to 95.8% among those with an in situ disease, 69.5% in localized disease, and only 4.6% in metastatic cancer [2]. Although 51% of all patients are diagnosed with carcinoma in situ, others are usually not suitable for radical treatment. Urothelial carcinoma (BLCA) is the most common histologic type of bladder cancer and constitutes approximately 90% of all cases [3]. The genetic

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Published online: 14 November 2022

abnormalities that accumulate during the progression of the disease may prevent the apoptosis of cancer cells and hinder the efficacy of systemic therapy [4]. In such cases, genomic profiling may be a key point to truly personalize care for bladder cancer patients. Therefore, finding new prognostic markers and therapeutic targets seems of great importance.

Tumor protein 53 (p53) is a major tumor suppressor encoded by the *TP53* gene located on human chromosome 17 [5]. P53 is post-translationally stabilized and activated in response to cellular stress, including DNA damage, hypoxia, and mitogenic oncogenes [6]. By intervening in the activity of its direct target genes, such as cyclin-dependent kinases, DNA repair genes, or apoptotic proteins, p53 alleviates cellular stress, maintains genome integrity, and prevents the initiation of carcinogenesis [5, 6]. TP53 is mutated in about half of human cancers. The hereditary loss of p53 function is associated with the occurrence of aggressive cancers, especially in young patients [7]. Therefore, p53 has become a potential therapeutic target. Recently, its newly discovered interactions with cyclin-dependent kinases have shed new light on how its activity may influence the early steps of tumorigenesis [8].

Cyclin-dependent kinases are a family of kinases that must bind to their regulatory proteins, cyclins, to gain enzymatic activity [9]. Cyclin-dependent kinase 9 (CDK9) is a transcription-regulating protein that has recently gained attention after promising in-vivo and in-vitro trials in multiple human cancers [10]. CDK9 binds to cyclin T, forming positive transcription elongation factor-B (P-TEFb), and stimulates transcription through the activity of RNA polymerase II (RNA POL II). Its overexpression may cause the accumulation of anti-apoptotic proteins, such as MYC or Mcl-1, disrupt cellular homeostasis, and promote the immortalization of abnormal cells [8, 10]. As a central regulatory hub of transcription, CDK9 is required for cell proliferation, differentiation, and apoptosis. It is also believed to partake in tumor growth via the p53-related pathway [11, 12]. Currently, two isoforms are known: 42-kDa and 55-kDa; they may differ functionally and prognostically. The upregulation of CDK9 42-kDa was recently associated with increased cell proliferation and survival, while no such activity of CDK9 55-kDa was detected [13, 14]. Instead, the 55-kDa isoform seems to mediate DNA repair through the Ku70-associated pathway, suggesting its potential role in maintaining genomic stability [15].

Recently, two novel drug regimens, immune checkpoint inhibitors, and fibroblast growth factor receptor tyrosine kinase inhibitors, have been approved for the treatment of bladder cancer. Nevertheless, frequent chemoresistance and low response rates prompt further research for novel therapeutic targets. In this work, we evaluate the prognostic value of p53 in urothelial carcinoma and investigate its possible correlations with CDK9 expression.

Materials and methods

Patients and tissue samples

The study included paraffin-embedded blocks containing tissue samples that were collected from urothelial carcinoma patients treated in the Department of Urology. The research group consisted of 67 bladder cancer samples, while 32 normal urothelial mucosa samples were used as a control group. All samples were collected during either transurethral resection of bladder tumor (TURBT) or radical cystectomy (RC). Clinical data, including age, sex, tumor grade and stage, cancer invasiveness, lymph node metastases, tumor size, the occurrence of progression and recurrence, as well as overall survival time were obtained (Table 1). The study was conducted following the Declaration of Helsinki, and the protocol was approved by the Bioethics Committee (KB881/2019).

Sample staining

A retrospective immunohistochemical analysis of p53 comprised 67 formalin-fixed, paraffin-embedded tissue blocks derived from 67 bladder cancer patients. The tissue block was cut into 5 µm sections, attached to a glass slide, and incubated at 60 °C for 2 h. IHC staining was performed on the Ventana Benchmark Ultra platform according to NordiQC operating procedure. A primary p53 monoclonal antibody (Bp53-11) was used for staining.

The expression of CDK9 was determined using IHC assays according to the protocol described in Buchholz et al. study [16]. In the beginning, standardization and optimization of the IHC method were performed on a recommended tissue, based on the antibody datasheet and reference sources (The Human Protein Atlas: https://www.proteinatlas.org; [17]). In brief, 3 µm thick sections of the tissue arrays were baked for 1 h at 60 °C before xylene deparaffinization and subsequent rehydration through graded ethanol (99.8, 96, 90 and 80%). Tissue sections were incubated with a primary rabbit monoclonal anti-CDK9 antibody (1:200, 40 min; ab76320, Abcam). Primary antibodies were visualized using either the UltraView Universal DAB Detection Kit (Roche Diagnostics/Ventana) followed by color development using 3,3-diaminobenzidine. The slides were counterstained with Hematoxylin II for 12 min and Bluing Reagent for 4 min. Finally, tissue sections were dehydrated in increasing ethanol concentrations (80, 90, 96, and 99.8%), cleared in xylenes (I–IV), mounted using a mounting medium, and examined.

Image acquisition and immunohistochemical analysis

The immunohistochemically stained slides were scanned by Ventana DP 200 Slide scanner (Roche Diagnostics). For each

Table 1 Clinicopathological characteristics of the study group

Variables	*n* (%)
Age (mean)	73.37 years (range 45–88 years)
Median follow-up time	60 months (range 5–60 months)
Sex	
Female	11/67 (16.42%)
Male	56/67 (83.58%)
Grade	
Low (G1)	32/67 (47.76%)
High (G2. G3)	35/67 (52.24%)
Stage	
Ta-T1	36/67 (53.73%)
T2	19/67 (28.36%)
T3	8/67 (11.94%)
T4	4/67 (0.06%)
Lymph node invasion	
N0	56/67 (83.58%)
N1-3	9/67 (13.43%)
Unknown	2/67 (0.03%)
Distant metastasis	
No	57/67 (85.07%)
Yes	7/67 (10.45%)
Unknown	3/67 (4.48%)
Size	
< 3 cm	31/67 (46.27%)
≥ 3 cm	36/67 (53.73%)
Invasiveness	
NMIBC	33/67 (49.25%)
MIBC	33/67 (49.25%)
Unknown	1/67 (1.5%)
Type of procedure	
TURBT	33/67 (49.2%)
RC	29/67 (43.28%)
Unknown	5/67 (7.46%)
Progression	
No	32/67 (47.76%)
Yes	16/67 (23.88%)
Unknown	19/67 (28.36%)
Recurrence	
No	7/67 (10.48%)
Yes	23/67 (24.33%)
Unknown	37/67 (55.22%)
Mean recurrence time	13.0 months (range 0–60 months)
Disease course	
Alive	40/67 (59.7%)
Dead	27/67 (40.3%)

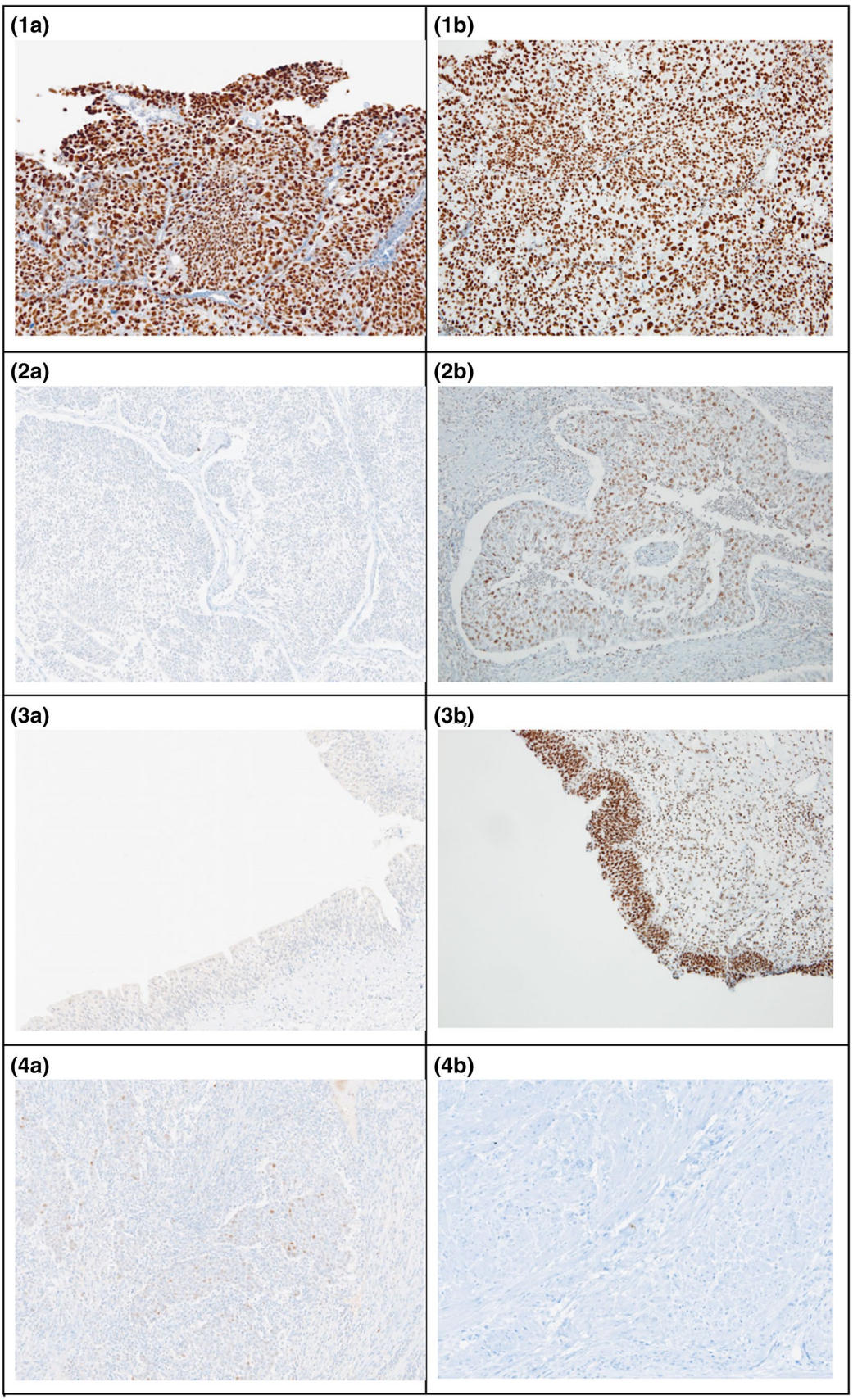
sample, two experienced pathologists selected the most representative regions and captured images at x10 magnification with a VENTANA Image Viewer v. 3.2.0. The analysis was performed using the ImageJ 1.53j version (NIH, Bethesda, Maryland) (Java 1.8.0_172) and the IHC profiler plugin. The expressions of p53 and CDK9 were assessed by following the standard protocol designed by Varghese et al. [18]. The highly positive zone was found to be ranging from 1 to 60; 61 to 120 for the positive zone; 121 to 170 for the low positive zone; and 181 to 220 for the negative zone, respectively. The intensity values ranging from 221 to 255 predominantly represent fatty tissues, stroma, or background artifacts that do not contribute to pathological scoring and were therefore excluded from the score determination zones. For each sample, the expression of p53 and CDK9 was obtained by calculating the H-Score. H-score was assigned using the formula [1 × (% cells low positive) + 2 × (% cells positive) + 3 × (%cells high positive)], obtaining a value from 0 to 300.

In silico analysis

The analysis was carried out using the data gathered from The Human Pathology Atlas (www.proteinatlas.org), cBioPortal [19] and The Cancer Genome Atlas (TCGA) database [20]. The TCGA cohort consisted of 406 patients diagnosed with urothelial carcinoma. The TCGA RNA-seq data were mapped using the Ensembl gene id available from TCGA, and the FPKMs (Fragments Per Kilobase of exon per Million reads) for TP53 and CDK9 were used to perform the quantitative analysis of their expression. The patients were classified into two expression groups based on the FPKM value. The best cutoffs were chosen using the Cutoff Finder web app [21]. Cancers with an expression of TP53 lower than 23.5 FPKM were considered low-TP53 and those with an expression equal to or higher than 23.5 FPKM were classified as high-TP53. Similarly, if the expression of CDK9 was lower than 13, the tumors were classified as low CDK9, otherwise were considered high-CDK9.

Statistical analysis

All statistical analyses were performed using Statistica version 13.3 (Statsoft) and Microsoft Excel 2019. The p value was considered statistically significant if $p < 0.05$. Continuous variables were tested for normality by the Kolmogorov–Smirnov test. The relations between groups of categorical variables were analyzed in the Mann–Whitney U Test or the ANOVA Kruskal–Wallis test. Correlations between clinicopathological features and p53 expression were evaluated using Pearson's correlation coefficient or Spearman's rank correlation coefficient. Univariate and multivariate analyses of potential predictors of overall survival were performed using Cox proportional hazard regression. Results were expressed as hazard ratio (HR) and 95% confidence interval (CI). The two-sided p value of < 0.05 was considered to indicate statistical significance. The relation between p53 expression with overall survival was evaluated


(1a)
(1b)
(2a)
(2b)
(3a)
(3b)
(4a)
(4b)

◂**Fig. 1** Representative cross-sectional staining patterns of **1a** bladder cancer with high p53 expression; **1b** bladder cancer with high CDK9 expression; **2a** bladder cancer with low p53 expression; **2b** bladder cancer with low CDK9 expression; **3a** normal mucosa with low p53 expression; **3b** normal mucosa with high CDK9 expression and positive reaction in the cells of the stromal inflammatory infiltration; **4a** p53 negative control; **4b** CDK9 negative control

with a log-rank test and presented using the Kaplan–Meier estimate.

Results

Patients characteristics

The research group consisted of 11 female and 56 male patients; their mean age was 71.5 years (range 45–88 years) and the median follow-up time was 5 years. Among 67 patients, 32 (47.76%) were diagnosed with low-grade tumors and 35 (52.24%) were diagnosed with high-grade tumors. 36 (53.73%) tumors were low-stage (Ta/T1), while 31 were high-stage (46.27%; T2–T4). At the time of diagnosis 9 (13.43%) patients had lymph node metastases and 7 (9.72%) had distant organ metastases. The mean 5-year overall survival time was 45.26 months, ranging from 5.0 to 60.0 months. The characteristics of this cohort are summarized in Table 1 (Fig. 1).

P53 is overexpressed in urothelial carcinoma

Immunohistochemical staining was evaluated in all samples in the study and the control group. The immunoreactivity observed in bladder cancer samples was significantly higher than in the control group (median H-SCORE = 46 vs. 5, respectively; $p = 0.00001$), and the results retained significance in both high-stage and low-stage tumors (Fig. 2a, b). The expression of p53 was then classified into low and high p53 expression groups with the cutoff being set at 90 H-Score.

P53 expression and clinical features of urothelial carcinoma

We evaluated the correlations between p53 expression and clinical features of BLCA. P53 levels were significantly higher in tumors with distant metastases than in non-metastatic tumors ($p = 0.02$) (Fig. 2c). There were no differences in p53 expression between groups of various stages, grades, invasiveness, tumor size, and lymph node invasion ($p > 0.05$) (Table 2).

The prognostic value of p53 was evaluated separately in muscle-invasive bladder cancer (MIBC) and non-muscle-invasive bladder cancer (NMIBC) patients. In the NMIBC group, patients with high p53 expression had significantly lower overall survival rate (94.44 vs. 57.14%, respectively; $p = 0.015$), lower progression-free survival rate (91.74 vs 52.85%, $p = 0.013$) and higher risk of reduced disease-free survival (HR = 9.63 [1.06–87.67); $p = 0.04$) than patients with low p53 expression after 5 years of follow-up. Univariate analysis revealed that low p53 expression (HR = 0.107 [0.012–0.96]; $p = 0.046$), low tumor grade (HR = 0.15 (0.03 − 0.093), $p = 0.04$) and a lack of distant metastases (HR = 0.06 [0.01–0.37]; $p = 0.002$) were favorable prognostic factors for longer patients' survival in NMIBC (Table 3). P53 was not prognostic of patients' survival in the MIBC group.

CDK9 is overexpressed in bladder cancer and correlates with longer survival

CDK9 staining intensity was measured in normal tissue and bladder cancer samples. CDK9 was overexpressed in the cancer group when compared to the control (196 vs. 166 H-Score, respectively). The expression of CDK9 was also higher in low-grade, non-muscle-invasive, and lower-stage tumors compared to high-grade, muscle-invasive, and high-stage tumors, respectively ($p < 0.05$). The samples were then classified into high-CDK9 and low-CDK9 expression groups, with the cutoff point being 219 H-Score. Patients with high CDK9 expression had a significantly higher 5-year survival rate than patients with low CDK9 tumors (76.19 vs. 51.93%; $p = 0.04$).

Correlations between the expression of p53 and CDK9

We examined correlations between the expression of p53 and CDK9. Tumors with high CDK9 expression showed significantly higher p53 expression than those with low CDK9 (mean H-SCORE 79.5 vs 39, respectively; $p < 0.05$) (Table 2); however, no significant correlation between p53 and CDK9 expressions in the research group was found (Pearson's correlation coefficient $k = 0.14$; $p > 0.05$). In tumors with high CDK9, higher p53 expression was detected in high-grade and muscle-invasive cancers compared to low-grade and non-muscle-invasive tumors ($p < 0.05$) (Fig. 3).

In silico analysis of CDK9 and p53 correlations in urothelial carcinoma

To validate our findings, in silico analysis of p53 and CDK9 expression was performed. We accessed The Human Pathology Atlas (www.proteinatlas.org) and gathered the corresponding data from The Cancer Genome Atlas (TCGA) database [31]. The TCGA cohort consisted of 406 patients diagnosed with urothelial carcinoma. The basic patient characteristics are summarized in Table 4. The median age of

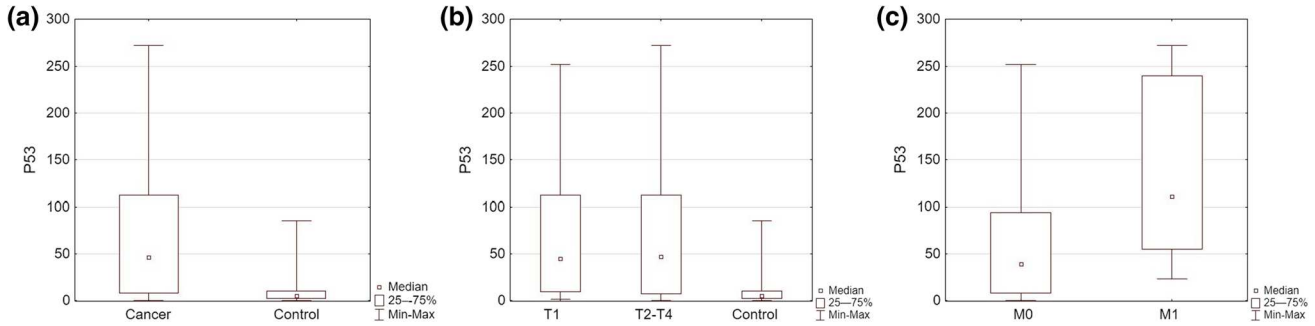


Fig. 2 P53 expression: **a** cancer vs. control ($p=0.00001$), **b** T1 and T2–T4 vs. control ($p=0.0001$), **c** in non-metastatic cancers vs. cancers with distant metastasis (M0 vs M1; $p=0.02$)

Table 2 Correlations between p53 expression and clinicopathological features

Clinical data	Total *N*	Median P53 expression (min–max)	Q1	Q3	Statistical differences between groups ($p<0.05$)
Cancer group	67	46 (0–272)	8	112	$p=0.00001$
Control group	26	5 (0–85)	2	10	
Low-grade	32	47.5 (1–252)	11	95.5	$p=0.84$
High-grade	35	40 (0–272)	7	129	
T1	36	45 (1–252)	9	112	$p=0.88$
T2–T4	31	47 (0–272)	7	112	
NMIBC	33	49 (1–210)	11	113	$p=0.51$
MIBC	33	39 (0–272)	7	73	
N0	56	46.5 (0–272)	9	113.5	$p=0.89$
N1–N3	9	55 (0–252)	23	63	
M0	57	39 (0–252)	8	94	$p=0.021$
M1	7	111 (23–272)	55	240	
Progression	16	70.5 (7–272)	31	187	$p=0.19$
No progression	32	45 (1–252)	11	113.5	
Low CDK9	49	39 (0–272)	7	80	$p=0.027$
High CDK9	18	77 (5–252)	13	207	

NMIBC non-muscle-invasive bladder cancer; *MIBC* muscle-invasive bladder cancer; *N0, N1-N3* lymph nodes metastasis, *M0, M1* distant metastasis, *Q* quartile

Table 3 Univariate analysis of patients' overall survival in non-muscle-invasive bladder cancer

Variable	HR	95% CI	*p* value
Age (< 70 vs. > 70)	0.53	0.06–4.79	0.57
Sex (F vs. M)	0	0–0	0.995
Stage (T1 vs. T2–T4)	0.14	0.015–1.27	0.08
Grade (low vs. high)	0.15	0.03-0.093	0.04
Metastasis (M0 vs. M1)	0.06	0.01–0.37	0.002
Tumor size (< 3 cm vs. > 3 cm)	0.37	0.06–2.2	0.27
Recurrence (Y/N)	0.52	0.05–5.73	0.59
CDK9 (low vs. high)	1.05	0.18–6.29	0.96
P53 (low vs. high)	0.11	0.01–0.96	0.046

patients was 69 years (range 34–90 years) and the median follow-up was 1.46 years (Table 4). The patients were dichotomized into low and high-expression groups. In the TCGA cohort, high CDK9 expression correlated with longer overall survival and favorable clinical features of urothelial carcinoma [22]. We found that tumors with no lymph node metastasis showed higher TP53 levels than those with lymph node metastasis (median FPKM 21.35 vs 18.00; $p<0.05$) (Fig. 4a). The expression of TP53 was not prognostic of patients' survival in this group and did not differ between tumors of different stages, grades, or distant metastatic status ($p>0.05$). There was also no difference in TP53 expression between tumors with mutated and non-mutated TP53.

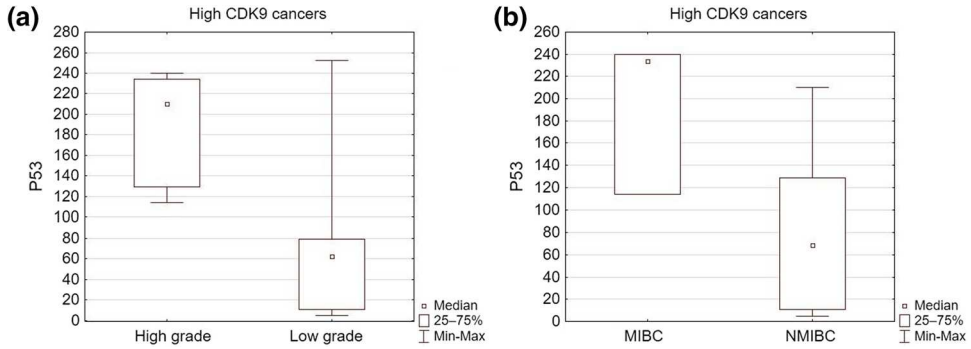


Fig. 3 P53 expression in high CDK9 urothelial cancers depending on: **a** tumor grade ($p = 0.02$), **b** tumor invasiveness ($p = 0.037$) *MIBC* muscle-invasive bladder cancer, *NMIBC* non-muscle-invasive bladder cancer

Table 4 The basic characteristics of The Cancer Genome Atlas cohort

Clinical data	Total $n = 406$ (%)
Sex	
Male	299 (73.65%)
Female	107 (26.35%)
Stage	
I	2 (0.49%)
II	129 (31.77%)
III	140 (34.48%)
IV	133 (32.76%)
Grade	
Low	20 (4.93%)
High	379 (93.35%)
Distant metastasis	
M0	193 (47.54%)
M1	11 (2.71%)
Lymph node metastasis	
N0	234 (57.64%)
N1	126 (31.03%)
Disease course	
Alive	227 (55.91%)
Dead	179 (44.09%)
TP53	
Not mutated	210 (51.72%)
Mutated	196 (48.28%)
Age (median)	69 (range 34–90)
Median follow-up time (months)	17.57 (range 0.43–168.3)

In samples with mutated TP53, the median expression of CDK9 was significantly higher than in samples without mutation (FPKM 18.85 vs 20.00; $p < 0.05$) (Fig. 4b).

The analysis of the TCGA cohort was broadened to investigate the potential correlation with other proteins associated with p53 and CDK9 biology. However, no correlation between the expressions of CDK9, TP53, MYC, Mcl-1, CDKN1A (p21 coding gene), and CDKN2A (p16 coding gene) was statistically significant (correlation coefficients $k < 0.2$) [23–28].

Discussion

We found that p53 is overexpressed in urothelial carcinoma tissues (Table 2, Fig. 2). P53 expression was significantly higher in tumors with distant metastasis when compared to non-metastatic tumors ($p < 0.05$). P53 has turned out to be prognostic in the NMIBC cohort, which seems to be in line with reports regarding the prognostic role of p53 in bladder cancer [29, 30].

In the early stages of BLCA, the overexpression of CDK9 and p53 seems to be a common occurrence. However, even in bladder cancers with high CDK9 expression, which seems to be a feature of less aggressive disease, high expression of p53 is associated with muscle-invasive, high-grade and metastatic cancers [22]. Those results suggest an interplay between CDK9 and p53, which may affect the progression of the disease, especially in its early stages.

The role of p53 in urothelial carcinoma

In this study, p53 was overexpressed in urothelial carcinoma and its levels were higher in high-stage, high-grade, muscle-invasive, and metastatic disease. Those results are consistent with the recent meta-analysis published by Liao et al. and reports regarding the prognostic value of p53 in NMIBC [31, 32]. TP53 mutation is more frequent in muscle-invasive tumors when compared to non-invasive tumors (35 vs 70%), and correlates with tumor grade, stage, and disease recurrence [33–35]. The p53 loss of function often leads to the accumulation of nonfunctional p53 and manifests as overexpression in various stages of carcinogenesis [36]. Although TP53 polymorphism influences the risk of bladder cancer initiation, the overexpression of p53 is consistently associated with an increased risk of T1 NMIBC progression. Given the importance of early treatment and diagnosis, p53 overexpression may be considered an indication for more aggressive treatment [29].

Nuclear p53 phosphoprotein is a regulator of cell proliferation, cell cycle arrest, and apoptosis [37]. While its normal expression suppresses proliferation, in response to cellular stress p53 is upregulated, accumulates in the nucleus, and

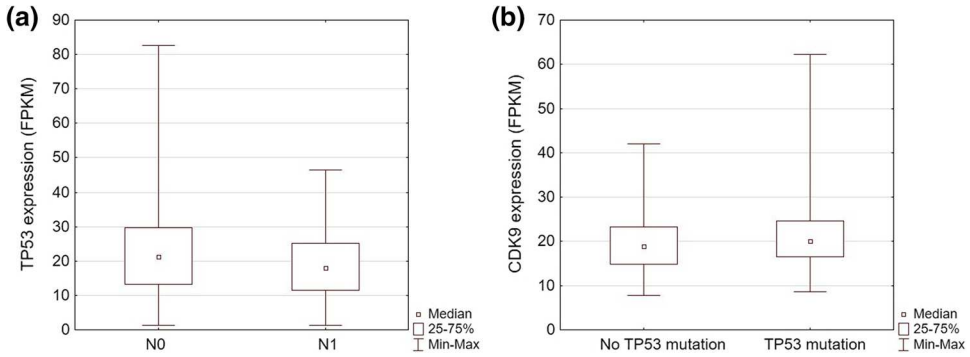


Fig. 4 Statistically significant results of the TCGA cohort analysis: **a** TP53 expression in the TCGA cohort depending on the status of lymph node invasion ($p=0.012$), **b** CDK9 expression depending on the presence of TP53 mutation ($p=0.012$)

can initiate cell death [38]. Furthermore, wt-p53 (wild-type p53) downregulates vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) production, limiting angiogenesis [39]. Its mutation often alters related signaling pathways and could drive the initiation and progression of bladder cancer [40]. In most cases, the inactivation of the TP53 gene is caused by a sporadic loss of function mutation or negative regulation of TP53 activity. More than 75% of TP53 mutations lead to an emergence of a nonfunctional wild-type p53. It not only cannot induce cell cycle arrest, DNA repair, and apoptosis, but can also gain tumorigenic properties and drive proliferation, invasion, and survival of cells, facilitating cancer progression [37, 41, 42]. Alternatively, p53 activity can be diminished by the upregulation of its inhibitors. MDM2, an E3 ubiquitin-protein ligase, mediates the ubiquitination and degradation of p53. Therefore, deregulation of the p53/MDM2 axis may impact patients' survival, accelerate the occurrence of immune resistance and reduce the efficacy of therapy [33, 43].

The role of CDK9 in urothelial carcinoma

The overexpression of CDK9 is frequently reported in cancers and is often associated with unfavorable prognoses. However, in some malignancies, such as PNET and neuroblastoma, its levels increase in line with cell differentiation grade [44]. In our recent study, CDK9 was overexpressed in all clinical stages of bladder cancer, while its levels decreased in line with grade and stage. Moreover, high CDK9 expression measured immunohistochemically correlated with longer patient survival. Those results were subsequently confirmed in The Cancer Genome Atlas cohort [22]. On the contrary, in Antonova et al. Study, CDK9 was upregulated in muscle-invasive bladder cancer samples when compared to non-muscle-invasive samples [45]. Rui et al. identified a novel long noncoding RNA (lncRNA) named GAS6-AS2 that contributed to the progression of bladder cancer cells through the GAS6-AS2/miR-298/CDK9 axis [46]. In this study, GAS6-AS2 knockdown in cancer cells induced G_1 cell cycle arrest, proliferation, endothelial–mesenchymal transition and metastasis, while its overexpression correlated with worse prognosis in BLCA patients. GAS6-AS2 increased the expression of CDK9, while CDK9 knockdown antagonized the effects of GAS6-AS2 on cell migration and proliferation [46].

At first, the initiation of transcription was deemed the main checkpoint of transcriptional regulation. However, as it became apparent that RNA POL II is paused at the promoter-proximal regions of most genes in a strictly regulated manner shortly after the initiation of transcription, the control of transcription elongation gained more attention. CDK9-cyclin T1, as a key part of the PTEF-b complex required to overcome the pause and continue elongation, is now considered the central hub for transcriptional control [47, 48]. As a relatively short-lived protein, with a half-life $T_{1/2}$ of 4–7 h, consistently expressed throughout the cell cycle, CDK9 mediates the production of anti-apoptotic proteins and enables cell division [49]. The CDK9-cyclin T1 activity seems crucial in preventing cell death in the setting of replication stress. There, the functional distinctiveness between CDK9 isoforms seems crucial. The depletion of $CDK9_{55}$ induces double-strand DNA breaks and apoptosis, while no such activity has been reported for the $CDK9_{42}$ isoform. $CDK9_{55}$ interactions with Ku70, a protein partaking in the non-homologous end-joining pathway, might play a role in double-strand DNA break repair. Presumably, cyclin K, but not T is engaged in this process [15]. In addition, CDK9 forms a complex with cyclin K, which functionally substitutes for positive transcription factor b (P-TEFb) and partakes in DNA damage response as a transcriptional target for p53 [50, 51]. In the presence of DNA damage, the depletion of CDK9 and cyclin K, but not cyclin T, hinders cell cycle progression [15].

Thus, CDK9 may play a key role in preventing genome instability in the early stages of carcinogenesis. Yu D.S. et al. observed no changes in proliferation and apoptosis when CDK9 signaling was silenced in the absence of DNA damage. However, in the setting of exogenous stress, CDK9 knockdown was associated with replication fork instability and breakdown. Since only the deficit in cyclin K, but not

cyclin T1 or cyclin T2, hindered the cell cycle recovery, cyclin K seems the more likely mediator of the genome-stabilizing CDK9 activity [50]. Interestingly, the role of cyclin K in DNA damage response seems ambiguous. The overexpression of cyclin K in 98G and U373MG glioblastoma cell lines and SW480 colorectal cancer cell lines suppressed cell growth after being targeted for transcription with p53 [51]. Its interplay with CDK12 seems crucial to maintaining genomic stability; the absence of cyclin K/CDK12 signaling induces spontaneous DNA damage and causes early embryonic lethality in mice [52]. On the other hand, degradation of CCNK/CDK12 in colorectal cancer inhibits cancer cell proliferation and growth in vivo [53]. Therefore, the biological effects of cyclin K activity may differ depending on the presence of exogenous DNA damage, disease stage, and the expression of its co-units.

The prospects of p53 and CDK9 interplay

In settings of cellular stress, p53 recruits various mediators, such as cyclin K, which control the transcription of DNA damage response genes and protect cells from genomic instability [51, 52]. Cyclin T and cyclin K, forming complexes with CDK9, act independently. Therefore, the differences in signaling activity determine whether the cell will survive or undergo apoptosis [50, 54]. CDK9/cyclin T1 and p53 form a regulatory feedback loop, in which CDK9 phosphorylates the C-terminal domain of p53, activating it, while p53 binds to and activates the CDK9 promoter at the N-terminal domain [54, 55]. This mechanism seems to explain why the expression of p53 is higher in high-CDK9 tumors (Fig. 3a). Furthermore, wt-p53 might play a pivotal role in the anti-cancer activity of CDK9 inhibitors. CDK9 phosphorylates MDM2, an E3 ubiquitin-protein ligase which mediates the ubiquitination and degradation of wt-p53 [56]. The inhibition of CDK9 is capable of restoring wild-type p53 activity in tumor cells through the inhibition of MDM2 signaling [57]. However, the outcome depends on the degree of CDK9 blockade. Complete CDK9 inhibition seems to diminish the residual activity of wt-p53, while partial CDK9 blockade has the potential to restore wt-p53 function [12]. CDK9 inhibition has also been reported to limit the activity of iASPP, a preferential inhibitor of p53's pro-apoptotic activity. In hepatocellular carcinoma cells, the overexpression of iASPP has been associated with even worse patients' overall survival than MDM2 overexpression [11]. Given that CDK9 is involved in the regulation of two main p53 inhibitors, its blockade may lead to the restoration of wild-type p53 functions, which has been reported to suppress tumor growth in tumors with a low frequency of p53 mutations. Therefore, CDK9 inhibitors might be most effective in lower-grade bladder cancers, where p53 mutations are still rarer and the genome is more stable than in high-grade tumors [11, 12, 58].

Conclusion

P53 is overexpressed in bladder cancer and its high expression correlates with the occurrence of metastasis. In non-muscle-invasive bladder cancer, p53 is a predictor of shorter overall survival, and shorter progression-free survival, while its expression increases in line with cancer grade. CDK9 is overexpressed in bladder cancer and correlates with favorable clinical features and longer patient survival. Although we found no correlations between the expression of p53 and CDK9, the levels of p53 were higher in cancers with high CDK9 expression. In high-CDK9 cancers, p53 was associated with high-grade and muscle-invasive cancers. Since the inhibition of CDK9 in other malignancies was reported to downregulate the expression of two main p53 inhibitors, MDM2 and iASPP, then its concurrent blockade may be an interesting approach to reactivate wild-p53 activity. Nevertheless, to this day, no clinical trials regarding the use of CDK9 inhibitors in bladder cancer have been conducted.

Author contributions Conceptualization: JB, KS, MM, writing—original draft preparation: JB, KS, MM, MB, ŁS, writing original draft: JB, KS, MM, ŁS, Data curation: PS, Methodology: MB, MZ, HA, JŁ-M, PR, Supervision: MB, ŁS, Visualization: JB, MZ, PR, MM, Validation: JB, HA, JŁ-M, KS; Resources: PS, MZ, HA, JŁ-M, PR, MB, ŁS.

Funding This study received no funding.

Data availability The results from the CDK9 group have been published in our recent article and are available on request from the corresponding author; reference [16]. The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions. This study includes publicly available data from The Cancer Genome Atlas database; references: [23–28].

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval The study was conducted following the Declaration of Helsinki. The protocol was approved by the Bioethics Committee (KB881/2019).

Informed consent The requirement for patient consent was waived by the Ethics Committee due to the retrospective nature of the study.

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Rozdział 4. Publikacje

CDK9 inhibitors in multiple myeloma: a review of progress and perspectives

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Medical Oncology. 2022 Jan 29;39(4):39.

doi: 10.1007/s12032-021-01636-1.

IF: 3.738 MNISW: 70

Medical Oncology (2022) 39:39
https://doi.org/10.1007/s12032-021-01636-1

REVIEW ARTICLE

CDK9 inhibitors in multiple myeloma: a review of progress and perspectives

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Received: 7 October 2021 / Accepted: 21 December 2021

Abstract

Currently, multiple myeloma is not yet considered a curable disease. Despite the recent advances in therapy, the average patient lifespan is still unsatisfactory. Recently, CDK9 inhibitors emerged as a suitable agent to overcome resistance and prolong survival in patients with poor diagnoses. Downregulation of c-MYC, XIAP, Mcl-1 and restoration of p53 tumor-suppressive functions seems to play a key role in achieving clinical response. The applicability of the first generation of CDK9 inhibitors was limited due to relatively high toxicity, but the introduction of novel, highly selective drugs, seems to reduce the effects of off-target inhibition. CDK9 inhibitors were able to induce dose-dependent cytotoxicity in Doxorubicin-resistant, Lenalidomide-resistant and Bortezomib-resistant cell lines. They seem to be effective in cell lines with unfavorable prognostic factors, such as p53 deletion, *t*(4; 14) and *t*(14; 16). In preclinical trials, the application of CDK9 inhibitors led to tumor cells apoptosis, tumor growth inhibition and tumor mass reduction. Synergistic effects between CDK9 inhibitors and either Venetoclax, Bortezomib, Lenalidomide or Erlotinib have been proven and are awaiting verification in clinical trials. Although conclusions should be drawn with due care, obtained reports suggest that including CDK9 inhibitors into the current drug regimen may turn out to be beneficial, especially in poor prognosis patients.

Keywords CDK9 · Myeloma · Resistance · Synergism · p53 · Bortezomib

Introduction

Multiple myeloma (MM) is the second most common hematological malignancy characterized by monoclonal plasma cell growth leading to the production of non-functional immunoglobulins [1]. MM is characterized by over 138.000 cases per year worldwide and an approximately 2 per 100,000 incidence rate [2]. Multiple myeloma derives from monoclonal gammopathy of undetermined significance (MGUS) transformed plasma cells. Recent studies suggest that the early genetic changes leading to MGUS transformation are related to cyclin D protein dysregulation which can be observed in nearly 50% of cases [3]. The overexpressed cyclin D1 was connected with better chemotherapy response in newly diagnosed MM. MYC and RAS gene mutations are other common findings in multiple myeloma [4]. Interestingly, c-MYC expression is increased in myeloma cells in relation to MGUS thus suggesting it to be the key player in MGUS to MM transition [5]. Mcl-1 and Bcl-2 dysregulations are subsequent molecular changes enabling MM cells to escape apoptotic mechanisms and promote progression [6].

Due to the introduction of novel drugs, such as Bortezomib (BTZ), the estimated survival rate of MM patients improved significantly. For newly diagnosed patients receiving an autologous stem cell transplant (ASCT), the 3-year overall survival rate has increased from 45% in 1992–1998 to 80% in 2014–2016 [7]. However, acquired drug resistance results in limited long-term survival [1]. The outcomes are highly dependent on the presence of karyotype changes. Translocation of *t*(11:14) is deemed a favorable marker, while *t*(14:16) and *t*(14:20) are predictors of poor prognosis. Del (17.13) is another poor prognostic factor, bound with

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Published online: 29 January 2022

resistance to Bortezomib. It is presumably related to the loss of TP53 tumor-suppressing functions, which is also an independent prognostic factor [1, 8]. The advances and possibilities of their use in the treatment of multiple myeloma will be discussed later. As for now, the disease is treatable, but not yet curable [9].

Cyclin-dependent kinases

Cyclin-dependent kinases (CDK) are a family of enzymes regulating the cell cycle and transcription. Together with cyclins, another protein group, they form active complexes that control cell survival and proliferation [10]. Depending on their functions, CDKs can be divided into two main subgroups, namely transcriptional and cell cycle regulators. CDKs 1–6 and 14–18 control cell cycle, whereas CDKs 7–13 regulate transcription [11]. Recent studies have shown a potential clinical benefit of targeting certain proteins from the CDK family in multiple neoplasms [12]. (Fig. 1) CDK7 inhibitors are tested as single agents or in combination with fulvestrant in small cell lung cancer and breast cancer (NCT04247126). Several CDK 4/6 inhibitors, such as abemaciclib, palbociclib and ribociclib were recently approved by FDA and EMA in the treatment of HR+/HER2− mBC/ABC breast cancer. These drugs are currently ongoing in multiple clinical trials in other types of breast cancer as well as in other neoplasms such as head and neck squamous cell carcinoma and glioblastoma [13, 14].

CDK9 is a member of the transcriptional cyclin-dependent kinases family which can be found in two isoforms $CDK9_{42}$ and $CDK9_{55}$ [15]. Together with cyclins T1, T2a, and T2b it forms a Positive Transcription Elongation Factor (P-TEFb). Most of the cellular P-TEFb is inactive, sequestrated by the 7SK snRNA complex, but can be mobilized through BRD4 binding. Together, P-TEFb and BRD4 are capable of phosphorylating RNA pol II, sustaining transcription [15, 16]. Although $CDK9_{42}$ and $CDK9_{55}$ share the ability to phosphorylate RNA pol II there seems to be some difference between their function. Recent studies have shown a correlation between increased cell proliferation and upregulation of the $CDK9_{42}$, whereas the $CDK9_{55}$ seems not to have that relationship [17]. Furthermore, $CDK9_{55}$ was suggested to take part in DNA repair mechanisms via the Ku70 associated pathway [18]. A similar variation in function was observed between CDK9 related cyclins. Cyclin T was deemed necessary for the differentiation of multiple cell lines including monocytes, lymphocytes and adipocytes [19]. Whereas Cyclin K was shown to be upregulated through p53 activation suggesting its role in DNA repair due to stress [20]. The role of the CDK9 in cancer pathogenesis is not fully established yet, but several studies proved it to be a poor prognostic factor in various cancers [12]. CDK9 was shown to take part in c-MYC oncogene activation and Mcl-1 and Bcl-2 protein overexpression. Since those proteins were proved to have an important role in the progression of

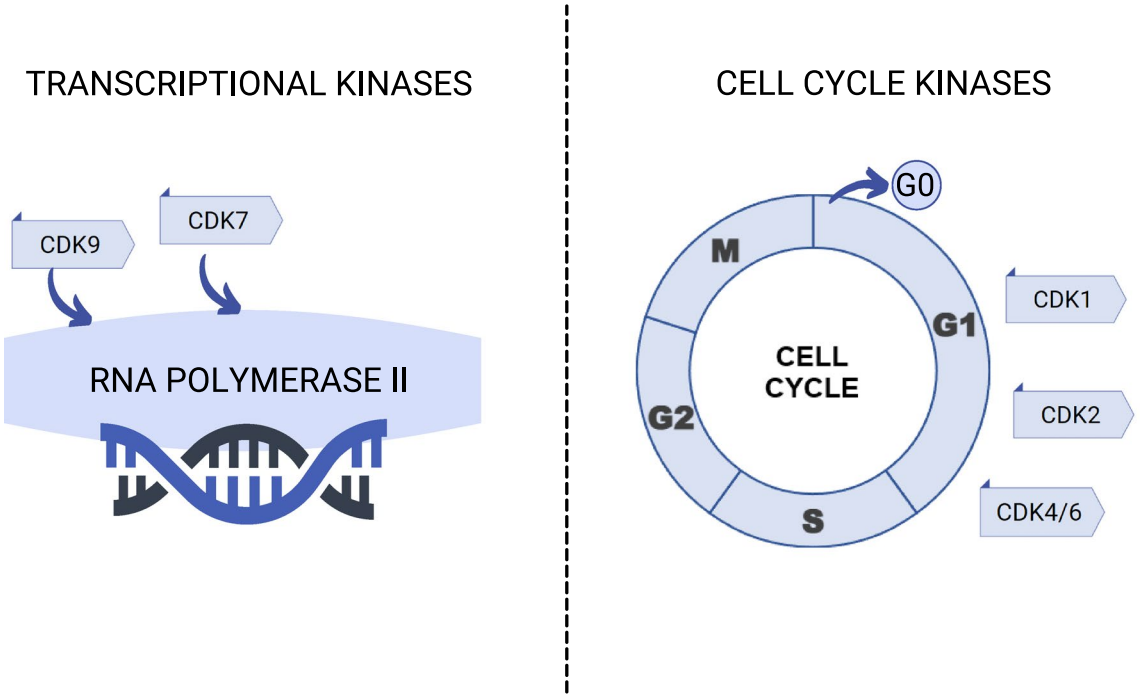

Fig. 1 Role of main cyclin-dependent kinases

hematological malignancies, CDK9 inhibitors are currently widely researched in those diseases [15].

Novel CDK9 inhibitors in multiple myeloma

CDK9 inhibitors recently gained more attention due to the application of Flavopiridol in breast cancer therapy and its synergistic effect with Trastuzumab [21, 22]. These drugs are currently undergoing thorough investigation in multiple hematological diseases (Table 1). Inhibition of CDK9 downregulates key metabolic pathways required for malignant cell survival and proliferation, for example, decreasing Mcl-1, XIAP and MYC expression [10]. Interestingly, p53 target genes function through CDK9-mediated transcription, while CDK9 inhibition downregulates p53 transcription and can increase the concentration of p53. The outcome depends on the degree of CDK9 blockade. Incomplete CDK9 blockade may trigger reactivation of residual CDK9 activity and overrun initial inhibition [10, 23]. Štětková et al. suggested that this effect can also be related to the inhibition of p53-opposing factors, such as mouse double minute 4 (MDM4) overexpression in tumors [24]. Moreover, CDK9 inhibitors downregulate the inhibitor of apoptosis-stimulating protein of p53 (iASPP), restoring p53 tumor-suppressing functions and opening a new perspective for the treatment of patients with loss of p53 function [25] (Fig. 2).

Despite recent progress in MM treatment, most patients develop resistance to therapy. Immunoresistance and inevitable relapse seems to be among the urgent challenges [26]. Although some clinicians proposed that MM is curable cancer [27], others argue that these patients lose over 25 years of life when compared to a healthy population and

Table 1 Clinical trials of CDK9 inhibitors in hematologic malignancies

Drug	Neoplasm	Phase	clinicaltrial.gov
Dinaciclib	Chronic Lymphocytic Leukemia	III	NCT01580228
AT-7519	Chronic Lymphocytic Leukemia	II	NCT01627054
P276-00	Mantle cell lymphoma	II	NCT00843050
AZD-4573	Hematological malignancies	I/II	NCT04630756
Alvocidib/Flavopiridol	Chronic lymphocytic leukemia	II	NCT00464633
CYC065	Solid tumors or lymphomas	I	NCT02552953
Atuveciclib	Acute leukemia	I	NCT02345382
BAY-1251152	Hematological malignancies	I	NCT02745743
Voruciclib	Hematological malignancies	I (recruiting)	NCT03547115
GFH009	Hematological malignancies	I (not yet recruiting)	NCT04588922

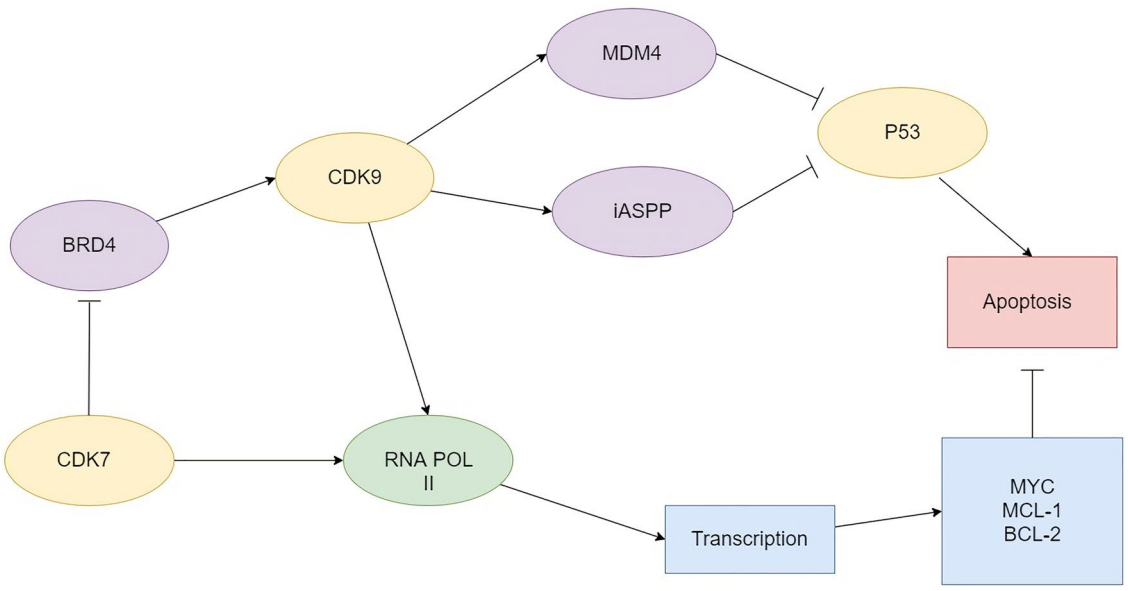

Fig. 2 Role of CDK9 in transcription and apoptosis. *CDK* cyclin-dependent kinase, *BRD4* bromodomain containing 4, *MDM4* mouse double minute 4, *iASPP* inhibitor of apoptosis-stimulating protein of p53

still need continuous therapy, which makes the use of the word "curable" ungrounded [9].

The pathomechanism of resistance in MM is complex. Bone marrow stromal cells (BMSCs) can prevent apoptosis by adhesion-mediated drug resistance, upregulating the expression of anti-apoptotic Bcl-2 family proteins and promoting the autocrine loop to facilitate proliferation and progression [26, 28, 29]. CDK9 inhibitors can potentially induce apoptosis, overcome resistance in Melphalan-, Bortezomib- and Doxorubicin-resistant cell lines and resensitize the "survivor" cells to re-treatment [30–32].

Multiple novel CDK9 inhibitors are currently used as either single agents or co-therapeutics in multiple myeloma (Tables 2 and 3). The first generation of CDK9 inhibitors (Flavopiridol and Seliciclib) were pan-CDK inhibitors, prone to cause off-target toxicity. As the understanding of

Table 2 Clinical trials of CDK9 inhibitors in multiple myeloma

Drug	Selectivity	Clinical trials	Phase	Status	Study completion date
AZD-4573	CDK9	NCT03263637	I	Recruiting	N/A
P276-00	CDK9-T1, CDK4-D1 and CDK1-B	NCT00882063	I/II	Completed	May 2012
		NCT00547404	I	Withdrawn	July 2010
AT-7519 + Bortezomib	CDK1,2, 4, 5, 6 and 9	NCT01183949	I/II	Completed	March 2015
SNS-032	CDK2, 7, 9	NCT00446342	I	Completed	December 2009
RGB-286638	CDK 1–9	NCT01168882	I	Withdrawn (sponsor decision)	N/A
Dinaciclib	CDK1, 2, 5 and 9	NCT01096342	II	Completed	December 2012
		NCT00871663	I	Completed	October 2012
		NCT00871910	I	Completed	February 2010
		NCT01711528	I	Completed	November 2016
		NCT02684617	I	Terminated	N/A

Table 3 Preclinical trials of CDK9 inhibitors

Drug	Trial setup	Inhibited targets	Clinical effect
P276-00	MM xenograft; SCID murine model [33]	CDK9, Mcl-1; RNA polymerase II, cyclin T1	26% tumor mass reduction; 63% growth inhibition
	MM xenograft; SCID murine model [34]	CDK9, Cyclin D1, pRB, CDK4,	Tumor cell apoptosis; tumor growth arrest; 10% mice mass reduction
SLM-6 [35]	MM xenograft; SCID murine model	CDK9, c-Myc, cyclin D1, RNA polymerase II, c-Maf	60–80% MM cells apoptosis; tumor mass reduction; no signs of systemic toxicity;
AT751 [36]	MM xenograft; SCID murine model	CDK9, cyclin D1, cyclin A, cyclin B1, Mcl-1, XIAP	MM cells growth suppression; 45,5% longer overall survival time; tumor mass reduction;
AAP1742 [37]	In-vitro, MM cell lines	CDK9, Mcl-1, Bcl-2, XIAP, RNA pol II,	Apoptosis and growth arrest of MM cells
MC180295 [32]	MM xenograft; murine model	CDK9	Delayed sensitization; increase in the sub-G1 subpopulation; improved mouse survival; PD-L1 downregulation; downregulation of EMT transcription factors;
AZD-4573 [38]	In-vitro; cell line- and patient-derived xenograft models in-vivo	CDK9, Mcl-1, CD45 +	Regression of MML for all treated mice (> 125 days) and 55% tumor volume reduction; AML tumor growth inhibition
RGB-286638 [39]	MM xenograft murine model	Mcl-1; XIAP;	Induction of p53-independent apoptosis; reduced transcription in mutant-p53 MM cells; induction of apoptosis in MM cells lines with mutant-p53

MM multiple myeloma, *SCID* severe combined immunodeficiency, *EMT* epithelial-mesenchymal transition, *PD-L1* programmed death ligand-1, *AML* acute myeloid leukemia

CDK9 biology grew, more selective CDK inhibitors were introduced. In the following part, we will discuss recent advances in the use of CDK9 inhibitors for multiple myeloma therapy.

AZD-4573

Non-selective inhibitors act in an ATP-competitive manner; therefore, they tend to target multiple CDKs, limiting their use as therapeutic agents. Due to the lack of selectivity, it was unclear whether therapeutic effects in previous trials are related to CDK9 inhibition alone [40]. AZD-4573 is a novel, highly selective CDK9 inhibitor that binds to CDK9 in complex with cyclin T1 near the αC-helix of CDK9, with no direct interaction with the ATP binding site nor the ligand [41]. Although it can block other CDKs, the inhibitory effect is > 25-fold more selective for CDK9 (IC_{50} (μM) a [ATP] 5 mM < 0.004) over CDK1, CDK2, CDK4, CDK6, and CDK7 upon short-term treatment of MCF7 cells. AZD-4573 can limit the activity of GSK-3α, GSK-3β, Jnk1 and DYRK2, but in much higher concentration [38]. It has an estimated $t_{1/2}$ of 1.6 h in humans [41].

AZD-4573 was successful in downregulating the phosphorylated RNA pol II, MCL-1 and Myc expression with no effect on the total RNA pol II levels [38]. Furthermore, in combination with ARV825, bromodomain proteolysis targeting chimeric molecule (PROTAC), AZD-4573 showed further decrease in MCL-1, Myc, RNA pol II, BRD2, BRD3 and BRD4 expression. A higher apoptosis rate was observed in MM cell lines treated with both drugs in comparison to monotherapy. A synergistic effect has been shown in in-vivo MM xenograft models with no significant side effects, apart from minor weight loss (< 10%) [42]. AZD-4573 was able to cause apoptosis in T-cell lymphoma and AML xenograft models, showing the synergistic effect with Venetoclax [38]. According to Su-Lin Lim et al. study AZD-4573 can inhibit the proliferation of multiple myeloma cell lines in-vitro, including Bortezomib- and Lenalidomide-resistant cell lines [42].

Despite promising anti-cancer activity in-vivo and in-vitro, and high selectivity, there are currently no reports from clinical trials that can validate the effect in humans. AZD-4573 was recently undergoing a phase I clinical trial in relapsed/refractory hematologic malignancies (NCT03263637). Although the study ended in September 2020, the results have not been posted yet.

MC180295

In the pursuit of epigenetic drugs that can reverse the silencing of tumor suppressor genes in cancer, Zhang et al. investigated the role of CDK9 in gene silencing in cancers. They discovered MC180295, a potent CDK9 inhibitor that binds to the C-terminal part of CDK9 through the norbornyl group, whose selectivity results from the subtle structural variation in the active site. The drug's potency toward CDK9 (IC_{50} = 5 nM) is over 22-fold stronger than for other CDKs. It also seems to downregulate GSK-3a and GSK-3b via non-gene activation mechanisms. The inhibition of CDK9, induced by MC180295 led to dephosphorylation of BRG1, which contributed to the restoration of tumor suppressor gene expression [32].

MC180295 inhibits the proliferation of numerous multiple myeloma cell lines. Even though MC180295 showed higher selectivity toward CDK9 than AZD-4573, it was not as potent [42]. MC180295 downregulated Myc and Mcl-1 in mantle cell lymphoma cell lines, as well as in Ibrutinib- and Venetoclax-resistant cell lines. A synergistic effect of Venetoclax and MC180295 was observed [43]. When compared to SNS-032 in NSG mice injected with SW48 cells, MC180295 slowed tumor growth slower and improved mouse survival without causing overt toxicity [32].

The broad MC180295 anti-cancer activity in-vivo and in-vitro seems promising, although the lack of toxicity and higher selectivity goes hand in hand with relatively lower potency toward CDK9 when compared to AZD-4573.

SLM-6

Sangivamycin was originally isolated from Streptomyces rimosus, and subsequently tested in phase I clinical trial in the 1960s [44]. It showed anti-tumor and anti-retroviral properties, safety in humans; however no further studies were conducted [35, 44]. Sangivamycin-Like Molecules (SLM) are nucleoside analogs of sangivamycin, which possess the same anti-tumor properties and were previously tested in preclinical models of colon cancer to overcome hypoxia-induced resistance to apoptosis [45]. Recently, Dolloff et al. reported that MM cells are sensitive to SLMs and identified SLM-6 as a lead compound with good tolerability and the most activity to inhibit growth and induce apoptosis of MM tumors [35].

SLM-6 inhibits phosphorylation of CDK9, critical to the kinase activity of P-TEFb, with preference to 55-kDa isoform of CDK9 [35, 46]. Unlike Flavopiridol, SLM-6 did not affect the phosphorylation of RNA polymerase II at Ser5, a CDK7 specific site. However, it was found to bind an autophosphorylation site of CDK9 at Thr186, a place critical to the kinase activity of P-TEFb [46]. in-vitro analysis showed that SLM-6 inhibits CDK9/cyclin K and CDK9/cyclin T1 with IC_{50}'s of 280 nmol/L and 133 nmol/L, respectively. SLM-6 inhibits CDK1 and CDK2 (both IC_{50}'s < 300 nmol/L), but only its activity against CDK9 induced MM cell death. The effect was similar when the authors treated MM cells with various CDKs inhibitors. Only the drugs with activity toward CDK9 were capable of downregulating c-Myc, c-Maf and cyclin

D1 in RPMI-8226 cells suggesting the pivotal role of CDK9 inhibition in SLMs anti-MM activity. Another sangivamycin analog, SM-3, was able to induce dose-dependent apoptosis of MM cells but did not affect cell lines from other types of tumors. MM cells turned out to be more sensitive to SLM3 than to other nucleoside analogs, namely 5′-fluorouracil, gemcitabine, and cladribine. That resulted in a rapid reduction of MM cells viability, measured histologically [35].

In in-vivo studies, SLM-6 significantly reduced the size of MM tumors, while Flavopiridol showed no anti-MM activity at a dose 10 times higher than SLM-6 in the same model. The repeated dosing of SLM-6 in immunocompetent mice showed no signs of systemic toxicity and no effects on normal hematopoiesis, aside from modest thrombocytopenia. It may be the effect of direct inhibition of CDK9 in MM cells but not in any other cell lines. SLM-6 and bortezomib showed an additive therapeutic effect in NCI-H929 and CD138+ patients bone marrow cells. The combination of both agents was more effective in reducing MM cell viability than either of the drugs alone [35]. SLM-6 showed promising anti-cancer properties in-vivo and in-vitro, but to our best knowledge, there were no more reports regarding the use of SLMs in cancer studies.

AAP1742

AAP1742 is an analog of CAN 508 discovered in the library of arylazo-3,5-diaminopyrazoles that is active in RPMI-8226 MM cell lines [37]. Although the compound acts primarily through inhibition of CDK9 ($IC_{50} = 0.28$ μM) it shows activity toward other CDKs (CDK2 $IC_{50} = 0.549$; CDK4 $IC_{50} = 0.454$), but in higher concentrations. It decreases the phosphorylation of RNA polymerase II and induces MM cells apoptosis by downregulating anti-apoptotic proteins Mcl-1, Bcl-2, and XIAP in a dose- and time-dependent manner.

The treatment of RPMI-8226 cells with AAP1742 induced suppression of proliferation and apoptosis at 10 lM concentration. After 24 h of treatment with 40 IM dose, Bcl-2 mRNA level decreased to 10% of the control, and Mcl-1 mRNA to 62% of the control. Mcl-1 downregulation was considered the event that initiated apoptosis in treated MM cells, while the cytotoxic activity of AAP1742 was attributed to cellular inhibition of CDK9 [37].

AT-7519

AT-7519 is an ATP-competitive multi-CDK inhibitor with potent activity toward CDK1,CDK2,CDK4, CDK6,and CDK9 with IC_{50} values of 210,47,100,13,170, and < 10 nmol/L, respectively. It shows the selectivity for CDK9 and blocks RNA polymerase II phosphorylation, a CDK7/9 substrate, and glycogen synthase kinase 3β (GSK-3β) phosphorylation [36]. In-vitro and in-vivo studies showed its cytotoxicity toward MM cells, associated with in-vivo tumor growth inhibition and prolonged survival of mice. MM cell death occurred through the dephosphorylation of RNA pol II, which resulted in the inhibition of transcription [36]. AT-7519 anti-tumor properties were independent of p53 expression, while the drug was effective against HT29 and MDA-MB-468 cell lines expressing a mutant form of p53 [47].

Dose-dependent cytotoxicity of AT-7519 was determined in MM cell lines sensitive and resistant to Doxorubicin and Melphalan. Moreover, AT7951 partially overcomes the proliferative effects of bone marrow stromal cells (BMSCs), IL-6 and IGF-1, reducing resistance to Doxorubicin and Bortezomib. Prolonged exposure of MM cells to AT-7519 did not show additional cytotoxicity, suggesting maximum effect at 48 h. Starting from 2 h after the first dose of AT7591, Bcl-2 family proteins, cyclin D1, cyclin A, and cyclin B1 were downregulated. Moreover, AT-7519 did not induce cytotoxicity in peripheral blood mononuclear cells from five healthy volunteers [36, 48]. In mice, AT-7519 inhibited tumor growth when compared with controls ($P < 0.05$). The median overall survival of animals treated was significantly prolonged (39 days vs. 27.50 days respectively) [36].

AZT7519 was recently tested in combination with Bortezomib in patients with previously treated multiple myeloma [49]. The treatment was well-tolerated, and the maximum doses for both AZT7519 and Bortezomib were achieved (21 mg/m^2 and 1.3 mg/m^2, respectively). No significant efficacy was observed after treatment with AT7519M alone, but the combination of AT7519M with Bortezomib resulted in significant rate (33% ≥ partial remission) responses.

P276-00

P276-00 is a flavone that arrests cells in the G1/S phase of the cell cycle. It shows selectivity toward inhibiting CDK9-T1, CDK-4-D1 and CDK1-B with IC_{50} values at 20 nM, 63 nM and 79 nM, respectively [33, 50]. It competes with ATP in the active site of CDKs causing either cell cycle arrest or apoptosis, but its efficacy is dose-dependent and cell-type dependent [50]. P276-00 acts mainly through inhibition of CDK9-T1, affecting primarily transcription of mRNA with short half-lives, such as Mcl-1 [33].

P276-00 inhibits tumor cell growth in culture 2 to 3 times stronger than Flavopiridol because of its higher selectivity toward CDK9. Hence, it's less toxic than Flavopiridol but remains more potent in inhibiting tumor cell growth [33]. The treatment of myeloma cell lines with P276-00 caused transcription inhibition and a significant decline in Mcl-1 protein levels prior to MM cells death [33, 34]. P276-00-induced downregulation of Mcl-1 seems to switch the balance

toward apoptosis, overcoming programmed cell death evasion in MM cells [33]. Treating cell lines for 3 h and 6 h resulted in a rapid, time and dose-dependent decrease in CDK9 and Mcl-1 expression. Other proteins from the Bcl-2 family and cyclin D1 with longer half-lives were significantly downregulated at the 24 h time-point. An increase in PARP cleavage and caspase-3 activity suggested the activation of apoptotic pathways [33, 34, 51]. An anti-MM synergistic effect of P276-00 and Bortezomib was observed in-vitro at a wide range of tested concentrations. P276-00 overcomes the growth and survival stimulation mediated by cytokines and bone marrow stem cells, alleviating the resistance to Bortezomib [34]. However, cyclin D1 overexpression may render the response to P276-00 therapy by making the MM cells more responsive to proliferative stimuli [34]. A synergistic effect of P276-00 and Doxorubicin was also reported in non-small cell lung carcinoma [42].

To confirm the in-vivo activity, P276-00 was administered intraperitoneally into RPMI-8226 xenograft for 15 days and reached the growth inhibition of 63% [33]. Reduction of the tumor mass and significant survival benefit in mice, compared to the control group, was observed after 30 days of P276-00 administration [34]. P276-00 was tested in phase I/II clinical trials to assess the safety and efficacy in patients with refractory multiple myeloma, but the results have not been published (Table 2).

P276-00 affects transcription of short half-live proteins, switching the in-cell balance toward apoptosis. The drug showed effectiveness in-vitro, in-vivo and enhanced the efficacy of Bortezomib, but the overexpression of cyclin D1 and other pro-apoptotic proteins may render its activity.

RGB-286638

RGB-286638 is a non-selective CDK inhibitor with activity against CDK 1, 2, 3, 4, 5, 6, 7, 9 and has the highest potency toward CDK9 ($IC_{50} = 1$ nM). It has shown the ability to downregulate other serine-threonine and tyrosine kinases, such as JAK2, AMPK, TAK1, MEK 1 and GSK-3β [39].

RGB-286638 can effectively inhibit the transcription to total blockage after 24 h of exposition of in-vitro multiple myeloma cell lines. It inhibits both RNA and DNA, downregulating their synthesis by 50% and 60% respectively. Furthermore, accumulation of p53, MM associated mi-RNAs and NAD/NADH reduction was observed after application of RGB-286638. Treatment after 12 h and 24 h caused an increase in apoptosis of MM cells by 25% and 45%, respectively [39]. in-vivo examination showed significant multiple myeloma growth suppression and improved survival time in SCID mice (43 days vs 24 days in the control group). It also triggered dose-dependent cytotoxicity in Melphalan-resistant, Doxorubicin-resistant and steroid-resistant MM cells [52].

In phase I clinical trials RGB-286638 treatment resulted in stabilization of the disease by up to 14 months. However, some side effects emerged during the treatment, with the most significant being hypotension, tachycardia, troponin T and liver enzyme elevation. This led to recommended administration for phase II is suggested to be 120 mg/d for 5 days every 28 days [53].

Dinaciclib

Dinaciclib interacts with acetyl-lysine recognition sites of bromodomains, primarily inhibiting CDK1, CDK2, CDK5, and CDK9 (($IC_{50} = 3$, 1, 1, and 4 nM, respectively). Its high selectivity is probably associated with the binding interactions in the ATP site of CDKs [54].

In phase I clinical trial for patients with advanced malignancies, Dinaciclib suppressed the proliferation of stimulated lymphocytes and reduced Rb phosphorylation. Inhibition of CDK9 blocked the transcription of both *CCND1* and *hDM2*, leading to a reduction in cyclin D1 and increased p53 expression [55]. Dinaciclib enhanced the response to Doxorubicin in RPMI-8226 MM cells [56].

Dinaciclib, as a single agent led to a prolonged remission in 3 out of 27 patients (11%), and minimal response in 2 patients with relapsed MM. The overall response in refractory/relapsed multiple myeloma was 18.5% and was the highest in the patients treated with a 40 mg/m^2 dose. The most common side effects were diarrhea (87%), fatigue (67%), thrombocytopenia (60%), and nausea (53%), but the treatment was overall well-tolerated [57].

Ghia et al. reported results of the only phase III study regarding the use of Dinaciclib when compared with Ofatumumab, an anti-CD20 antibody, in 44 patients with chronic lymphocytic leukemia (CLL) resistant to either fludarabine or chemoimmunotherapy [58]. Even though the patients assigned to the Dinaciclib group had more advanced disease (Rai stage IV 65% vs. 31,8%) compared to the Ofatumumab group, the Dinaciclib group achieved longer median PFS (13.7 vs. 5.9 months), longer OS (21.2 vs. 16.7 months) and higher ORR (40% vs. 8.3%). Interestingly, these differences increased significantly in patients with p53 deletion (median PFS 17.2 vs. 2.4 months; median OS 21.2 vs. 5.4 months), suggesting that CDK9 inhibitors might be beneficial for patients with refractory/relapsed disease and unfavorable in cytogenetic changes (Table 4).

Dinaciclib is currently under investigation in combined therapy with Bortezomib and Dexamethasone in the treatment of relapsed multiple myeloma (NCT01096342). It can potentially reduce exposure to cytotoxic chemotherapy and minimize side effects, by enhancing the activity of other drugs, such as Doxorubicin and Bortezomib [55].

Table 4 Clinical effects of Dinaciclib and Ofatumumab in 44 patients with chronic lymphocytic leukemia [58]

Drug	Medium PFS (months)		Medium OS (months)		ORR	
	Overall	P53 deletion	Overall	P53 deletion	Overall	P53 deletion
Dinaciclib	13.7	17.2	21.2	21.2	8/20 (40%)	N/A
Ofatumumab	5.9	2.4	16.7	5.4	2/24 (8.3%)	N/A

PFS progression-free survival, *OS* overall survival, *ORR* overall response rate

*The deletion of p53 was present in seven patients

Seliciclib

Seliciclib is a roscovitine derivative, multipotent, ATP-competitive pan-CDK inhibitor with most activity against CDK2, CDK7 and CDK9 (IC_{50} = 0.1, 0.36 and 0.81 µm, respectively). Seliciclib can effectively kill MM cells in-vitro even with added protective factors such as Interleukin 6, VEGF and IGF-1. It showed an anti-tumor effect in multiple neoplastic cell lines and xenografts including non-small cell lung cancer, hepatocellular carcinoma and multiple myeloma [51]. The effect of 8 h Seliciclib infusion persisted up to 72 h, reducing the three different cell lines by a minimum of 50% and a reduction in Mcl-1 level was observed. These changes were mainly obtained via transcription inhibition thus suggesting the key role of CDK9 and CDK7 inhibition in the process.

Seliciclib might be a potent additional treatment to Bortezomib and Doxorubicin-based MM protocols due to the synergistic effect [59]. Zhang et al. revealed that "survivors" of CDK9 inhibition were also more sensitive to re-treatment, which may prove crucial in case of recurrence and long-term therapy [32].

SNS-032

SNS-032 was previously described as a selective CDK2 inhibitor that possesses anti-tumor activity in animal models. Subsequent research revealed that it possesses the greatest potency toward CDK9 (IC_{50} = 4 nM) and weaker activity toward other kinases such as CDK2, CDK7 and GSK-3α (IC_{50} 38–48 nM, 62 nM and 230 nM, respectively) [60]. It inhibits phosphorylation of mTOR proteins, completely blocking the activity of mTORC1 and mTORC2 in HL-60 and KG-1 cells (IC_{50} = 200 and 400 nM), achieving a slight degradation of mTOR expression [61].

In RPMI-8226 MM cells SNS-032 transiently inhibited transcription and decreased the concentration of VEGF, XIAP and Mcl-1 transcripts within 2 h after infusion. Evaluation of CDK9 inhibition and PARP cleavage established a temporal association between CDK inhibition, down-regulation of survival proteins, and apoptosis. In human plasma, SNS-032 kept its anti-MM activity and remained fivefold more potent than Flavopiridol [60]. H929 MM cells co-cultured with the bone marrow stromal cell line HS-5 showed resistance to SNS-032, which suggests that bone marrow stroma may play a pivotal role in the development of primary resistance to CDK9-targeted treatment. in-vitro exposure of patient-derived MM cells showed that SNS-032 induces apoptosis of CD138+ cells, but it's only mildly toxic to CD138- MM population and does not prevent the formation of CD34+ colonies derived from normal bone marrow [62].

SNS-032 was examined in phase I clinical trial (NCT00446342) in patients with advanced CLL and MM. Dose-limiting toxicities were not observed, while maximum-tolerated dose was not established due to the early closure of the study. In the MM group, 78% of patients experienced grade 3 to 4 neutropenia, thrombocytopenia or anemia. Other grades 3 to 4 adverse events were sporadic. The most common grade 1 to 2 adverse were nausea, vomiting, constipation, and diarrhea. The treatment was well-tolerated, but the efficacy was limited. As all patients in this study had two or more prior therapies, a better clinical response may be observed in the earlier-stage disease [63].

Flavopiridol

Flavopiridol (Alvocidib) is a flavonoid alkaloid and the first pan-CDK inhibitor to enter clinical trials [64]. Its anti-cancer activity was originally attributed to its ability to induce cell cycle arrest at G1 and G2/M checkpoints through ATP-competitive inhibition of CDK1 and CDK4/6. Later it was found to be most effective against CDK7 and CDK9 (IC_{50} < 300 nM), but also able to inhibit both EGFR and PKA kinases (IC_{50} 21 and 122 µM, respectively) [65].

Flavopiridol downregulated the expression of anti-apoptotic proteins in ANBL-6, ARP1 and RPMI-8226 MM cells lines in-vitro. The decrease in Mcl-1, Bcl-XL and XIAP correlated with early apoptosis of MM cells, but the effect differed in various cell lines. Flavopiridol induced rapid apoptosis of MM cell lines, but Mcl-1 overexpression was able to limit Flavopiridol-induced cell death [66].

In phase II clinical trials of relapsed/refractory multiple myeloma flavopiridol showed no indication of anti-myeloma effects in any patient. The subsequent in-vitro study showed that although significant anti-myeloma effects were noted after 12 h to 24 h, no response was observed after 4 h of exposure. The results were then confirmed in another phase

I clinical study [67]. Flavopiridol turned out to be unable to cause long-term anti-myeloma effects [68].

Recently, attempts to use Flavopiridol in MM have been resumed. Zhou et al. reported that Flavopiridol enhanced the efficacy of Venetoclax in MM cell lines that were primarily less responsive or unresponsive to Venetoclax-induced apoptosis. The synergistic effect was present in either U266, H929 and RPMI-8226 cell lines, as well as multiple other cell lines with unfavorable karyotypes [del 13, del 17p, *t*(11; 14)]. The combination has the potential to overcome MM-related and microenvironment-driven drug resistance by downregulating MCL-1 and upregulating BIM, proteins mediating resistance to Venetoclax. In both NOD/SCID-ɣ and immunocompetent mice Flavopiridol achieved longer survival than mice treated with Venetoclax (79 vs 63 days and 69 vs 49 days, respectively) [69].

While further trials with Flavopiridol as a single drug in MM seems inexpedient, the ability of CDK9 inhibitors to overcome resistance to therapy is well-grounded in literature [6, 28, 30, 66, 70]. In this case, Flavopiridol may prove effective, but it's being replaced by more selective drugs.

Potential synergistic combinations with CDK9 inhibitors in multiple myeloma

Most of the reports regarding the use of CDK9 inhibitors in MM pertain to refractory or relapsed patients, in which previous treatment regimens turned out to be ineffective. In those settings, CDK9 inhibitors were primarily examined as co-therapeutics to alleviate the resistance to other drugs and enhance anti-tumor properties. Therefore, not much data are available regarding the clinical outcomes in patients with better prognosis. Furthermore, accurate safe doses, therapeutic doses, bioavailability and pharmacokinetics of individual drugs are still not clearly determined. Nonetheless, many authors suggest that CDK9 inhibitors may complement current treatment regimens and will be discussed below (Table 5).

Bortezomib, a proteasome inhibitor, has revolutionized the treatment of MM, but despite its high initial response rate, Bortezomib loses efficacy over time [35]. Dai et al. suggested that a combination of Flavopiridol and Bortezomib acts synergistically through induction of mitochondrial damage, caspase activation, and apoptosis [72]. CDK inhibitors downregulate the transcription, reducing the number of anti-apoptotic proteins, while proteasome inhibition blocks the degradation of pro-apoptotic proteins. Hence, the combination of both drugs changes the intracellular balance to favor apoptosis. P276-00 was tested together with Bortezomib in myeloma cells and showed marked synergism [34], which coincides with the results of SLM-6 [86] and Dinaciclib [87]. The combination of Doxorubicin, Bortezomib and either P276-00 [34] or Seliciclib [59] were also deemed effective. Zhang et al. showed that Mcl-1 was upregulated in all tested MM lines, including the Bortezomib-resistant lines. Moreover, Mcl-1 overexpression significantly reduced Bortezomib cytotoxicity, indicating a functional role for Mcl-1 in Bortezomib resistance. CDK9 inhibition substantially potentiated the susceptibility of Bortezomib-resistant cells to both proteasome inhibitors and BH-3 mimetics [30]. On the other hand, Zabihi et al. study showed no significant enhancing effect of AT-7519 together with Bortezomib in KG-1 cells, suggesting that CDK9 inhibitors do not act by the activation of the proteasome pathway [88].

Venetoclax is a BH-3-mimetic that blocks the Bcl-2 protein, leading to cell apoptosis [89]. Voruciclib [90], A-1467729 and A-1592668 [79] were recently proven synergistic with Venetoclax via CDK9 inhibition. Treatment of mice with A-1592668, a selective CDK9 inhibitor, led to a significant increase in survival (median survival 24.5 days, $P < 0.0001$) compared to the control group (median survival 13.5 days). There was no significant weight loss, and the decrease in lymphocyte burden did not impact hemoglobin, neutrophil, or platelet counts. Venetoclax was substantially less active and did not provide any survival benefit. However, co-treatment of mouse lymphoma #4242 cell line tumors in-vitro with A-1592668 and Venetoclax extended the median survival from 30.5 to 41 days [91]. Voruciclib was reported to downregulate Mcl-1 and c-Myc, enhancing Venetoclax activity in AML models. However, the effect is transient and the drug needs to be administered repeatedly [90]. Similarly, AZD-4573 together with Venetoclax achieved prolonged regressions in 100% of treated mice, with all eight mice remaining tumor-free till day 63. The only notable side-effect was minimal bodyweight loss, suggesting that the combination was well-tolerated [38].

BRD4 is a member of the human BET protein family that binds acetylated histones during mitosis to maintain chromatin structure and ensure early re-initiation of transcription after mitosis [92]. BRD4 recruits P-TEFb and promotes the elongation of transcription. When used together, CDK9 and BRD4 inhibitors impede transcription of anti-apoptotic genes and c-MYC oncogene, suppressing tumor proliferation. Combination of ARV825 and AZD-4573 caused apoptosis of 67% of KMS11 cells and 71% of RPMI-8226 cells, significantly slowing MM tumor growth ($P < 0.001$) [42].

A synergy between CPI-203, a novel bromodomain inhibitor, and either Bortezomib or Lenalidomide was also observed [86, 87]. Lenalidomide contributes to overcoming resistance to Bortezomib via inhibition of IRF4, which leads to MYC downregulation [93]. CPI-203 represses MYC gene transcription and has a cytostatic effect on MCL cells in-vivo, while the cytotoxicity in peripheral blood from healthy donors was below 25%, indicating the drug's selectivity. Lenalidomide alone partially reduced MYC and RF4

Table 5 Potential combinations of CDK9 inhibitors with other agents [71]

Drug	Potential synergy	Mechanism of synergy/clinical effect	Type of effect
P276-00 [33, 51]; SLM-6 [35], AT-7519 [36]; Roscovitine [72]; Seliciclib [59]; Flavopiridol	Bortezomib, Carfilzomib	Cyclins and CDKs are substrates of proteasomes, which accumulation may lead to resistance to therapy; CDK9 inhibitors prevent drug resistance, obstruct the accumulation of anti-apoptotic proteins, diminish proteasomal protein degradation, promote cancer cells apoptosis and activate alternate apoptotic signaling cascades [34]	Additive/synergistic
P276-00 [73]; Dinaciclib [56]; Roscovitine[74]	Doxorubicin	Inhibition of doxorubicin-induced chemoresistance, involving reduction of (LPS)-induced NF-kB [75], inflammatory genes transcription[76] and TNF expression [77]	Synergistic
Flavopiridol [69]; Dinaciclib [78]; AZD-4573 [38]	BH-3 mimetics: Venetoclax; [79]	CDK9 decreases the transcription of Bcl-2 family proteins and upregulated BH-3 proteins expression; BH-3 mimetics block remaining Bcl-2 proteins activity, leading to apoptosis	Synergistic
SNS-032; Dinaciclib [80]; AZD-4573, MC180295 [42]	BET inhibitors; OTX015; ARV825, CPI-203 [81]	Targets of both drugs are positive regulators of P-TEFb; inhibition of transcription and tumors growth; cell cycle arrest, increased in MM cells apoptosis; c-MYC transcription inhibition	Synergistic
Dinaciclib [82]; Flavopiridol [83]	Ofatumumab, Rituximab, Cyclophosphamide	Mechanism remains unclear	Not defined
PHA-767491 [84]	Erlotinib—inhibitor EGFR	PHA-767491 overcomes the resistance to EGFR- based therapy; induction of apoptosis, G2-M cell cycle arrest, inhibition of DNA replication	Synergistic
Inhibition of CKIα, CDK7 and CDK9 [85]	Lenalidomide	Leukemia cells apoptosis by triggering DNA repair response and augmenting p53 activation; p53 stabilization; preservation of hematopoiesis	Synergistic

expression, but together with CPI-203 the expression of genes was almost completely abrogated. The combination of CPI-203 and lenalidomide induces programmed cell death in MCL, inhibits the growth of bortezomib-resistant cells in-vivo and reduces tumor volume [93]. While this combination has not been tested in multiple myeloma models Minzel et al. [85] showed that CDIα inhibition, which co-targets CDK7/9, underlines the therapeutic effect of lenalidomide in a pre-leukemia syndrome through p53 activation and stabilization [85]. Since the synergies between BET inhibitors and CDK9 inhibitors, as well as BET inhibitors and lenalidomide were confirmed, the addition of CDK9 inhibitors to standard lenalidomide-based therapy could prove beneficial.

CDK9 degradation or CDK9 inhibition?

Currently, all CDK9 inhibitors that have advanced to the second phase of clinical trials are non-selective, reversible and require continuous target occupancy to maintain CDK9 inhibition [15, 94]. As those agents bind to the CDK9/cyclinT1 complex in an ATP-competitive manner, the CDK9 blockade may be prone to be overrun by residual CDK9 activity, limiting their clinical effectiveness [10, 12, 15, 23]. Due to the lack of human clinical trials with selective CDK9 inhibitors and the off-target toxicity of the first generation CDK9 inhibitors, an alternative method to blocking the CDK9 activity was sought. Recently, the degradation of CDK9 has been suggested as an alternative to CDK9 inhibition [15, 94–97]. CDK9, as an endogenous protein is stabilized by a chaperone pathway, which helps in forming a stable cyclin T1/CDK9 complex. The excess of CDK9 becomes very unstable and is rapidly degraded by the proteasome [95]. Robb et al. demonstrated that chemical degradation of CDK9 in HCT116 can be successfully induced by proteolysis targeting chimera (PROTAC) [96].

The PROTACs are bivalent chemical protein degraders that link specific endogenous proteins with a component of E3 ubiquitin ligase. In this way, the protein is polyubiquitinated and degraded [98]. Ubiquitination is associated with the functionality of the CRBN gene, whose product is a receptor of E3 ubiquitin ligase; hence, CRBN expression may affect the therapeutic effectiveness of protein degraders [94, 99]. This strategy seems promising, especially in the degradation of CDK 9–13, which are not associated with the cell cycle [100].

Olson et al. reported that THAL-SNS-032, a selective CDK9 degrader, together with NVP-2, a CDK9 inhibitor, induced rapid degradation of CDK9 without affecting the levels of other CDKs [94]. THAL-SNS-032 inhibited proliferation of MOLT4 cells at lower concentrations (IC_{50} = 50 nM) than SNS-032 (IC_{50} = 173 nM) 11 different leukemia cancer cell lines. However, THAL-SNS-032 was less potent than the selective CDK9 inhibitor NVP-2 (IC_{50} = 9 nM). The anti-proliferative activity of THAL-SNS-032 was nearly 100 times weaker in CRBN negative cells than in CRBN positive cells, while CDK9 inhibitors activity was independent of CRBN status.

The in-vivo ability to degrade CDK9 via PROTAC molecules was examined by Qiu et al. who introduced PROTACs based on Pomalidomide and a selective CDK9 inhibitor, BAY-1143572. In their study, PROTAC B03 showed 20-fold stronger anti-proliferative activity in MV4-11 cells than BAY-1143572 alone, resulting in strong cancer cell inhibition in BALB/c nude mice bearing MV4-11 xenograft [97].

CDK9 degraders show prolonged pharmacodynamic effects compared to CDK9 inhibitions and high on-target selectivity. They can contribute to achieving an irreversible inhibition via CDK9 degradation, overcoming treatment resistance caused by target mutation and limiting effects of off-target toxicity [98]. However, the lack of clinical trials with CDK9 degraders in multiple myeloma makes the final comparison of these methods a matter of the future.

Perspectives and limitations

CDK9 inhibitors showed broad anti-cancer activity in-vivo and in-vitro, but the results from clinical trials are still uncertain. The first generation of CDK9 inhibitors (Flavopiridol and Seliciclib) targets multiple CDKs and acts in an ATP-competitive manner, which was the main reason for their off-target toxicity and lack of clinical relevance [16, 57]. Novel CDK9 inhibitors are designed to improve tolerance and compliance for patients undergoing treatment [91]. Their systemic toxicity, cytotoxicity to peripheral blood [36] and adverse effects rate [58] seems to be acceptable.

The use of CDK9 inhibitors as a standalone medication is not supported by much evidence. Despite the encouraging results of preclinical studies, their efficacy in clinical trials was mediocre. Only dinaciclib showed encouraging results as a single agent in patients with relapsed multiple myeloma [57]. Flavopiridol showed no anti-MM activity in patients, while the activity of SNS-032 in phase I clinical trial was limited [63, 67]. Although Dinaciclib and RGB-286638 were able to either achieve prolonged remission or stabilize the disease due to their lack of selectivity there is no certainty that this effect was caused by the inhibition of CDK9 [53, 57]. Nevertheless, the combination of CDK9 inhibitors with either Bortezomib, Doxorubicin or Venetoclax seems to overcome resistance to therapy and cause increased apoptosis of MM cells. This effect was observed in most preclinical studies of examined drugs and was later confirmed in clinical trials with AT-7519 and is currently examined in MM patients treated with Dinaciclib, Bortezomib and Dexamethasone (NCT01096342) [49]. CDK9 inhibitors have an established mechanism of synergy with numerous drugs (Table 5). In this scenation CDK9 inhibitors are used

as co-agent, the use of more selective inhibitors may reduce systemic toxicity and alleviate resistance to therapy.

CDK9 degraders appeared because of the inability to induce selective CDK9 inhibition [96, 97]. THAL-SNS-032 and PROTAC B03 induced rapid degradation of CDK9 without affecting the levels of other CDKs [94, 97]. Both of them showed higher potency than CDK9 inhibitors, but their activity seems to depend on the expression of CRBN-mediated genes. THAL-SNS-032 was nearly 100 times weaker in CRBN negative cells, while CDK9 inhibitors work independently of CRBN status [94]. CDK9 degraders show prolonged pharmacodynamic effects compared to CDK9 [98]. This is a major advantage over older CDK9 inhibitors, which requires longer and repetitive infusions [90]. Novel, orally active CDK inhibitors, such as voruciclib, started to emerge to improve the compliance with patients, but were not tested in MM treatment yet [91].

Noteworthy, CDK9 inhibitors were tested only in pretreated patients with relapsed/refractory multiple myeloma or unfavorable cytogenetics. Even in those disadvantageous settings their clinical effect in lifting resistance was noticeable. [49] Furthermore, CDK9 inhibitors act independently of p53, causing MM cell apoptosis even in p53-mutated cell lines [47]. P53 target genes function through CDK9-mediated transcription, while CDK9 inhibition can downregulate p53 transcription or increase the concentration of p53. The outcome depends on the degree of CDK9 blockade, which can be overrun by residual CDK9 activity [10, 23]. Moreover, CDK9 inhibitors can restore p53 tumor-suppressing functions by downregulating iASPP [25]. The nuances of p53 and CDK9 interactions are not clearly explained yet, but opens a new perspective for the treatment of patients with loss of p53 function (Fig. 2).

The last decade has significantly improved the understanding of CDK9 and MM biology. While the first generation of CDK9 inhibitors turned out to be lacking as single agents, they seem to potentiate the efficacy of other therapeutics [84, 93]. More selective inhibitors are less toxic and are usually well-tolerated [15]. The mechanism of synergy between CDK9 inhibitors and Bortezomib, Doxorubicin or Venetoclax is established and prompts the incorporation of CDK9 inhibitors into current drug regimens in further clinical trials (Table 5). However, the need for a long drug infusion or the lack of pharmacokinetic data are still obstacles that need to be addressed.

Author contributions J.B. and K.S. conceptualized the work, performed literature search and data analysis. Ł.S. critically reviewed and N.A. made recommendations and proofread the work. All authors contributed to and approved the final manuscript.

Funding The authors did not receive support from any organization for the submitted work.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Rozdział 4. Publikacje

CDK9: Therapeutic Perspective in HCC Therapy

Borowczak J, Szczerbowski K, Stec E, Grzanka D, Szylberg Ł.

Current Cancer Drug Targets. 2020;20(5):318-324.

doi: 10.2174/1568009620666200212124357.

IF: 3.428 MNISW: 70

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Current Cancer Drug Targets, 2020, *20*, 1-7

REVIEW ARTICLE

CDK9: Therapeutic Perspective in HCC Therapy

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ARTICLE HISTORY

Received: September 23, 2019
Revised: November 27, 2019
Accepted: December 06, 2019

DOI:
10.2174/1568009620666200212124357

Abstract: CDK9 is an important cell-cycle control enzyme essential in transcription, elongation, and mRNA maturation. Overexpression of CDK9 has been reported in several diseases, including acute lymphoblastic leukemia, chronic lymphocytic leukemia, and malignant melanoma. Recent research revealed that CDK9-inhibitors have a major impact on the induction of apoptosis in hepatocellular carcinoma (HCC) cell lines. Despite surprisingly promising results in *in vitro* and *in vivo* research, no CDK9 related therapy is currently allowed in cases of HCC. Furthermore, due to their high specificity, the inhibitors had no effects on unaltered hepatocytes and no toxic effects were shown. Considering that they were well tolerated and showed relatively few severe side-effects in mice, CDK9-inhibitors would seem to be promising targets in HCC biomarker-guided immunotherapy. Studies have verified that CDK9 has a pivotal role in c-Myc-mediated tumor growth and CDK9 inhibitors inhibit not only its progression but diametrically decrease both the mass and size of HCC nodules. CDK9-inhibitors seem to be a promising target in HCC treatment.

Keywords: CDK9, c-Myc, HCC, inhibitors, P-TEFb, therapy.

1. INTRODUCTION

CDKs are a family of proline-directed serine/threonine kinases that work in association with another family of proteins called cyclins. Heterodimers, which they form with each other, are responsible for cell proliferation, differentiation and apoptosis [1].

Among the many proteins from the CDK family, the one that currently seems to be gaining attention is CDK9, which is thought to be responsible for the regulation of transcription in normal and elevated stimulation conditions. Recent studies suggest, that CDK9 is not only an RNA polymerase II transcription elongation factor, but also becomes important as a central hub of transcription regulation. Embryonic stem cells require CDK9 activation to activate the transcription of genes responsible for cell differentiation, while under reduced stimulation conditions, a decrease in the activity of CDK9 (through its incorporation into the 7SK snRNP complex) was observed. Therefore, CDK9 activation requires its release from snRNP complexes, either by direct interaction or by CDK9 releasing factors [2].

Malfunctions of CDK9 have been confirmed in various diseases, including acute lymphoblastic leukemia [3], pancreatic cancer [4], prostate cancer [5, 6] and breast cancer [7] (Table **1**). Recent research shows that the altered function of CDK9 also has a major impact on the induction of apoptosis in hepatocellular carcinoma (HCC) cell lines [1-3].

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Presently, two isoforms of CDK9 are known: a 42-kDa isoform and a 55-kDa isoform, the expression of which depends on the type of tissue and cell cycle phase [1, 8]. Increased expression of the 55-kDa CDK9 isoform may be observed in many cases of HCC cell lines. Proteins from the CDK family, including CDK9, interact with cyclin T1 or cyclin T2, forming heterodimers and leading to autophosphorylation of the T-loop, which is necessary to form Positive Transcription Elongation Factor (P-TEFb). A model has been proposed, in which CDK9 is required to release previously stopped Pol II, initiating transcription. Aside from the direct pro-transcriptional activity, it is suspected, that CDK9 is able to induce transcription by the enhancer-promotor loop [8-10]. Physiological regulation of CDK activity is necessary to maintain cell homeostasis [11, 12]. Moreover, Claudio *et al.* [13] showed that a positive feedback loop exists between CDK9 and p53, enabling a mutual increase of transcriptional activity. It is worth mentioning that CDK9 inhibition in the treatment of synovial inflammation resulted in the loss of Mcl-1 expression [14], a protein from the BCL-2 family, which is often overexpressed in HCC and enhances cell survival by inhibiting apoptosis [15].

Due to overexpression of the c-Myc oncogene and increased Mcl-1 activity, which are responsible for increased proliferation and impaired apoptosis respectively [1, 7, 16] studies have concluded that the CDK9 signaling pathway might be an interesting target for future therapy for patients with HCC.

2. ROLE OF CDK9 IN HCC

HCC has been one of the most common cancer types worldwide in recent decades and its incidence and mortality

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rates have been increasing [17-19]. Despite recent advances in the therapeutic approach towards HCC, the prognosis is still poor, due to long asymptomatic progress and late diagnosis [20, 21]. Most known cases of HCC are associated with chronic hepatitis [22]. Major risk factors include liver cirrhosis, HBV and HCV infection, alcohol abuse and nonalcoholic fatty liver disease [17]. The occurring inflammation leads to both reactive oxygen species (ROS) mediated DNA injury, and the initiation and progression of carcinogenesis [23]. HCC is a highly heterogeneous tumor and multiple genetic alterations have been identified [24], therefore no leading mutation has been found [12].

Although HCC is not widely considered to be an immunogenic tumor [21], there is no doubt, that this cancer is able to escape immune surveillance [25] by affecting its microenvironment, recruiting tumor-associated cells, [26] and by secreting immunosuppressive cytokines [27]. Recent studies have proven that inhibition of CDK9 suppresses the NF-kB mediated inflammatory pathway, and therefore leads to the reduction of inflammation [14, 28]. Moreover, Haider at al. 2013 [29] showed that CDK9 is involved in cancer cell invasion and that suppressing the activity of the CDK9/P-TEFb complex can prevent cell migration and invasion. Commonly occurring Myc oncogene overexpression in cells with uninhibited CDK9 (a fundamental component of P-TEFb) leads to abnormal proliferation of affected cells, affecting different metabolic pathways [11,12,16]. Furthermore, due to frequent inactivation of suppressor genes (p16, p21, p53, pRB) or abnormal CDK activation, CDK9 activity in HCC cells is up-regulated [30, 31]. Physiologically, T-loop autophosphorylation of CDK9/cyclin T1 complex leads to direct activation of tumor suppressor proteins p53 [13] and pRB [32] which are essential for proper control at cell cycle checkpoints [2]. The existence of a positive feedback loop between p53 and CDK9 suggests that in order to initiate the neoplastic process, an inactivating mutation of both is needed. The disfunction of DNA repair mechanisms is followed by the uncontrolled expression of proto-oncogenes, such as c-Myc and Mcl-1. C-Myc is also responsible for the recruitment of positive transcription factors which, in the presence of dysfunctional suppressor proteins, leads to positive feedback and overexpression of anti-apoptotic genes [33]. Under physiological conditions, CDK9 activity is related to c-Myc expression and Myc overexpression, which is a common finding in HCC [34]. Prolonged inhibition of CDK9 is thought to prevent the proliferation and maintenance of cells and is associated with Myc overexpression and tumor involution. The suggested mechanism involves reducing the intracellular concentration of anti-apoptotic proteins and inducing cell cycle arrest [1, 33, 35]

Chemoresistance is one of the major factors that hinder HCC treatment [36]. While the impact of HCC chemoresistance oriented on CDK9 inhibition has not yet been fully explored, there have been clinical trials that have demonstrated a directly proportional correlation between CDK9 activity and tumor chemoresistance, for instance in pancreatic cancer [4]. Some studies have shown that inhibition of CDK9 may reduce the resistance of tumors to apoptosis by decreasing Mcl-1 and Myc overexpression, thereby modulating chemoresistance. The possibilities and limitations of CDK9 inhibitors, as used in HCC treatment, will be discussed further.

3. CDK9 RELATED TARGETS IN HCC TREATMENT

The overall survival rate in HCC has not improved in the recent 18 years [37]. Current HCC therapies are still deemed unsatisfactory due to the estimated median survival time which varies from 11 to 20 months [18, 38]. Despite surprisingly promising results in *in vitro* and *in vivo* research (Table **2**), no CDK9 related therapy is currently allowed in hepatocellular carcinoma [39]. Nevertheless, many potential therapies and therapeutic goals have been identified and will be discussed below.

3.1. miR-206

miR-206 is a miRNA particle from the miR1 family and has a role in the suppression of tumor growth [40]. It has been found to be downregulated in many diseases, including prostate cancer [41], pancreatic cancer [42], and hepatocellular carcinoma (Table **1**). Inhibition of miR-206 leads to the up-regulation of E-cadherin, down-regulation of N-cadherin, and the promotion of cell invasion *in vitro* [41]. *In vitro* trials have suggested that miR-206 mediated therapy, aimed at increasing intracellular miR-206 levels, may lead to the decreased invasive and metastatic potential of cancers [5]. Recent studies have shown that the antineoplastic properties of miR-206 were caused by its inhibitory effects on the mRNA of CDK9, in turn leading to the downregulation of hepatocellular carcinoma cell line proliferation and the downregulation of VEGF expression [43]. These findings indicate that miR-206 is a strong candidate as a future target in HCC therapy [5, 40].

3.2. PHA-767491

PHA-767491 is an inhibitor of cell division cycle kinase (Cdc7) and cyclin-dependent kinase 9. Recent research by Liu *et al.* [44] has shown that it might also be an inhibitor of the NRF-2 mediated antioxidant response. There appears to be a synergistic effect with 5-fluorouracil, which reduces chemoresistance and intensifies apoptosis in hepatocellular carcinoma cell lines. Moreover, PHA-767491 seems to have a chemosensitizing effect in other neoplasms such as AML [45] (Table **2**).

3.3. BA-12 and BP-14

BA-12 and BP-14 are novel Roscovitine derivatives, specifically antagonizing CDK 1, CDK 2, CDK 5, CDK7 and CDK9. In rats, Bp-14 has been shown to be a highly lipophilic molecule with extremely low intestinal absorption and rapid distribution to the adipose tissues, where it reaches its highest concentration [46]. According to the research of Haider *et al.* [29], not only BA-12 but also BP-14 significantly reduce the phosphorylation of RNA polymerase II at CDK 7 and 9 related points in hepatocellular carcinoma cell lines. Growth reduction and DNA synthesis inhibition were observed. BA-12 and BP-14 showed significantly lower cytotoxicity to hepatocytes than was observed in hepatocellular cell lines (Table **2**). *In vivo*, tests have established that there may be a reduction in the size of tumor nodules, without obvious side effects or the induction of chemoresistance. Furthermore, Bp-14 together with Everolimus was shown to inhibit the growth of anaplastic thyroid cancer cells [47].

Table 1. Effects of CDK9 mediated treatment in various neoplastic diseases [3-5, 7].

-	Triple-Negative Breast Cancer	Prostate Cancer	Pancreatic Cancer	Acute Myeloid Leukemia
MYC expression suppression	+	+	+	+
Cell arrest induction	+	+	+	+
Cell proliferation inhibition	+	+	+	+
Tumor growth suppression	+	+	+	+
Apoptosis induction	+	+	+	+

Table 2. Clinical effect of CDK9 inhibitors *in vitro* and *in vivo* [29-31, 35, 35, 40, 43, 44, 47, 70].

	miR-206	PHA767491	BA12&BP14	Xylocydine	Ibulocydine
Inhibition of HCC cell lines	+	+	+	+	+
Toxicity against non-malignant hepatic cells	-*	-*	-	-	-
In vivo tumor growth suppression	-*	-*	+	+	+
In vivo observed toxic effect	-	-	-	-	-

*- no data available

3.4. Xylocydine

Xylocydine is a novel CDK inhibitor, an L-derivative of sangivamycin (a microorganism isolated CDK inhibitor), which preferentially inhibits cyclin-dependent kinases 1,2,7 and 9. It has been suggested that it is better able to suppress cell growth in hepatocellular carcinoma than other CDK inhibitors, such as Roscovitine or Olomoucine, and that it can induce apoptosis in those cells [30]. It has also been suggested that Xylocydine can reduce the levels of phosphonucleolin and phospho-RB (CDK1 and CDK2 activity indicators), RNA Polymerase II-CTD (a CDK7 and CDK9 activity indicator) and also the antiapoptotics Bcl-2 and XIAP, resulting in apoptosis [31]. Furthermore, Xylocydine also increases the levels of p53 and Bax through the stabilization of p53, thereby inducing cell cycle arrest at the G1/S, intra-S and G2/M checkpoints [48, 49]. *In vivo*, Xylocydine shows significant suppression of tumor growth and intensification of apoptosis, without liver cell apoptosis, in Xenograft Balb/C-nude mice (Table **2**) [30, 31].

3.5. Ibulocydine

ItIbulocydine is a novel prodrug of the CDK inhibitor BMK-Y101, which has a specific influence on not only CDK7 and CDK9, but also on CDK1, and CDK2. It has strong inhibitory effects on the growth of hepatocellular carcinoma cell lines, probably via the blockage of carboxyl-terminal domain phosphorylation, mediated by CDK7/9, resulting in the reduction of Mcl-1 and XIAP mRNA and protein levels [35]. Furthermore, Ibulocydine seems to sensitize HCC cells to TRAIL-induced apoptosis. A study by Park *et al.* suggested that Ibulocydine can be useful in Bcl-xL overexpressing HCC, where it overcomes the influence of Bcl-xL on TRAIL-induced apoptosis [50]. It has been shown to be more effective than other CDK inhibitors, such as Olomoucine and Roscovitine, and has no toxic effect on hepatocytes. It has also been reported that Ibulocydine inhibits growth and induces apoptosis in HCC Xenografts (Table **2**). However, the bioavailability of the drug in Sprague-Dawley rats was not satisfactory and was found to be only 34% by oral absorption and 58% by peritoneal injection [50]

3.6. Olomoucine and Roscovitine

Olomoucine and Roscovitine are both older representatives of the CDK-inhibiting drugs, which act through direct competition for ATP-binding sites [51]. Olomoucine has been shown to inhibit CDKs 1, 2, 5 and 7. Roscovitine has shown similar specificity for CDKs with stronger inhibitory action on CDK 1 than that of Olomoucine and with additional activity on CDK9 [30]. Both drugs showed an inhibitory effect on tumor cells and, because of that, are used as a baseline against which the effectiveness of newer drugs, such as Xylocydine and Ibulocydine, is measured [29, 52]. Moreover, Roscovitine is being tested as a treatment in various diseases such as B-cell lymphoma, non-small cell lung cancer, viral diseases (HIV and HSV infections), and in inflammatory diseases such as arthritis [31].

3.7. Xylocydine and Ibulocydine vs Roscovitine and Olomoucine (New vs Old)

Recent studies have suggested that Xylocydine and Ibulocydine are superior to the older CDK inhibitors - Olomoucine and Roscovitine. Studies have shown that both novel drugs reduced the growth of hepatocellular carcinoma cell lines significantly more effective than older drugs. Furthermore, noticeable effects of the inhibition were observed with lower dosages of either Xylocydine or Ibulocydine than when using Olomoucine or Roscovitine [30, 35].

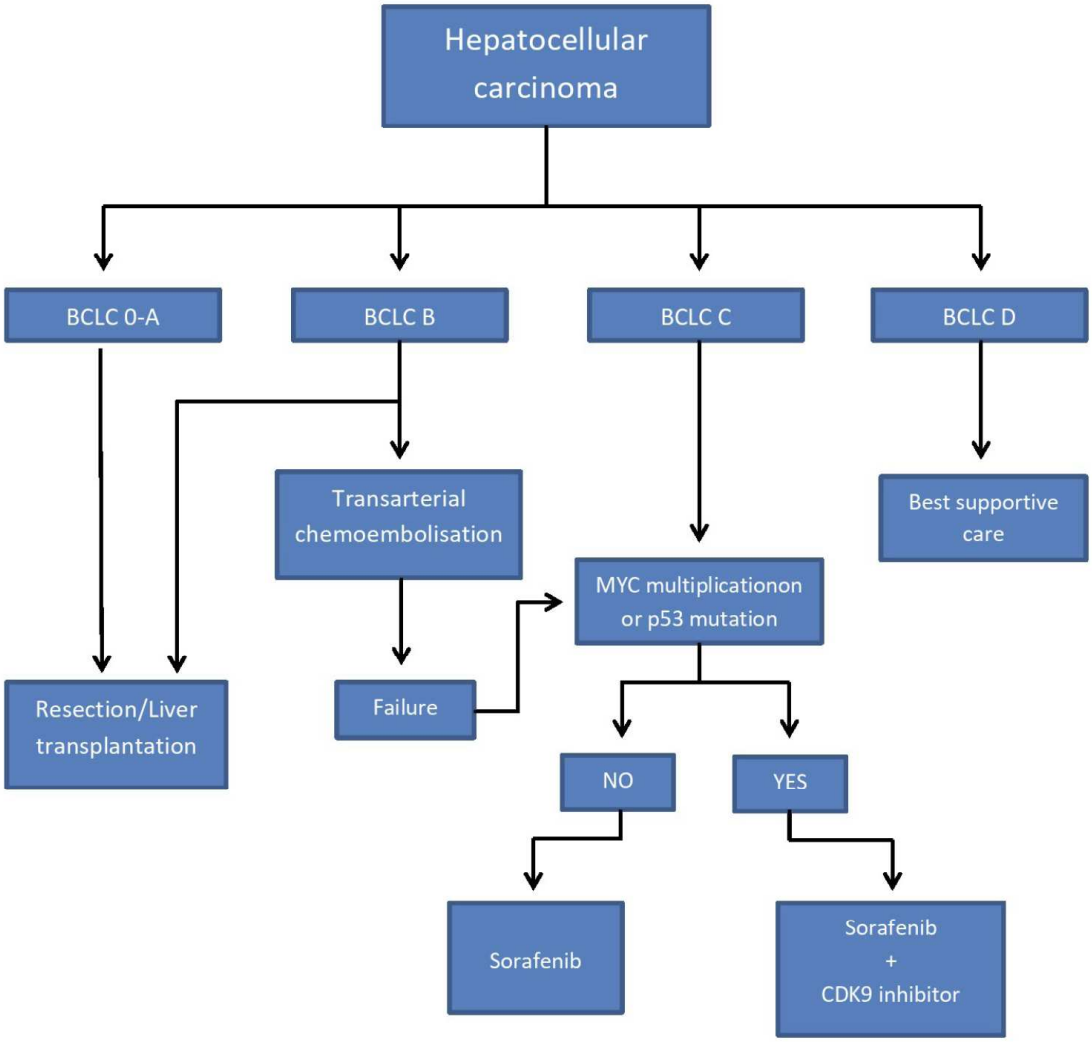

Fig. (1). Suggested place of CDK9 inhibitors' application in current algorithm for HCC treatment options depending on BCLC stage [39, 68].

4. THERAPEUTIC PERSPECTIVES FOR THE APPLICATION OF CDK9 INHIBITORS IN THE CURRENT HCC TREATMENT ALGORITHM

CDK9 inhibitors have yet to be tested in patients with hepatocellular carcinoma despite having been demonstrated to be effective in HCC treatment in both *in vitro* and *in vivo* research, thus enabling their use in clinical trials [29-31, 33, 35, 39, 40, 44, 50]. According to a recent publication from ESMO Clinical Practice Guidelines [39], no survival rate improvement is brought about by the use of chemotherapy in advanced HCC (BLCB C) [53-56] and only a few drugs, such as Sorafenib [39, 57, 58] and Lenvatinib [39, 59] are currently used in systemic therapy. However, the optimal treatment strategy is yet to be defined. In intermediate HCC treatment (stage BLCB B), trans-arterial chemoembolization (TACE) is widely accepted as the first-line treatment [60-64]. Nevertheless, the results of clinical trials have shown no improvement in median overall survival, or median progression-free survival, through the use of TACE with systemic treatment [65-67]. Taking into consideration the ESMO recommendations [39], TACE should be discontinued after its second failure. For such patients, CDK9 inhibition together with Sorafenib has been shown to be exceptionally effective when p-53 mutation and c-Myc overexpression occur [13, 16, 34]. Consequently, detection of p-53 mutations or c-Myc overexpression should be the primary selection criterion for these patients. Chun Hsu *et al.* [68] confirmed that CDK9 inhibitors can significantly increase Sorafenib's efficacy. Therefore, the combination of CDK9 inhibitors with Sorafenib may turn out to be an effective systemic treatment for patients with advanced HCC.

5. DISCUSSION

Inhibition of HCC cell lines and tumor growth suppression was observed after the application of each of the CDK9 inhibitors presented above. Furthermore, owing to their high specificity, they had no effects on unaltered hepatocytes and no toxic effects were observed in either HCC cell lines or *in vivo* in mice (Table **2**) [3, 5, 7, 12, 29-31, 44] The antitumor properties of miR-206 have been recognized in multiple studies. It is possible that the increased intracellular levels of miR-206, brought about by regulation of E-cadherin and N-cadherin, and by VEGF expression, can suppress progression and metastasis in HCC. The synergistic effects of PHA-767491 with 5-fluorouracil may enhance the clinical effectiveness of HCC treatment schemes based on 5-fluorouracil

(5-fluorouracil, mitoxantrone, and cisplatin included), by reducing chemoresistance and by stimulating apoptosis. MinKe He *et al.* [69], in their recent study, revealed significantly longer overall median survival time in patients treated with a combination of Sorafenib and hepatic arterial infusions of oxaliplatin, fluorouracil, and leucovorin systemic therapy, in comparison to Sorafenib alone. Together with the discovery of the synergistic effects of PHA-767491 with 5-fluorouracil, this has opened up new perspectives for the application of CDK9 inhibitors in the systemic treatment of HCC. Those drugs whose application did not result in chemoresistance in mice are currently undergoing thorough evaluation for use in highly chemoresistant tumors, such as HCC. Neither BA-12 nor BP-14 induced chemoresistance, even after prolonged treatment, and their therapeutic effect is considered significant (Table **2**). Nevertheless, further trials are needed to determine their pharmacokinetics and to ensure their safety for usage *in vivo*. The new generation CDK9-inhibitors, Xylocydine and Ibulocydine, reduce the intracellular levels of anti-apoptotic proteins such as BCL, leading to increased apoptosis of tumor cells. Moreover, they seem to be especially effective in HCC with c-Myc overexpression. Studies have verified that CDK9 has a key role in c-Myc-mediated tumor growth and that CDK9 inhibitors not only inhibit progression but diametrically decrease both the mass and size of HCC nodules. As previously noted, Xylocydine stabilizes p53 and induces cell cycle arrest, while the activity of Ibulocydine was unrestricted by Bcl-xL overexpression. Targeting CDK9 is, therefore, worth consideration, especially in the immunotherapy of HCC in cases with diagnosed p53 mutation and BCL2 overexpression, the activity of which is regulated by the CDK9/P-TEFb complex. It is possible that impaired cell cycle control can be compensated for by decreased levels of pro-proliferative proteins. Additionally, Xylocydine has been shown to increase the expression of functional p53 and to further intensify the suppression of tumor growth. Suggested prognostic markers for CDK9-targeted therapy require further examination but, based on their dependence on CDK activity, a reasonable approach would be to track the cellular responses to drugs by measurement of c-Myc, Mcl-1 and Bax expression in tumor cells. Treatment based on CDK9 inhibition is associated with potential prognosis improvements and with survival time extension. Such treatment may also amplify the therapeutic effects of other drugs (such as Sorafenib). By adopting appropriate entry criteria (paragraph 4), it is likely that CDK9 inhibitors will show a synergistic effect when used together with Sorafenib or Lenvatinib, even in the treatment of advanced HCC. Taken together, CDK9-inhibition is a promising target in HCC treatment, due to low overall toxicity and strong antiproliferative effect. Despite many promising studies, many details have yet to be examined. The pharmacokinetics and possible side effects *in vivo*, as well as possible interactions with other drugs, have yet to be defined. In summary, although further investigation is needed, CDK inhibition is a promising therapeutic target for the systemic treatment of advanced HCC.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors would like to thank the reviewer for constructive feedback.

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Rozdział 5. Podsumowanie

W powyższych badaniach wykazaliśmy, że CDK9 ulega nadekspresji w raku urotelialnym pęcherza moczowego, a jej wysoki poziom związany jest z niższym stopniem złośliwości histopatologicznej nowotworu. Charakter tej zależności jest jednak odmienny niż w większości dotychczas przebadanych nowotworów. W przeciwieństwie do raka jajnika czy kostniakomięsaka, wysoka ekspresja CDK9 w BLCA była związana z dłuższym okresem przeżycia i wyższym odsetkiem pięcioletnich przeżyć [22,24]. Weryfikacja otrzymanych danych, poprzez analizę kohorty pochodzącej z bazy The Cancer Genome Atlas, pozwala zakładać, że uzyskane wyniki są reprezentatywne dla omawianego nowotworu. W niniejszej pracy po raz pierwszy opisana została relacja pomiędzy ekspresją CDK9 a rokowaniem pacjentów z BLCA. Z tego powodu nie mamy możliwości skontrastowania naszych wyników z pracami innych autorów. Niemniej jednak, wynik zaskakująco odstający od konwencji przyjętej w literaturze światowej wymaga drobiazgowej analizy.

5.1. Znaczenie nadekspresji kinazy CDK9 w raku urotelialnym pęcherza moczowego

Dotychczas jedynie dwie inne prace poruszyły zagadnienie ekspresji CDK9 w BLCA. Antonova i wsp. wykazali nadekspresję CDK9 w inwazyjnym raku pęcherza moczowego w porównaniu z rakiem nieinwazyjnym [44]. W naszej grupie to raki nieinwazyjne miały statystycznie wyższą ekspresję CDK9. Poziom CDK9 był najwyższy w guzach o wysokim stopniu dojrzałości histologicznej i niskim stopniu zaawansowania klinicznego. Zjawisko to można wyjaśnić dwojako. Z jednej strony, uzyskane wyniki zdają się potwierdzać, że komórki lepiej zróżnicowane są w większym stopniu zależne od endogennych szlaków regulujących podział i śmierć komórki [26]. Zakładając, że na wczesnym etapie karcynogenezy komórki nowotworowe nie zostały jeszcze genetycznie przeprogramowane i zachowały przynajmniej częściową aktywność genów supresorowych, nadekspresja CDK9 może sprzyjać produkcji białek antyapoptotycznych i zapobiegać śmierci komórki [19,20]. Tym samym, ekspresja CDK9

maleje, kiedy choroba postępuje, a komórki stopniowo wyswobadzają się spod kontroli organizmu. Z drugiej strony, istnienie pętli dodatniego sprzężenia zwrotnego pomiędzy p53 a CDK9 wskazuje na możliwość kompensacyjnej nadekspresji CDK9 w guzach, które utraciły funkcjonalność p53 [45].

Niestety, brak jest obecnie badań oceniających skutki owej kompensacji. Hipotetyzujemy, że jej aktywność biologiczna warunkowana jest interakcjami pomiędzy guzem a jego mikrośrodowiskiem oraz ekspresją cyklin tworzących z CDK9 funkcjonalne kompleksy. Wyciszenie aktywności ścieżki sygnałowej CDK9 w komórkach linii kostniakomięsaka (U2OS) opóźniło przejście tych komórek do fazy S cyklu komórkowego i spowolniło ich proliferację, ale tylko w obecności czynnika uszkadzającego DNA [28]. Zastosowanie technologii ”knockdown” w celu dezaktywacji szlaków sygnałowych CDK9 wiązało się także ze spontanicznym uszkodzeniem DNA i zmniejszeniem zdolności komórki do rekonwalescencji po przejściowym zatrzymaniu cyklu komórkowego. Również uszkodzenie DNA po wcześniejszym wyciszeniu aktywności CDK9 powodowało niestabilność widełek replikacyjnych, do której doszło pomimo nieobecności substancji genotoksycznych [46,47]. Yu i wsp. zbadali także rolę cykliny K, która alternatywnie do cykliny T tworzy kompleksy z CDK9. Co ciekawe, jedynie deficyt cykliny K, ale nie cykliny T, zaburzał regenerację komórek, implikując udział cykliny K w odpowiedzi na stres replikacyjny [28]. W myśl tej zasady wysoka ekspresja cykliny K może przyczyniać się do utrzymania stabilności genetycznej i naprawy DNA w warunkach zwiększonej podatności na jego uszkodzenia, podczas gdy nadekspresja cykliny T sprzyjać będzie narastaniu immunooporności i zwiększonemu przeżyciu komórki nowotworowej [17,28].

Zagadką pozostaje również dlaczego ekspresja CDK9 w BLCA ma odmienne znaczenie prognostyczne niż w innych nowotworach złośliwych. Jedna z hipotez zakłada, że zmniejszanie się ekspresji CDK9 wraz z postępem choroby jest przejawem niestabilności genomowej. Jedną z najczęstszych aberracji genetycznych w raku urotelialnym jest utrata chromosomu 9, występująca w ponad 50% guzów pęcherza niezależnie od ich stopnia zaawansowania [48]. W zaawansowanych nowotworach delecja krótkiego ramienia chromosomu 9 (9q) ma miejsce częściej niż delecja krótkiego chromosomu (9p) [49,50]. Utrata heterozygotyczności 9q uznawana jest za bardzo wczesną zmianę w patogenezie BLCA, która poprzedza serię zmian liczby kopii DNA i prowadzi do szybkiego narastania niestabilności genomowej [48]. Delecje

9q33 i 9q34 wiążą się z bardziej agresywnym przebiegiem choroby [50]. Gen *CDK9* mieści się na chromosomie 9q34, możliwe jest zatem, że zmniejszona ekspresja kinazy CDK9 w guzach bardziej zaawansowanych powstała na skutek utraty funkcjonalnego *CDK9* h i odzwierciedla stopień destabilizacji genomu [51,52]. Oznaczałoby to, że w rakach z niską częstotliwością mutacji somatycznych aktywność CDK9 nie jest ograniczana niestabilnością genetyczną i wysoka ekspresja tej kinazy może przewidywać złe rokowanie. Natomiast w nowotworach z większym nagromadzeniem mutacji somatycznych, a zwłaszcza wczesną utratą 9q, ekspresja CDK9 może mieć odmienne znaczenie prognostyczne. Model ten zdaje się być przynajmniej częściowo prawdziwy dla BCLA i raka płuca, charakteryzujących się większą częstotliwością mutacji somatycznych i związkiem wysokiego CDK9 z dłuższym przeżyciem pacjentów [53–55]. W tych nowotworach niska ekspresja CDK9 koreluje również cechami histologicznej złośliwości nowotworu, wskazując na potencjalne korzyści tej grupy pacjentów z bardziej agresywnej terapii.

5.2. Zależności pomiędzy ekspresjami białka p53 i CDK9 w raku urotelialny pęcherza moczowego

W kolejnym etapie pracy oznaczyliśmy ekspresję białka p53 w raku urotelialnym pęcherza moczowego oraz oceniliśmy korelację z cechami histopatologicznej złośliwości nowotworu i ekspresją CDK9. Wykazaliśmy, że białko p53 ulega nadekspresji w BCLA, a jego wysoki poziom został stwierdzony w rakach inwazyjnych, rakach o niższym stopniu dojrzałości histologicznej oraz rakach z przerzutami. Nadekspresja p53 jest często związana z mutacją punktową szczególnie konserwatywnego regionu genu *TP53* i wynika z nagromadzenia niefunkcjonalnego białka [56,57]. Tłumaczy to wyższą ekspresję zmutowanego *TP53* w porównaniu z genem niezmutowanym oraz związek wysokiego poziomu p53 z wystąpieniem przerzutów odległych u pacjentów wchodzących w skład naszej grupy badanej. Wśród pacjentów z inwazyjnymi BLCA, wysoka ekspresja p53 wiązała się z istotnie niższym współczynnikiem pięcioletnich przeżyć (94.44% vs. 57.14%; p=0.015), krótszym czasem do progresji (91.74% vs 52.85%, p=0.013) oraz ryzykiem szybszej progresji (HR=9.63 [1.06–87.67); p=0.04). Natomiast niska ekspresja p53 była markerem prognostycznym

dłuższego przeżycia pacjentów (HR=0.107 [0.012–0.96]; p=0.046). Dane te znajdują odzwierciedlenie w literaturze, ponieważ mutacja p53 stanowi czynnik ryzyka progresji nowotworu nieinwazyjnego do nowotworu inwazyjnego, charakteryzującego się znacznie krótszym czasem przeżycia [34].

W warunkach stresu komórkowego p53 stanowi mechanizm ochronny, który aktywuje szlaki sygnałowe indukowane uszkodzeniem DNA oraz ich liczne mediatory, takie jak cyklinę K [58,59]. Cykliny K i T tworzą kompleksy z CDK9 niezależnie od siebie, a ich wzajemny stosunek i aktywność warunkują przeżycie lub śmierć komórki [28,45]. Pomiędzy kompleksem CDK9/cyklina T1 i p53 istnieje pętla sprzężenia zwrotnego, w której wzajemna fosforylacja CDK9 i p53 prowadzi do modyfikacji i wzmocnienia ich aktywności [45]. Mechanizm ten zdaje się wyjaśniać, dlaczego guzy z wysoką ekspresją CDK9 mają także wysoką ekspresję p53.

Chociaż współwystępowanie wysokiej ekspresji CDK9 i p53 może zaistnieć na wczesnych etapach rozwoju BLCA, w naszej pracy nie znaleźliśmy istotnej korelacji pomiędzy obydwoma białkami. Wysoki poziom p53 w niskozróżnicowanych i inwazyjnych rakach pęcherza moczowego o wysokiej ekspresji CDK9 sugeruje nie tylko związek narastającej niestabilności genetycznej ze wzrostem histopatologicznej agresywności nowotworu, ale także świadczy o skomplikowanych interakcjach między obydwoma białkami.

CDK9 aktywuje białko mouse double minute 4 (MDM4) i inhibitor białka p53 stymulującego apoptozę (iASPP), które antagonizują p53 i sprzyjają akumulacji zmian genetycznych i narastaniu niestabilności genomowej [30,60]. Rui i wsp. potwierdzają te doniesienia w swoich badaniach. Obniżenie aktywności CDK9 hamuje proliferację komórek nowotworowych i przeciwdziała powstawaniu przerzutów raka pęcherza moczowego [61]. Zmniejszenie aktywności CDK9 może być również bezpośrednią konsekwencją mutacji p53, utraty jego funkcji i rozregulowania pętli sprzężenia zwrotnego pomiędzy p53 i CDK9. W tym sensie CDK9 może być wskaźnikiem funkcjonalności p53 [40,45]. Przypuszczamy, że w dobrze zróżnicowanych rakach urotelialnych, nadekspresja CDK9 może tłumić aktywność białka p53 i ułatwiać rozwój choroby. Natomiast w rakach niskozróżnicowanych, cechujących się większą niestabilnością genomową, p53 jest często zmutowane i nieaktywne. W tej sytuacji wysoka aktywność CDK9 traci znaczenie jako mechanizm przeciwdziałający apoptozie, a jej wyciszenie na kolejnych etapach rozwoju choroby mogłaby pozwolić na ograniczenie wpływu cykliny K na integralność genomu i mechanizmy naprawy DNA [28,62].

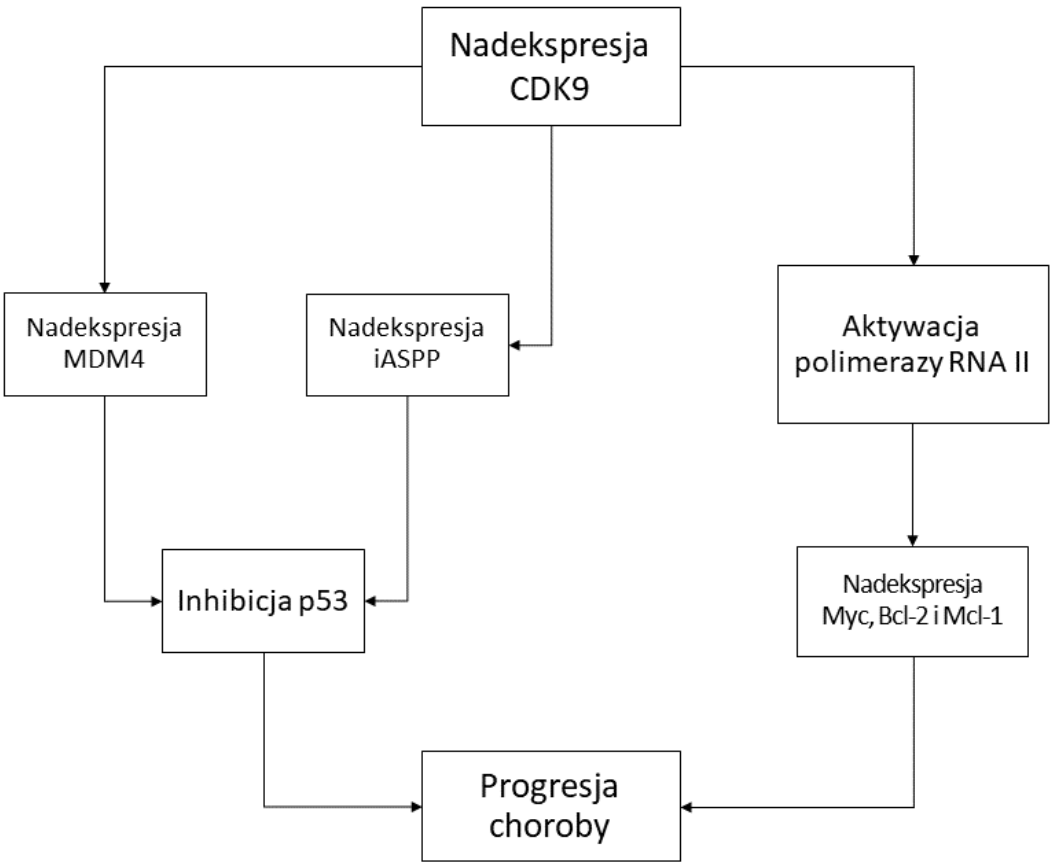
5.3. Znaczenie kliniczne wysokiej ekspresji CDK9 i białka p53 w raku urotelialnym pęcherza moczowego

Zważywszy, że wpływ CDK9 na rozwój raka pęcherza moczowego nie został ostatecznie zdeterminowany, niniejszy projekt może znacząco wpłynąć na przebieg dalszych badań. Po pierwsze, ustaliliśmy, że znaczenie prognostyczne wysokiej ekspresji CDK9 w BLCA jest odmienne niż w innych nowotworach. Po drugie, największa aktywność kinazy przypada na wczesne stadia rozwoju tego raka, stawiając pod znakiem zapytania jej hamowanie w zaawansowanym stadium choroby. Finalnie, efekt biologiczny aktywności CDK9 zależy nie tylko od ekspresji jej izomerów i tworzących z nią kompleksy cyklin, ale także od funkcjonalności genów supresorowych i nasilenie niestabilności genomowej, co zaprezentowaliśmy na przykładzie białka p53.

Istotne jest także uwzględnienie powyższych danych w szerszym kontekście, przedstawionym w pracach przeglądowych. Największą wadą pierwszej generacji inhibitorów CDK9 jest ich niska selektywność i mechanizm działania polegający na konkurencji z ATP w miejscu allosterycznym kinazy CDK9. Uważa się, że jest to główny powód ich systemowej toksyczności oraz zasadnicza przeszkoda w ich wprowadzeniu do praktyki klinicznej[11,63]. Pomimo obiecujących wyników prób przedklinicznych i akceptowalnej toksyczności drugiej generacji inhibitorów CDK9, jedynie dinaciclib przeszedł do trzeciej fazy badań klinicznych [36,64]. Obecnie za główną zaletę blokady CDK9 uważa się jej potencjał do przełamywania immunooporności nowotworów. Inhibitory CDK9 wykazują synergistyczne działanie z venetoklaksem, bortezomibem, czy doksorubicyną, co uczyniło je potencjalnymi lekami w terapii nawrotowego i opornego na leczenie szpiczaka mnogiego [65–67].

Mając na względzie rolę p53 w przeciwnowotworowej aktywności inhibitorów CDK9, wykazanie zależności pomiędzy ekspresjami CDK9 i p53 stanowi pierwszy krok w kierunku terapii pacjentów z niekorzystnymi zmianami cytogenetycznymi. Kinaza CDK9 fosforyluje i aktywuje białko MDM2 oraz MDM4, które odpowiadają za ubikwitynację i degradację p53 [68]. Blokada aktywności CDK9 umożliwia przywrócenie funkcji niezmutowanego p53 w komórkach nowotworowych poprzez hamowanie jego inhibitorów, MDM4 oraz iASPP

(Rycina 3) [30,60]. Stwarza to potencjał do zastosowanie inhibitorów CDK9 w niezaawansowanych BLCA, w których mutacje p53 są rzadsze, ale aktywność p53 jest ograniczana przez inne mechanizmy [40,60]. Wydaje się także, że ostateczny efekt zależy od stopnia blokady CDK9. Całkowite wyciszenie jej aktywności niweluje resztkową aktywność p53, natomiast częściowa blokada ją przywraca [40].



Rycina 3. Przewidywany wpływ nadekspresji CDK9 na progresję raka urotelialnego [69]. Nadekspresja CDK9 zwiększa aktywność inhibitorów p53, MDM4 i iASPP, uniezależniając komórkę nowotworową od mechanizmów regulujących cykl komórkowy. Aktywność CDK9 umożliwia również transkrypcję białek antyapoptotycznych, takich jak Myc, Bcl-2 i Mcl-1, przez polimerazę RNA II. Obydwa mechanizmy przyczyniają się do progresji choroby nowotworowej.

Ze względu na możliwość stosunkowo prostego wkomponowaniu inhibitorów CDK9 do obecnych schematów terapeutycznych oraz ich obiecująca aktywność in-vitro i in-vivo,

próby kliniczne w HCC wydają się jedynie kwestią czasu [41,42,70]. W momencie ukazania się naszego artykułu, jedynie sorafenib i lenvatinib wydłużały czas przeżycia chorych z zaawansowanym rakiem wątrobowokomórkowym [42,71–73]. Od tego czasu atezolizumab w połączeniu z bewacyzumabem wykazały istotnie wydłużony czas całkowitego przeżycia w porównaniu z sorafenibem, co zaowocowało zarekomendowaniem ich w marcu 2021 roku jako pierwszej linii terapii przez Europejskie Towarzystwo Onkologii Klinicznej [74,75]. Niemniej jednak 20% pacjentów nie odpowiada na terapię atezolizumabem i bewacyzumabem, dlatego dyskusja na temat drugiej linii leczenia jest nadal otwarta [74,76]. Tym samym sorafenib pozostaje alternatywną pierwszą linią terapii dla pacjentów po nieudanej procedurze przeztętniczej chemoembolizacji lub z rozsiewem choroby [74]. Jako że inhibitory CDK9 łagodzą oporność na sorafenib i zwiększają jego efektywność, zachowały one swój potencjał do pozostania lekami uzupełniającymi pierwszą linię terapii [43,77]. W kombinacji z sorafenibem wykazały one szczególną skuteczność wobec komórek z mutacją p53 i nadekspresją c-Myc, wskazując że właśnie pacjenci z tymi zmianami mogą odnieść największe korzyści terapeutyczne [78,79].

Nierozstrzygniętą kwestią pozostaje skuteczność alternatywnych form blokowania aktywności CDK9, takich jak leki skoniugowane. Jednym z nich jest THAL-SNS-032, cząsteczka selektywnie degradująca CDK9, która składa się z inhibitora CDK9 (SNS-032) oraz ligandu związanego z pochodną talidomidu. THAL-SNS-032 umożliwia szybką degradację CDK9 i nie wpływa na aktywność innych kinaz cyklinozależnych. Chociaż sugeruje to jego dużą selektywność, efekt biologiczny zdaje zależeć od ekspresji genu CRBN, współtworzącego kompleks ligazy ubikwityny. Utrata CRBN może zatem uodpornić komórki nowotworowe na terapię THAL-SNS-032 [39]. Niewątpliwą zaletą degradatorów CDK9 jest natomiast wydłużony czas półtrwania w porównaniu z inhibitorami CDK9, co pozwala uniknąć powtarzalnych infuzji i zwiększa umożliwia stabilizację stężenia terapeutycznego leku [80,81]. Nowe, doustne inhibitory CDK9, takie voruciclib, mogą poprawić tolerancję terapii przez pacjentów, ale dostępne dane nie pozwalają na wyciągnięcie jednoznacznych wniosków [80].

Należy zaznaczyć, że spora część badań z użyciem inhibitorów CDK9 została przeprowadzona wśród pacjentów wcześniej leczonych, u których wystąpił nawrót choroby, pojawiła się oporność na leczenie, lub którzy mieli niekorzystny profil cytogenetyczny. Pomimo to, zaobserwowano efekt kliniczny wynikający z przełamania lekooporności oraz

skuteczność wobec komórek ze zmutowanym p53 [82,83]. Sugeruje to, że zarówno pacjenci z prawidłową, jak i zmutowaną wersją tego genu mogą odnieść korzyści z leczenia.

Choć niniejszy projekt usystematyzował postępy w dziedzinie terapii celowanej na CDK9 i postawił pierwszy krok w kierunku pierwszych badań klinicznych w raku urotelialnym pęcherza moczowego, wiele pytań nadal pozostaje bez odpowiedzi. Nie ulega jednak wątpliwości, że kolejne badania powinny analizować CDK9 w szerokim kontekście jej skomplikowanych interakcji.

Rozdział 6. Wnioski

1. Wysoka ekspresji CDK9 koreluje z cechami mniejszej złośliwości raka urotelialnego pęcherza moczowego, takimi jak brak naciekania mięśniówki właściwej, wyższy stopień histologicznej dojrzałości i niższy stopień zaawansowania klinicznego.
2. CDK9 stanowi potencjalny marker prognostyczny w raku urotelialnym pęcherza moczowego, a jego wysoka ekspresja związana jest z dłuższym czasem przeżycia chorych.
3. Wysoka ekspresja p53 w nieinwazyjnym raku urotelialnym pęcherza moczowego stanowi niekorzystny czynnik prognostyczny związany z krótszym przeżyciem całkowitym i krótszym czasem do wystąpienia progresji.
4. Wartość predykcyjna p53 w rakach nieinwazyjnych może wynikać ze zwiększonego ryzyka progresji do raka inwazyjnego.
5. Ekspresje CDK9 i p53 nie wykazują liniowej korelacji, ale współistnieje ich nadekspresji sugeruje dysregulację obu szlaków już na wczesnych etapach karcynogenezy.
6. CDK9 stanowi potencjalny cel terapeutyczny w raku urotelialnym pęcherza moczowego, a terapie ukierunkowane na CDK9 powinny uwzględniać jej biologię oraz odmienność prognostyczną zależną od nowotworu.
7. Ograniczoną skuteczność inhibitorów CDK9 w monoterapii i ich dużą efektywność w znoszeniu lekooporności sprawia, że stanowią one potencjalne uzupełnienie powszechnie już stosowanych schematów terapeutycznych.
8. Inhibitory CDK9 mogą przywracać aktywność niezmutowanego p53 oraz wykazują skuteczność wobec linii komórkowych posiadających jego zmutowaną formę. Z tego powodu pacjenci z niekorzystnymi zmianami cytogenetycznymi oraz nawrotowymi i lekoopornymi nowotworami mogą odnieść z ich zastosowania szczególne korzyści kliniczne.

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Cyclin-Dependent Kinases, in Human Tumor Cell Lines. *Mol. Cancer Ther.* **2009**, *8*, 324–332.

Rozdział 8. Streszczenie w języku polskim

W ostatnich dekadach nastąpił znaczący postęp w leczeniu nowotworów i opiece onkologicznej. Niestety, obecnie stosowane schematy leczenia systemowego posiadają liczne ograniczenia, skłaniając do poszukiwań nowych markerów prognostycznych i celów terapeutycznych. Jednym z efektów tych poszukiwań zostało wprowadzenie inhibitorów kinaz cyklinozależnych 4/6 (CDK) do terapii zaawansowanego raka piersi. Obecnie za jeden z najbardziej obiecujących celów terapeutycznych uważa się CDK9, która stanowi centralny ośrodek regulacji transkrypcji. Jej nadaktywność zwiększa ekspresję białek antyapoptotycznych, takich jak Bcl-2 i Mcl-1, a także niweluje działanie białka supresorowego p53, czym sprzyja onkogenezie. Niniejszy projekt miał na celu określenie znaczenia prognostycznego CDK9 w nowotworach złośliwych, ocenę możliwości zastosowania inhibitorów CDK9 w praktyce klinicznej i wskazanie grupy pacjentów mogącej uzyskać największe korzyści z terapii. Analiza poziomu ekspresji CDK9 w raku urotelialnym pęcherza moczowego (BLCA) wykazała, że jej wysoka ekspresja korelowała z wyższym stopniem histologicznej dojrzałości guza, brakiem naciekania błony mięśniowej oraz mniej zaawansowaną chorobą. Zarówno analiza naszej grupy badanej, jak i kohorty The Cancer Genome Atlas wykazały, że nadekspresja CDK9 jest predyktorem dłuższego przeżycia w BLCA, co odbiega od doniesień z innych nowotworów. W naszej grupie badanej wysoka ekspresja p53 wiązała się z niekorzystnym rokowaniem w BCLA nienaciekającym mięśniówkę właściwą, a raki z wysoką ekspresją CDK9 cechowały się również wysoką ekspresją p53. Nie zaobserwowaliśmy jednak jednoznacznej korelacji pomiędzy ekspresjami p53 i CDK9. Ze względu na niską skuteczność w monoterapii, możliwość zastosowania blokady aktywności CDK9 w terapii guzów litych nadal nie została w pełni określona. Odpowiedź na to pytanie mogą przynieść badania kliniczne przeprowadzone z użyciem nowej generacji selektywnych inhibitorów CDK9, leków degradujących CDK9, oraz stosujące inhibitory CDK9 w celu uzupełnienia obecych schematów terapeutycznych. Ze względu na synergistyczny efekt przeciwnowotworowy sorafenibu oraz inhibitorów CDK9, badania kliniczne w raku wątrobowokomórkowym wydają się jedynie kwestią czasu. Dane z literatury światowej sugerują, że pacjenci z mutacją p53 mogą osiągnąć z terapii największe korzyści. Pomimo obiecujących wyników badań przedklinicznych, wszelkie założenia teoretyczne powinny

zostać zweryfikowane w badaniach klinicznych.

Rozdział 9. Streszczenie w języku angielskim

The last decades have brought immense progress in cancer therapy and patient care. Unfortunately, current systemic therapies are not devoid of limitations and prompt the search for novel therapeutic targets and prognostic markers. As a result, the FDA and EMA recently approved cyclin-dependent kinases 4/6 (CDK) for treating advanced breast cancer. One of the most promising therapeutic targets is CDK9, considered a central transcription regulation hub. Its overexpression increases the level of anti-apoptotic proteins, such as Bcl-2 and Mcl-1, and diminishes the activity of p53, facilitating carcinogenesis. This project aims to determine the prognostic significance of CDK9 in human cancers, define the applicability of CDK9 inhibitors for clinical use, and identify patients that can achieve the most benefits from the therapy. The analysis of CDK9 expression in urothelial carcinoma (BLCA) revealed that high-CDK9 tumors were usually lower-grade, lower-stage, and non-muscle-invasive compared to low-CDK9 tumors. Furthermore, in both TMA and The Cancer Genome Atlas cohorts, high expression of CDK9 predicted longer patients' survival, which contrasts with the reports from other cancers. In our cohort, high expression of p53 predicted shorter survival in non-muscle-invasive bladder cancer. Tumors with high p53 also showed high levels of CDK9, but we found no linear correlation between the expressions of p53 and CDK9. The complex relationship between both proteins should be considered when preclinical trials in BLCA are conducted. Due to low efficacy in monotherapy, the utility of CDK9 inhibitors has yet to be entirely determined. Therefore, further trials should consider using more selective CDK9 inhibitors, CDK9 degraders, and their incorporation into current therapeutics regimens as complementary agents instead of monotherapy. Considering the synergistic effects between CDK9 inhibitors and sorafenib, clinical trials in hepatocellular seem only a matter of time. Literature data suggest that the efforts should focus on patients with the mutation of p53, as this group can achieve the most clinical benefits. Despite the recent advances, hopes for finding drugs that overcome resistance o therapy may be preemptive. Thus, all theoretical concepts should be proven in future trials.

Rozdział 10. Oświadczenia współautorów

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1) Borowczak, J., Szczerbowski, K., Stec, E., Grzanka, D. & Szylberg, Ł. CDK9: Therapeutic Perspective in HCC Therapy. Curr. Cancer Drug Targets 20, 318–324 (2020). doi: 10.2174/1568009620666200212124357

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- konceptualizacji pracy,
- analizie literatury,
- opracowaniu rycin i tabel,
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- nadzorze merytorycznym manuskryptu,
- koordynowaniu klinicznych aspektów pracy.

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Oświadczenie

Jako współautor artykułów:

1) Borowczak, J., Szczerbowski, K., Maniewski, M., Zdrenka, M., Słupski, P., Antosik, P., Kołodziejska, S., Sekielska-Domanowska, M., Dubiel, M., Bodnar, M. & Szylberg, Ł. The Prognostic Role of CDK9 in Bladder Cancer. Cancers 14, (2022). doi:10.3390/cancers14061492

2) Borowczak J, Szczerbowski K, Maniewski M, Zdrenka M, Słupski P, Andrusewicz H, Łysik-Miśkurka J, Rutkiewicz P, Bodnar M, Szylberg Ł. The prognostic role of p53 and its correlation with CDK9 in urothelial carcinoma. Clin Transl Oncol. 2023 Mar;25(3):830-840. doi: 10.1007/s12094-022-02994-6. Epub 2022 Nov 14. PMID: 36374405; PMCID: PMC9941229.

oświadczam, że mój wkład merytoryczny w przygotowanie i przeprowadzenie badań oraz ich przedstawienie w formie publikacji polegał na:

- ocenie zebranych preparatów,
- przygotowaniu manuskryptu.

Oświadczam, że udostępnienie utworów nie będzie naruszało praw autorskich osób trzecich. Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionych publikacji przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część wyżej wymienionych prac wykazuje indywidualny wkład lek. Jędrzeja Borowczaka, polegający na:

- opracowaniu koncepcji badania,
- analizie zebranych preparatów histopatologicznych i immunohistochemicznych,
- opracowaniu i interpretacji wyników,
- wykonaniu analizy in silico,
- przygotowaniu manuskryptu.

- Mateusz Maniewski .....

Bydgoszcz, dnia 25.05.2023r.

prof. dr hab. n. med. Mariusz Dubiel

Klinika Położnictwa, Chorób Kobiecych i Ginekologii Onkologicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Oświadczenie

Jako współautor artykułu:

1) Borowczak, J., Szczerbowski, K., Maniewski, M., Zdrenka, M., Słupski, P., Antosik, P., Kołodziejska, S., Sekielska-Domanowska, M., Dubiel, M., Bodnar, M. & Szylberg, Ł. The Prognostic Role of CDK9 in Bladder Cancer. Cancers 14, (2022). doi:10.3390/cancers14061492

oświadczam, że mój wkład merytoryczny w przygotowanie i przeprowadzenie badań oraz ich przedstawienie w formie publikacji polegał na:

- nadzorze merytorycznym manuskryptu.

Oświadczam, że udostępnienie utworu nie będzie naruszało praw autorskich osób trzecich. Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część wyżej wymienionej pracy wykazuje indywidualny wkład lek. Jędrzeja Borowczaka, polegający na:

- opracowaniu koncepcji badania,
- analizie zebranych preparatów histopatologicznych i immunohistochemicznych,
- opracowaniu i interpretacji wyników,
- wykonaniu analizy in silico,
- przygotowaniu manuskryptu.

.....

Bydgoszcz, dnia 25.05.2023r.

dr n. med. Marta Sekielska-Domanowska
Klinika Położnictwa, Chorób Kobiecych i Ginekologii Onkologicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Oświadczenie

Jako współautorka artykułu:

1) Borowczak, J., Szczerbowski, K., Maniewski, M., Zdrenka, M., Słupski, P., Antosik, P., Kołodziejska, S., Sekielska-Domanowska, M., Dubiel, M., Bodnar, M. & Szylberg, Ł. The Prognostic Role of CDK9 in Bladder Cancer. Cancers 14, (2022). doi:10.3390/cancers14061492

oświadczam, że mój wkład merytoryczny w przygotowanie i przeprowadzenie badań oraz ich przedstawienie w formie publikacji polegał na:

- przygotowaniu manuskryptu.

Oświadczam, że udostępnienie utworu nie będzie naruszało praw autorskich osób trzecich. Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część wyżej wymienionej pracy wykazuje indywidualny wkład lek. Jędrzeja Borowczaka, polegający na:

- opracowaniu koncepcji badania,
- analizie zebranych preparatów histopatologicznych i immunohistochemicznych,
- opracowaniu i interpretacji wyników,
- wykonaniu analizy in silico,
- przygotowaniu manuskryptu.

Dr n.med. Marta
Sekielska-Domanowska
lekarz specjalista
położnictwa i ginekologii

Bydgoszcz, dnia 25.05.2023r.

lek. Marek Zdrenka

Zakład Patologii Nowotworów i Patomorfologii

Centrum Onkologii im. prof. Franciszka Łukaszczyka w Bydgoszczy

Oświadczenie

Jako współautor artykułów:

1) Borowczak, J., Szczerbowski, K., Maniewski, M., Zdrenka, M., Słupski, P., Antosik, P., Kołodziejska, S., Sekielska-Domanowska, M., Dubiel, M., Bodnar, M. & Szylberg, Ł. The Prognostic Role of CDK9 in Bladder Cancer. Cancers 14, (2022). doi:10.3390/cancers14061492

2) Borowczak J, Szczerbowski K, Maniewski M, Zdrenka M, Słupski P, Andrusewicz H, Łysik-Miśkurka J, Rutkiewicz P, Bodnar M, Szylberg Ł. The prognostic role of p53 and its correlation with CDK9 in urothelial carcinoma. Clin Transl Oncol. 2023 Mar;25(3):830-840. doi: 10.1007/s12094-022-02994-6. Epub 2022 Nov 14. PMID: 36374405; PMCID: PMC9941229.

oświadczam, że mój wkład merytoryczny w przygotowanie i przeprowadzenie badań oraz ich przedstawienie w formie publikacji polegał na:

- ocenie zebranych preparatów,
- nadzorze nad wykonaniem i oceną przygotowanych zdjęć.

Oświadczam, że udostępnienie utworów nie będzie naruszało praw autorskich osób trzecich. Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienion7ch publikacji przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część wyżej wymienionych prac wykazuje indywidualny wkład lek. Jędrzeja Borowczaka, polegający na:

- opracowaniu koncepcji badania,
- analizie zebranych preparatów histopatologicznych i immunohistochemicznych,
- opracowaniu i interpretacji wyników,
- wykonaniu analizy in silico,
- przygotowaniu manuskryptu.

Cambridge, 25.05.2023r.

Navid Ahmadi, M.D.
Department of Cardiothoracic Surgery
Royal Papworth Hospital, Cambridge, UK

Oświadczenie

Jako współautor artykułu:

1) Borowczak, J., Szczerbowski, K., Ahmadi, N. & Szylberg, Ł., CDK9 inhibitors in multiple myeloma: a review of progress and perspectives. Med. Oncol. 39, 39 (2022). doi:10.1007/s12032-021-01636-1

oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz ich przedstawienie w formie publikacji polegał na:

- krytycznej ocenie manuskryptu,
- korekcie językowej manuskryptu.

Wyrażam zgodę na przedłożenie wyżej wymienionej pracy przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych. Jednocześnie oświadczam, że udostępnienie utworu nie będzie naruszało praw autorskich osób trzecich.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część wyżej wymienionej pracy wykazuje indywidualny wkład lek. Jędrzeja Borowczaka polegający na:

- konceptualizacji pracy,
- analizie literatury,
- opracowaniu rycin i tabel,
- przygotowaniu manuskryptu.

.....

Bydgoszcz, dnia 26.05.2023r.

dr n. med. Paulina Antosik

Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Oświadczenie

Jako współautorka artykułu:

1) Borowczak, J., Szczerbowski, K., Maniewski, M., Zdrenka, M., Słupski, P., Antosik, P., Kołodziejska, S., Sekielska-Domanowska, M., Dubiel, M., Bodnar, M. & Szylberg, Ł. The Prognostic Role of CDK9 in Bladder Cancer. Cancers 14, (2022). doi:10.3390/cancers14061492

oświadczam, że mój wkład merytoryczny w przygotowanie i przeprowadzenie badań oraz ich przedstawienie w formie publikacji polegał na:

- przygotowaniu i opracowaniu zebranych próbek;
- barwieniu preparatów histopatologicznych.

Oświadczam, że udostępnienie utworu nie będzie naruszało praw autorskich osób trzecich. Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część wyżej wymienionej pracy wykazuje indywidualny wkład lek. Jędrzeja Borowczaka, polegający na:

- opracowaniu koncepcji badania,
- analizie zebranych preparatów histopatologicznych i immunohistochemicznych,
- opracowaniu i interpretacji wyników,
- wykonaniu analizy in silico,
- przygotowaniu manuskryptu.

Paulina Antosik

Bydgoszcz, dnia 25.05.2023r.

lek. Piotr Słupski

Klinika Urologii

Szpital Uniwersytecki nr 2 nr 2 im Jana Biziela w Bydgoszczy

Oświadczenie

Jako współautor artykułów:

1) Borowczak, J., Szczerbowski, K., Maniewski, M., Zdrenka, M., Słupski, P., Antosik, P., Kołodziejska, S., Sekielska-Domanowska, M., Dubiel, M., Bodnar, M. & Szylberg, Ł. The Prognostic Role of CDK9 in Bladder Cancer. Cancers 14, (2022). doi:10.3390/cancers14061492

2) Borowczak J, Szczerbowski K, Maniewski M, Zdrenka M, Słupski P, Andrusewicz H, Łysik-Miśkurka J, Rutkiewicz P, Bodnar M, Szylberg Ł. The prognostic role of p53 and its correlation with CDK9 in urothelial carcinoma. Clin Transl Oncol. 2023 Mar;25(3):830-840. doi: 10.1007/s12094-022-02994-6. Epub 2022 Nov 14. PMID: 36374405; PMCID: PMC9941229.

oświadczam, że mój wkład merytoryczny w przygotowanie i przeprowadzenie badań oraz ich przedstawienie w formie publikacji polegał na:

- zebraniu preparatów,
- przygotowaniu bazy danych klinicznych.

Oświadczam, że udostępnienie utworów nie będzie naruszało praw autorskich osób trzecich. Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionych publikacji przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część wyżej wymienionych prac wykazuje indywidualny wkład lek. Jędrzeja Borowczaka, polegający na:

- opracowaniu koncepcji badania,
- analizie zebranych preparatów histopatologicznych i immunohistochemicznych,
- opracowaniu i interpretacji wyników,
- wykonaniu analizy in silico,
- przygotowaniu manuskryptu.

Słupski Piotr

Bydgoszcz, dnia 25.05.2023r.

mgr Joanna Łysik-Miśkurka

Zakład Patologii Nowotworów i Patomorfologii

Centrum Onkologii im. prof. Franciszka Łukaszczyka w Bydgoszczy

Oświadczenie

Jako współautorka artykułu:

1) Borowczak J, Szczerbowski K, Maniewski M, Zdrenka M, Słupski P, Andrusewicz H, Łysik-Miśkurka J, Rutkiewicz P, Bodnar M, Szylberg Ł. The prognostic role of p53 and its correlation with CDK9 in urothelial carcinoma. Clin Transl Oncol. 2023 Mar;25(3):830-840. doi: 10.1007/s12094-022-02994-6. Epub 2022 Nov 14. PMID: 36374405; PMCID: PMC9941229.

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- przygotowaniu i barwieniu preparatów histopatologicznych.

Oświadczam, że udostępnienie utworu nie będzie naruszało praw autorskich osób trzecich. Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część wyżej wymienionej pracy wykazuje indywidualny wkład lek. Jędrzeja Borowczaka, polegający na:

- opracowaniu koncepcji badania,
- analizie zebranych preparatów histopatologicznych i immunohistochemicznych,
- opracowaniu i interpretacji wyników,
- wykonaniu analizy in silico,
- przygotowaniu manuskryptu.

Joanna Łysik-Miśkurka

Bydgoszcz, dnia 25.05.2023r.

mgr Hanna Andrusewicz

Zakład Patologii Nowotworów i Patomorfologii

Centrum Onkologii im. prof. Franciszka Łukaszczyka w Bydgoszczy

Oświadczenie

Jako współautorka artykułu:

1) Borowczak J, Szczerbowski K, Maniewski M, Zdrenka M, Słupski P, Andrusewicz H, Łysik-Miśkurka J, Rutkiewicz P, Bodnar M, Szylberg Ł. The prognostic role of p53 and its correlation with CDK9 in urothelial carcinoma. Clin Transl Oncol. 2023 Mar;25(3):830-840. doi: 10.1007/s12094-022-02994-6. Epub 2022 Nov 14. PMID: 36374405; PMCID: PMC9941229.

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Oświadczam, że samodzielna i możliwa do wyodrębnienia część wyżej wymienionej pracy wykazuje indywidualny wkład lek. Jędrzeja Borowczaka, polegający na:

- opracowaniu koncepcji badania,
- analizie zebranych preparatów histopatologicznych i immunohistochemicznych,
- opracowaniu i interpretacji wyników,
- wykonaniu analizy in silico,
- przygotowaniu manuskryptu.

Hanna Andrusewicz

Bydgoszcz, dnia 25.05.2023r.

lek. Ewa Stec

Szpital Uniwersytecki nr. 1 im. Antoniego Jurasza w Bydgoszczy

Oświadczenie

Jako współautorka artykułu:

1) Borowczak, J., Szczerbowski, K., Stec, E., Grzanka, D. & Szylberg, Ł. CDK9: Therapeutic Perspective in HCC Therapy. Curr. Cancer Drug Targets 20, 318–324 (2020). doi: 10.2174/1568009620666200212124357

oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz ich przedstawienie w formie publikacji polegał na:

- konceptualizacji pracy,
- analizie literatury,
- przygotowaniu manuskryptu.

Wyrażam zgodę na przedłożenie wyżej wymienionej pracy przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych. Jednocześnie oświadczam, że udostępnienie utworu nie będzie naruszało praw autorskich osób trzecich.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część wyżej wymienionej pracy wykazuje indywidualny wkład lek. Jędrzeja Borowczaka polegający na:

- konceptualizacji pracy,
- analizie literatury,
- opracowaniu rycin i tabel,
- przygotowaniu manuskryptu.

Ewa Stec
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Lekarz

Bydgoszcz, dnia 25.05.2023r.

mgr Sylwia Kołodziejska

Zakład Patomorfologii, Placentologii i Hematopatologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Oświadczenie

Jako współautorka artykułu:

1) Borowczak, J., Szczerbowski, K., Maniewski, M., Zdrenka, M., Słupski, P., Antosik, P., Kołodziejska, S., Sekielska-Domanowska, M., Dubiel, M., Bodnar, M. & Szylberg, Ł. The Prognostic Role of CDK9 in Bladder Cancer. Cancers 14, (2022). doi:10.3390/cancers14061492

oświadczam, że mój wkład merytoryczny w przygotowanie i przeprowadzenie badań oraz ich przedstawienie w formie publikacji polegał na:

- przygotowaniu, zabezpieczeniu i barwieniu materiałów histopatologicznych.

Oświadczam, że udostępnienie utworu nie będzie naruszało praw autorskich osób trzecich. Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część wyżej wymienionej pracy wykazuje indywidualny wkład lek. Jędrzeja Borowczaka, polegający na:

- opracowaniu koncepcji badania,
- analizie zebranych preparatów histopatologicznych i immunohistochemicznych,
- opracowaniu i interpretacji wyników,
- wykonaniu analizy in silico,
- przygotowaniu manuskryptu.

Sylwia Kołodziejska

Bydgoszcz, dnia 25.05.2023r.

mgr Paula Rutkiewicz

Zakład Patomorfologii, Placentologii i Hematopatologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Oświadczenie

Jako współautorka pracy:

1) Borowczak J, Szczerbowski K, Maniewski M, Zdrenka M, Słupski P, Andrusewicz H, Łysik-Miśkurka J, Rutkiewicz P, Bodnar M, Szylberg Ł. The prognostic role of p53 and its correlation with CDK9 in urothelial carcinoma. Clin Transl Oncol. 2023 Mar;25(3):830-840. doi: 10.1007/s12094-022-02994-6. Epub 2022 Nov 14. PMID: 36374405; PMCID: PMC9941229.

oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz ich przedstawienie w formie publikacji polegał na:

- przygotowaniu i barwieniu preparatów histopatologicznych.

Oświadczam, że udostępnienie utworu nie będzie naruszało praw autorskich osób trzecich. Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wydrębnienia część wyżej wymienionej pracy wykazuje indywidualny wkład lek. Jędrzeja Borowczaka polegający na:

- opracowaniu koncepcji badania,
- analizie zebranych preparatów histopatologicznych i immunohistochemicznych,
- opracowaniu i interpretacji wyników,
- wykonaniu analizy in silico,
- przygotowaniu manuskryptu.

Rutkiewicz Paula

Bydgoszcz, dnia 25.05.2023r.

prof. dr hab. Dariusz Grzanka

Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Oświadczenie

Jako współautor pracy:

1) Borowczak, J., Szczerbowski, K., Stec, E., Grzanka, D. & Szylberg, Ł. CDK9: Therapeutic Perspective in HCC Therapy. Curr. Cancer Drug Targets 20, 318–324 (2020). doi: 10.2174/1568009620666200212124357

oświadczam, że mój wkład merytoryczny w przygotowanie i przeprowadzenie badań oraz ich przedstawienie w formie publikacji polegał na:

- nadzorze merytorycznym manuskryptu.

Oświadczam, że udostępnienie utworu nie będzie naruszało praw autorskich osób trzecich. Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy przez lek. Jędrzeja Borowczaka jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wydrębnienia część wyżej wymienionej pracy wykazuje indywidualny wkład lek. Jędrzeja Borowczaka, polegający na:

- konceptualizacji pracy,
- analizie literatury,
- opracowaniu rycin i tabel,
- przygotowaniu manuskryptu.

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Rozdział 11. Zgoda komisji bioetycznej na prowadzenie badań

Uniwersytet Mikołaja Kopernika w Toruniu
Collegium Medicum im L. Rydygiera w Bydgoszczy
KOMISJA BIOETYCZNA
Ul. M. Skłodowskiej-Curie 9, 85-094 Bydgoszcz, tel.(052) 585-35-63, fax.(052) 585-38-11

KB 881/2019 Bydgoszcz, 22.11.2022 r.

Działając na podstawie art. 29 ustawy z dnia 5.12.1996 r. o zawodach lekarza i lekarza dentysty Dz.U. z 1997 r. Nr 28 poz. 152 (wraz z późniejszymi zmianami), rozporządzenia Ministra Zdrowia i Opieki Społecznej z dnia 11.05.1999 r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych (Dz.U. Nr 47 poz.480) oraz Zarządzenia Nr 21 Rektora UMK z dnia 4.03.2009 r. z późn. zm. w sprawie powołania oraz zasad działania Komisji Bioetycznej Uniwersytetu Mikołaja Kopernika w Toruniu przy Collegium Medicum im Ludwika Rydygiera w Bydgoszczy oraz zgodnie z zasadami zawartymi w DH i GCP

Komisja Bioetyczna przy UMK w Toruniu, Collegium Medicum w Bydgoszczy

(której skład podano w załączeniu) na posiedzeniu w dniu **22.11.2022 r.** przeanalizowała prośbę o:

- dołączenie do zespołu badawczego: lek. med. Jędrzeja Borowczaka;
- uaktualnienie afiliacji osób: dr hab. n. med. Łukasz Szylberg, prof. UMK - specjalista patomorfolog, profesor Uniwersytetu, dr hab. n. med. Magdalena Bodnar, prof. UMK – w trakcie specjalizacji z laboratoryjnej genetyki medycznej.

którą złożyła:

dr hab. n. med. Magdalena Bodnar, prof. UMK
Katedra Położnictwa, Chorób Kobiecych i Ginekologii Onkologicznej
Szpital Uniwersytecki nr 2 im. dr. J. Biziela w Bydgoszczy

w sprawie badania:

„Przygotowanie banku referencyjnego materiału tkankowego, DNA, RNA oraz białek, izolowanych od pacjentów operowanych z powodu nowotworów układu moczowo-płciowego -profilowanie pacjentów z nowotworami układu moczowo-płciowego".

Po zapoznaniu się ze złożonym dokumentem i w wyniku przeprowadzonej dyskusji oraz głosowania jawnego Komisja przyjęła do wiadomości podane informacje i wyraża zgodę na powyższe pod warunkami określonymi w uchwale Komisji podjętej w dniu 17.12.2019 r. oraz w ewentualnych aneksach do tejże uchwały.

Zgoda na kontynuowanie przedmiotowego badania obowiązuje do końca 2025 r.

Prof. dr hab. med. Karol Śliwka

Przewodniczący Komisji Bioetycznej

Otrzymuje:

Otrzymuje:
lek. med. Piotr Słupski
Klinika Urologii
Szpital Uniwersytecki nr 2 w Bydgoszczy

dr hab. n. med. Magdalena Bodnar, prof. UMK
Katedra Położnictwa, Chorób Kobiecych i Ginekologii Onkologicznej
Szpital Uniwersytecki nr 2 w Bydgoszczy

Lista obecności

na posiedzeniu Komisji Bioetycznej

w dniu 22.11.2022 r.

Lp.	Imię i nazwisko	Funkcja/ Specjalizacja	Podpis
1.	Prof. dr hab. med. Karol Śliwka	*medycyna sądowa*	
2.	Mgr prawa Joanna Połetek-Żygas	*prawniczka*	
3.	Prof. dr hab. med. Mieczysława Czerwionka-Szaflarska	*pediatra, alergologia i gastroenterologia dziecięca*	
4.	Prof. dr hab. med. Marek Grabiec	*położnictwo, ginekologia onkologiczna*	
5.	Prof. dr hab. n med. Maria Kłopocka	*choroby wewnętrzne, gastroenterologia*	
6.	Prof. dr hab. med. Zbigniew Włodarczyk	*chirurgia ogólna, transplantologia kliniczna*	
7.	Dr hab. n. med. Maciej Słupski, prof. UMK	*chirurgia ogólna, transplantologia kliniczna*	
8.	Dr hab. n. med. Katarzyna Sierakowska, prof. UMK	*anestezjologia i intensywna terapia*	
9.	Ks. dr hab. Wojciech Szukalski, prof. UAM	*duchowny*	
10.	Dr n. med. Radosława Staszak-Kowalska	*pediatria, choroby płuc*	
11.	Mgr prawa Patrycja Brzezicka	*prawniczka*	
12.	Mgr farm. Aleksandra Adamczyk	*farmaceutka*	
13.	Mgr Lidia Iwińska-Tarczykowska	*pielęgniarka*	