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IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

Nowe histomorfologiczne i immunohistochemiczne czynniki prognostyczne w czerniaku gałki ocznej

Tomasz Berus

ROZPRAWA DOKTORSKA

PROMOTOR
dr hab. n. med. Piotr Donizy
Katedra oraz Zakład Patomorfologii i Cytologii Onkologicznej
Wydział Lekarsko-Stomatologiczny
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

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II. Tomasz Berus, Anna Markiewicz, Przemysław Biecek, Jolanta Orłowska-Heitzman, Agnieszka Hałoń, Bożena Romanowska-Dixon, Piotr Donizy.: Clinical Significance of Nucleoli Cytomorphology Assessment in Patients With Uveal Melanoma

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III. Tomasz Berus, Anna Markiewicz, Katarzyna Kobylińska, Przemysław Biecek, Jolanta Orłowska-Heitzman, Bożena Romanowska-Dixon, Piotr Donizy.: Downregulation of Polo-like kinase-1 (PLK-1) expression is associated with poor clinical outcome in uveal melanoma patients

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2. Wstęp

Czerniak błony naczyniowej (uveal melanoma; UM) jest najczęstszym pierwotnym nowotworem oka osób dorosłych[1]. Zachorowalność w populacji europejskiej wynosi od mniej niż 2 do ponad 8 na milion rocznie i jest zbliżona do występującej w USA średniej 4,3 na milion[2,3]. Wiek w momencie rozpoznania UM wynosi przeciętnie 58-61,4 lat (±15 lat; zakres 3-100 lat)[4]. Nieznacznie wyższy współczynnik zachorowalności stwierdza się u mężczyzn[4]. W zdecydowanej większości przypadków UM występuje u pacjentów rasy białej (95-98%)[4].

Pomimo postępów w zakresie leczenia pierwotnego nowotworu, śmiertelność związana z UM wciąż pozostaje wysoka[1,5]. Choć UM stanowi zaledwie 5% wszystkich przypadków czerniaka, odpowiada za 13% zgonów związanych z tym nowotworem[6,7]. Wachlarz stosowanych opcji terapeutycznych – brachyterapia, terapia wiązką protonów, termoterapia przezźreniczna, koagulacja laserowa, endoresekcja, resekcja lokalna czy enukleacja – nie zmieniają faktu, że w blisko połowie przypadków UM dochodzi do powstania przerzutów, co może być spowodowane trudnym do wykrycia rozsiewem, który występuje już w momencie rozpoznania ogniska pierwotnego[8]. Szerzenie nowotworu następuje drogą krwi, a najczęstszą lokalizacją przerzutów jest wątroba (89%), rzadziej płuca (29%) oraz kości (17%)[9].

Powszechnie przyjętą praktyką, mającą na celu wykrycie ewentualnych przerzutów u pacjentów leczonych z powodu UM, jest wykonywanie co 6-12 miesięcy badań obrazowych i laboratoryjnych wątroby. Wystąpienie przerzutów, wobec niewielkiej skuteczności terapii adiuwantowej, związane jest ze złym rokowaniem. O ile 5-letnie przeżycia względne w UM wahają się od 77% do 84%[10], o tyle średni czas przeżycia od momentu rozpoznania przerzutu UM wynosi 3-4 miesiące, przeżycia 1-roczne dotyczą 10-15% pacjentów, a powyżej 5 lat są stwierdzane zaledwie u 1% chorych[9,11]. Chemioterapia, konwencjonalna oraz w postaci izolowanej perfuzji wątroby, leczenie chirurgiczne, a także zastosowanie inhibitorów punktów kontrolnych, cechują się bardzo ograniczoną efektywnością w UM w stadium rozsiewu. Z zastosowaniem tych metod udaje się uzyskać średni czas przeżycia wynoszący przeciętnie zaledwie 1,07 roku (zakres: 0,59-2,50 lat)[12].

Parametrami klinicznymi i histopatologicznymi mającymi wpływ na rokowanie pacjentów z UM są: zaawansowany wiek, lokalizacja w ciele rzęskowym, największa szerokość podstawy guza i jego wysokość, obecność zamkniętych pętli naczyniowych, typ nabłonkowaty,

podwyższony wskaźnik mitotyczny, intensywny naciek limfocytarny, szerzenie się guza poza gałkę oczną, a także: wzrost pigmentacji komórek nowotworowych, obecność płynu podsiatkówkowego oraz obecność krwotoku do wnętrza gałki ocznej[13–15]. Analiza tych parametrów pozwala na określenie przybliżonego rokowania co do wystąpienia przerzutów i przeżycia długoterminowego. Poszukiwanie nowych czynników prognostycznych i predykcyjnych, szczególnie tych związanych ze szlakami sygnałowymi funkcjonującymi w komórce nowotworowej, daje szansę na zwiększenie precyzji rokowania i określenie kolejnych punktów uchwytu, stanowiących potencjalnie cele terapeutyczne.

Jąderka są miejscem przemian rybosomalnego RNA od etapu transkrypcji do powstania cząstek prerybosomowych[16]. Dlatego też zwane są fabrykami rybosomów, odpowiedzialnymi za syntezę białek. Biogeneza rybosomów jest jednym z najważniejszych i najbardziej złożonych procesów metabolicznych, który pochłania do 80% zasobów energetycznych komórki[17,18]. Jej hyperaktywacja, do której może dojść m. in. w wyniku aktywacji onkogenów lub utraty genów supresorowych, ma kluczowe znaczenie dla inicjacji i progresji nowotworów. Dotyczy to zarówno mutacji wpływających bezpośrednio na budowę rybosomów, jak i szerokiego spektrum deregulacji procesu ich powstawania. Obniżanie aktywności produkcji rybosomów skutkuje zahamowaniem cyklu komórkowego i indukcją apoptozy, na drodze zależnej jak i niezależnej od białka p53, co może być wykorzystane jako potencjalna strategia terapeutyczna[19–21].

Nasilenie aktywności procesów metabolicznych toczących się w jąderkach w komórkach intensywnie proliferujących wpływa na ich cechy cytomorfologiczne (m.in.: wielkość, liczba w komórce)[22]. Zmiany w tym zakresie, które mogą być ocenione w rutynowym badaniu histopatologicznym, mogą mieć znaczenie prognostyczne w chorobach nowotworowych. Zwiększona liczba jąderek oraz występowanie makrojąderek (*prominent nucleoli*) obserwowane są w licznych nowotworach, w niektórych z nich stanowiąc negatywny czynnik rokowniczy[23,24]. Ocena wartości prognostycznej wybranych parametrów jąderek komórek nowotworowych UM w standardowym barwieniu hematoksyliną i eozyną może pozwolić na uzyskanie potencjalnie istotnych czynników rokowniczych w tym typie nowotworu.

Białko PLK1 (*polo-like kinase 1*) jest kinazą serynowo-treoninową o specyficznej budowie. Składająca się z 252 aminokwasów N-końcowa wysoce konserwatywna domena

kinazowa, uzupełniona jest w części C-końcowej przez tzw. polo-box domain (PBD) - dwie struktury polo-box połączone krótkim łącznikiem. W interakcji z peptydami, fosforylowanymi wcześniej przez inne kinazy biorące udział w cyklu komórkowym, dochodzi do zmiany konformacji PBD. Działając niczym klips powoduje ona dokowanie PLK1 w miejscu jej działania. Dzieje się to precyzyjnie w odpowiednich lokalizacjach w komórce i w odpowiedniej fazie podziału komórkowego[25,26], co stawia PLK1 w pozycji białka koordynującego przebieg mitozy i cytokinezy[27].

PLK1 bierze udział w dojrzewaniu centrosomu, tworzeniu dwubiegunowego wrzeciona kariokinetycznego, aktywacji kompleksu promującego anafazę APC/C, akumulacji protein wchodzących w skład punktu kontrolnego wrzeciona podziałowego SAC, rozdziale chromatyd siostrzanych, a także w cytokinezie[28–30]. Poza tym PLK1 odgrywa rolę w licznych innych procesach, w tym m. in. w przemianach mikrotubul, replikacji DNA, regulacji aktywności białka p53, czy wychodzeniu z punktu kontrolnego uszkodzeń DNA w fazie G2[31].

Wzrost ekspresji PLK1 jest stwierdzany w licznych nowotworach, a poziom ekspresji koreluje z nasileniem proliferacji i złą prognozą[32–38]. Ma to miejsce także w czerniaku skóry[39,40]. W ostatnim czasie pojawiły się doniesienia o działaniu PLK1 jako supresora rozwoju niektórych nowotworów[41], jednak nadal jest ona uznawana przede wszystkim za czynnik proonkogenny, działający między innymi poprzez wpływ na punkty kontrolne cyklu komórkowego i indukujący niestabilność genetyczną. Jako taki stanowi cel terapii przeciwnowotworowych[42]. Postuluje się próby ich zastosowania również w leczeniu UM[43]. Ocena ekspresji PLK1 oraz jej korelacji ze szczegółowymi parametrami kliniczno-patologicznymi i przeżyciem długoterminowym stanowić może punkt wyjścia do badań nad rolą tej kinazy w czerniaku błony naczyniowej oka.

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3. Założenia i cele pracy

Czerniak błony naczyniowej oka jest rzadkim nowotworem, a liczba doniesień o parametrach mających znaczenie dla rozwoju UM jest ograniczona. Stąd też pilna konieczność kliniczna poszukiwania nowych czynników, które z większą precyzją będą określały rokowanie u pacjentów z tym podtypem czerniaka.

Celami niniejszej rozprawy doktorskiej, składającej się z trzech powiązanych tematycznie artykułów pełnotekstowych opublikowanych w czasopismach z Listy Filadelfijskiej, są:

1. **Usystematyzowanie dostępnych danych na temat czynników rokowniczych w UM.**
   Zebranie informacji na temat parametrów klinicznych, histomorfologicznych, jak i cytogenetycznych, pozwoli na poznanie bieżącego stanu wiedzy w tym zakresie w możliwie szerokim spektrum.
2. **Ocena wartości rokowniczej cech morfologicznych jąderek komórek UM.**
   Parametry związane z jąderkami jąder komórkowych były jednymi z historycznie pierwszych branych pod uwagę w przewidywaniu przebiegu chorób nowotworowych. W wielu z nich okazywały się mieć znaczenie prognostyczne. Badanie będzie dotyczyło oceny znaczenia prognostycznego wybranych parametrów cytomorfologii jąderek (obecność, wielkość, liczba w pojedynczej komórce) u pacjentów z czerniakiem gałki ocznej.
   Uzyskane dane zostaną skorelowane z parametrami klinicznymi i histomorfologicznymi, a także z informacjami dotyczącymi przeżycia.
3. **Ocena wartości rokowniczej ekspresji kinazy PLK1 w komórkach UM.**
   Uzasadnienie badania stanowi pełnienie przez kinazę serynowo-treoninową PLK1 centralnej roli w koordynacji mitozy i cytokinezy, a co za tym idzie w proliferacji komórek. Dodatkowym argumentem są doniesienia o potwierdzonym związku PLK1 z rokowaniem w innych nowotworach, a także trwające badania nad stosowaniem inhibitorów PLK1, jako potencjalnych środków terapeutycznych w tych chorobach Celem badania będzie immunohistochemiczna ocena ekspresji białka PLK1 w komórkach nowotworowych UM oraz analiza korelacji parametrów ekspresji PLK1 ze szczegółowymi danymi kliniczno-histopatologicznymi oraz przeżyciem pacjentów.

4. Streszczenie

Badania przeprowadzono na grupie 164 pacjentów leczonych w latach 2002-2011 z zastosowaniem pierwotnej enukleacji w Klinice Okulistyki i Onkologii Okulistycznej Collegium Medicum Uniwersytetu Jagiellońskiego w Krakowie. Włączenie pacjentów do badania następowało na podstawie dostępnej dokumentacji i materiału tkankowego. Szczegółowe dane kliniczne uzyskano z archiwum Oddziału Klinicznego Okulistyki i Onkologii Okulistycznej oraz Poradni Onkologii Okulistycznej Szpitala Uniwersyteckiego w Krakowie. Zebrane informacje obejmowały: wiek i płeć, oko zajęte procesem chorobowym, wysokość i największą średnicę podstawy guza, stratyfikację guza zgodnie z AJCC (pT oraz stage), lokalizację guza w stosunku do równika gałki ocznej, zajęcie ciała rzęskowego, pigmentację oraz kształt guza, współistnienie jaskry i/lub odwarstwienia siatkówki, podtyp histologiczny, naciek twardówki i/lub nerwu wzrokowego, obecność martwicy w obrębie guza. Dodatkowo uwzględniono szczegółowe parametry histopatologiczne, takie jak: wskaźnik mitotyczny, obecność nacieku limfocytarnego (TILs, *tumor-infiltrating lymphocytes*), pseudoinkluzji jądrowych (NPIs, *nuclear pseudoinclusions*), bruzd wewnątrzjądrowych, wielojądrowych komórek olbrzymich oraz wylewów krwi, a także ilość barwnika w komórkach nowotworowych. Dla opisania wysokości i największej średnicy podstawy guza przyjęto wytyczne *American Joint Commitee on Cancer* (AJCC), a także dokonano podziału zgodnego z zastosowanym w badaniu *Collaborative Ocular Melanoma Study* (COMS).

Celem oceny cytomorfologii jąderek zastosowano trzy parametry. Obecność jąderek oceniono w relacji do całkowitej liczby jąder w preparacie UM stosując następujące kryteria grupowania: 0 – brak jąderek w komórkach czerniaka, 1 – niewielka liczba komórek z obecnością jąderek (≤ 20% komórek czerniaka w analizowanym pojedynczym preparacie guza pierwotnego barwionego metodą H&E), 2 – wysoki odsetek komórek z obecnością jąderek (> 20% komórek czerniaka w analizowanym pojedynczym preparacie guza pierwotnego barwionego metodą H&E). Rozmiar jąderka odnosił się do wielkości obserwowanych jąderek (0: brak jąderek w jądrach komórek czerniaka, 1: obecne mikrojąderka (*inconspicious nucleoli*), 2: obecne makrojąderka (*prominent nucleoli*). Liczba jąderek w jądrach komórek czerniaka była oceniona wg poniższego algorytmu: 0 – brak jąderek

w jądrach komórek czerniaka, 1 – pojedyncze mikro- lub makrojąderko w jądrze, 2 – dwa lub więcej jąderek w jednym jądrze komórki czerniaka.

W wyniku przeprowadzonej analizy statystycznej stwierdzono, że zarówno większa liczba jąderek, jak i obecność makrojąderek korelowały z większą średnicą podstawy guza. Zależność występowała zarówno dla podziału wg AJCC (odpowiednio $p = 0,042$ i $p = 0,019$), jak i COMS (odpowiednio $p = 0,0089$ i $p < 0,001$). Wysokość guza nie była związana z liczbą jąderek oraz ich wielkością. Większa liczba jąderek w jądrach komórek nowotworowych związana była z wyższym stopniem zaawansowania guza pierwotnego (pT), określonego zgodnie z protokołem *College of American Pathologists* ($p = 0,013$). Analiza wykazała również trend pomiędzy wielkością jąderek i pT, jednak nie był on istotny statystycznie ($p = 0,053$). Występowanie makrojąderek, jak i większa liczebność jąderek korelują z wyższym klinicznym stopniem zaawansowania (*stage*) (odpowiednio $p = 0,041$ i $p = 0,013$). Bardziej obwodowa lokalizacja guza związana jest z większą liczbą, jak i rozmiarem jąderek (odpowiednio $p = 0,029$ i $p = 0,0024$). Dodatnia zależność łączy także występowanie makrojąderek oraz zajęcie przez proces nowotworowy ciała rzęskowego ($p = 0,0069$). Ponadto obecność większych jąderek koreluje z bardziej nasiloną pigmentacją guza w ocenie klinicznej ($p < 0,001$). W zakresie korelacji z parametrami histopatologicznymi stwierdzono, że obecność makrojąderek i mnogich jąderek związana jest istotnie z podtypem nabłonkowatokomórkowym guza pierwotnego (odpowiednio $p < 0,001$ i $p = 0,013$), wysokim wskaźnikiem mitotycznym (odpowiednio $p = 0,0039$ i $p = 0,0041$), pleomorfizmem ($p < 0,001$ w obu przypadkach), występowaniem wielojądrowych komórek olbrzymich ($p < 0,001$ w obu przypadkach) oraz pseudoinkluzji wewnątrzjądrowych (NPIs) ($p < 0,001$ w obu przypadkach), a także brakiem bruzd wewnątrzjądrowych (odpowiednio $p < 0,001$ i $p = 0,0019$). Większa zawartość barwnika w komórkach nowotworowych również związana była w sposób istotny statystycznie z obecnością większych ($p = 0,021$) i mnogich jąderek ($p = 0,045$). W analizie Kaplana-Meiera zarówno występowanie makrojąderek, jak i mnogich jąderek związane było z istotnie krótszym przeżyciem wolnym od choroby oraz przeżyciem całkowitym.

Immunohistochemiczną (IHC) ocenę ekspresji PLK1 wykonano w 158 preparatach guzów pierwotnych. Skrawki parafinowe grubości 4 μm zostały wybarwione z użyciem króliczego przeciwciała monoklonalnego anty-PLK1 (208G4; # 4513; rozcieńczenie 1:100; Cell Signaling Technology, USA). Zastosowano barwienia kontrolne, pozytywne i negatywne.

Do oceny stopnia ekspresji PLK1 w komórkach UM zastosowano półilościową skalę wg Remmele i Stegner, przyznając punkty w zależności od intensywności reakcji (0-3 pkt) oraz odsetka komórek z pozytywną reakcją cytoplazmatyczną (0-4 pkt), a następnie mnożąc wynik tych dwóch parametrów, aby otrzymać ostateczny rezultat określany jako współczynnik IRS (*immunoreactive score*) o zakresie od 0 do 12 punktów.

Podwyższoną ekspresję PLK1 w komórkach nowotworowych, określaną jako IRS > 2, obserwowano w 70% (111/158) przypadków, podczas gdy w pozostałych 30% (47/158) przypadków stwierdzono brak lub niską ekspresję. PLK1 było nieobecne w 3,8% (6/158) guzów. Średni IRS dla ekspresji PLK1 w komórkach guza wyniósł 4 (mediana: 4). W analizie zależności z parametrami klinicznymi stwierdzono, że obniżona ekspresja PLK1 koreluje z większą średnicą podstawy guza pierwotnego ($p = 0,044$), jest związana z wyższym stopniem zaawansowania klinicznego pT ($p = 0,040$) i stage ($p = 0,037$) oraz z częstszym występowaniem odwarstwienia siatkówki towarzyszącemu UM ($p = 0,0076$). Zwiększona ekspresja PLK1 koreluje z wyższym wiekiem pacjenta ($p = 0,0019$). W relacji z parametrami histologicznymi wzrost ekspresji PLK1 był istotnie związany z obniżoną zawartością barwnika w komórkach nowotworowych ($p = 0,0019$) oraz obecnością bruzd wewnątrzjądrowych ($p = 0,017$). Z kolei obniżona ekspresja PLK1 wykazuje istotną korelację z obecnością NPIs w obrębie jąder komórek czerniaka ($p = 0,0071$). W analizie Kaplana-Meiera niska ekspresja PLK1 była związana z istotnie krótszym przeżyciem całkowitym ($p = 0,0058$). W zakresie przeżycia wolnego od choroby zaobserwowano podobną tendencję, choć nie został osiągnięty poziom istotności statystycznej ($p = 0,088$).

5. Summary

The research was carried out in 164 patients with uveal melanoma who underwent primary enucleation treated in 2002-2011 at the Department of Ophthalmology and Ocular Oncology, Jagiellonian University Medical College in Kraków. The inclusion criteria were positive health record verification and a histological diagnosis of ocular melanoma. The detailed clinical information was provided by the archives of the Department of Ophthalmology and Ocular Oncology and Ocular Oncology Outpatient Clinic, University Hospital in Kraków. The ascertained data included: patient age and sex, eye involved, tumour maximal basal diameter and thickness, tumour stratification as per the AJCC criteria (clinical and pathologic staging, pT), tumour location relative to the equator, ciliary body involvement, tumour pigmentation and shape, concomitant glaucoma and/or retinal detachment, histology subtype, scleral and/or optic nerve infiltration, as well as the histological evidence of necrosis within the tumour. Additional detailed histological parameters have also been identified and ascertained, such as: mitotic index, the presence of tumor-infiltrating lymphocytes (TILs), nuclear pseudoinclusions (NPIs), nuclear grooves, multinucleated giant cells (MGCs) and haemorrhages, as well as melanoma cell pigmentation level. The largest basal diameter and thickness of the tumour were described in line with the American Joint Commitee on Cancer (AJCC) guidelines and staged according to the Collaborative Ocular Melanoma Study (COMS).

Three parameters were applied to assessing the nucleolar morphology: the presence of nucleoli, their size and their number. In terms of nucleolus presence, the number of nucleoli was compared to the total number of nuclei in the specimen, and graded as: 0 – no nucleoli were detected in the evaluated tumour cells, 1 – a low percentage of tumour cells containing the nucleoli (≤ 20% of all uveal melanoma cell in a single H&E-stained primary tumour slide), 2 – a high percentage of tumour cells containing the nucleoli (> 20% of all uveal melanoma cells in a single H&E-stained primary tumour slide). The nucleolus size was graded as: 0: nucleoli absent, 1: presence of inconspicious nucleoli, 2: presence of prominent nucleoli. The number of nucleoli in uveal melanoma cells was assigned to one of the three categories: 0 – nucleoli absent, 1 – presence of a single inconspicious or prominent nucleolus in a nucleus, 2 – two or more nucleoli present in a single nucleus.

There was a positive correlation between the basal diameter of the tumour and both nucleolus number and the presence of prominent nucleoli, which remained significant for both AJCC ($p = 0.042$ and $p = 0.019$, respectively) and COMS ($p = 0.0089$ and $p < 0.001$, respectively) criteria. There was no correlation between tumor height and nucleolus number or size. The increased number of nucleoli in tumour cells positively correlated with primary tumor (pT) staging determined as per the College of American Pathologists (CAP) protocol ($p = 0.013$). There was also a trend towards an association between nucleolus size and pT, which was on the verge of significance ($p = 0.053$). There was a positive correlation between tumour staging and both the number of nucleoli as well as the prominent nucleoli ($p = 0.013$ and $p = 0.041$). There was a positive correlation between peripheral tumour location and both nucleolus number and size ($p = 0.029$ and $p = 0.0024$). Similarly, there was a positive correlation between the presence of prominent nucleoli and ciliary body involvement ($p = 0.0069$). Furthermore, the presence of macronucleoli correlated positively with clinically assessed darker tumour pigmentation ($p < 0.001$). The analysis of associations with histology parameters demonstrated that the presence of prominent nucleoli and multiple nucleoli significantly correlated with the epithelioid cell melanoma subtype ($p < 0.001$ and $p = 0.013$, respectively), high mitotic index ($p = 0.0039$ and $p = 0.0041$, respectively), pleomorphism ($p < 0.001$ for both comparisons), the presence of multinucleated giant cells (MGCs) ($p < 0.001$ for both comparisons) and nuclear pseudoinclusions (NPIs) ($p < 0.001$ for both comparisons), as well as the absence of the nuclear grooves ($p < 0.001$ and $p = 0.0019$, respectively). Furthermore, there was a positive correlation between the pigment content in tumour cells and the presence of larger ($p=0.021$) or multiple ($p=0.045$) nucleoli. In a Kaplan-Meier analysis, the presence of both prominent nucleoli and multiple nucleoli was associated with a significantly shorter disease-free survival and overall survival.

Immunohistochemical (IHC) staining was performed in 158 primary tumour specimens to detect the PLK-1 expression. The 4 μm thick paraffin-embedded sections were stained using the anti-PLK-1 rabbit monoclonal antibody (208G4; # 4513; diluted at 1:100; Cell Signaling Technology, USA). Control, positive and negative staining was carried out. The semi-quantitative immunoreactive score of Remmele and Stegner (IRS) was used to assess the PLK-1 expression in uveal melanoma cells, which assigns scores of 0-3 to the intensity of staining

and scores of 0-4 to the percentage of positive cells and then multiplies the two scores to yield the immunoreactive score of 0-12.

The enhanced PLK-1 expression in tumour cells, defined as IRS>2, was demonstrated in 70% (111/158) of cases. The remaining 30% (47/158) of cases were classed as either weak/mild or no expression. The PLK-1 was not detected in 3.8% (6/158) of analysed tumours. The mean IRS for PLK-1 expression in uveal melanoma cells was 4 (median: 4). In terms of clinical parameters, there was a negative correlation between PLK-1 expression and basal tumour diameter ($p = 0.044$), pT stage ($p = 0.040$), clinical stage ($p = 0.037$) as well as concomitant retinal detachment ($p = 0.0076$). The PLK-1 expression correlated positively with patient's age ($p = 0.0019$). In terms of histologic features, there was a positive correlation between PLK-1 and both reduced melanoma cell pigmentation ($p = 0.0019$) and the presence of nuclear grooves ($p = 0.017$). On the other hand, reduced PLK-1 expression significantly correlated with the presence of NPIs in the nuclei of uveal melanoma cells ($p = 0.0071$). In a Kaplan-Meier analysis, the weak/mild PLK-1 expression was associated with a significantly shorter overall survival. Furthermore, there was a tendency, albeit non-significant ($p = 0.088$), towards a shorter disease-free survival in cases with downregulated PLK-1 expression.

6. Publikacje

Tomasz Berus, Agnieszka Hałoń, Anna Markiewicz, Jolanta Orłowska-Heitzman, Bożena Romanowska-Dixon, Piotr Donizy.: Clinical, Histopathological and Cytogenetic Prognosticators in Uveal Melanoma - A Comprehensive Review
Anticancer Res. 2017 Vol.37 no.12; s.6541-6549

Tomasz Berus, Anna Markiewicz, Przemysław Biecek, Jolanta Orłowska-Heitzman, Agnieszka Hałoń, Bożena Romanowska-Dixon, Piotr Donizy.: Clinical Significance of Nucleoli Cytomorphology Assessment in Patients With Uveal Melanoma
Anticancer Res. 2020 Vol.40 no.6; s.3505-3512

Tomasz Berus, Anna Markiewicz, Katarzyna Kobylińska, Przemysław Biecek, Jolanta Orłowska-Heitzman, Bożena Romanowska-Dixon, Piotr Donizy.: Downregulation of Polo-like kinase-1 (PLK-1) expression is associated with poor clinical outcome in uveal melanoma patients
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Review

Clinical, Histopathological and Cytogenetic Prognosticators in Uveal Melanoma – A Comprehensive Review

TOMASZ BERUS[1], AGNIESZKA HALON[2], ANNA MARKIEWICZ[3], JOLANTA ORLOWSKA-HEITZMAN[4], BOZENA ROMANOWSKA-DIXON[3] and PIOTR DONIZY[2]

[1]The Clinical Ward of Ophthalmology, Fourth Military Clinical Hospital with Polyclinic, Wroclaw, Poland; [2]Department of Pathomorphology and Oncological Cytology, Wroclaw Medical University, Wroclaw, Poland; Departments of [3]Ophthalmology and Ocular Oncology, and [4]Clinical and Experimental Pathomorphology, Jagiellonian University, Medical College, Krakow, Poland

Abstract. *Uveal melanoma is the most prevalent primary intraocular cancer in adults. Although it accounts for only 5% of all melanomas, it is responsible for 13% of deaths due to this type of cancer. A wide variety of therapeutic options of primary tumor is available and progress in its management is noticeable. The fact still remains, however, that almost half of patients develop metastases which may be due to practically undetectable cancer spread present as early as at diagnosis of the primary focus. Metastatic disease is uniformly fatal despite systemic therapy. Prediction of metastasis is crucial for prognosis. It also allows targeting of emerging new therapeutic methods to the appropriate group of patients. The Authors reviewed literature concerning epidemiology and etiopathogenesis of uveal melanoma, and its clinical, histopathological and cytogenetic prognosticators.*

Uveal melanoma (UM) is a potentially lethal cancer but factors determining unfavorable course of this disease are still unknown. Severe form of UM requires intense therapy. On the other hand, unreasonable treatment affects negatively the quality of vision and whole life of the patients. The decision on the scope of treatment needs to precede uniformly fatal metastatic spread. Consideration of clinical, histopathological and cytogenetical prognosticators help to define group of patients with different prognosis in UM.

Correspondence to: Tomasz Berus, The Clinical Ward of Ophthalmology, 4th Military Clinical Hospital with Polyclinic, ul. Weigla 5, 50-981 Wroclaw, Poland. Tel: +48 261660449, Fax: +48 261660350. e-mail: tberus@4wsk.pl

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Epidemiology and Etiopathogenesis

Uveal melanoma (UM) is the most prevalent type of primary intraocular neoplasm in adults (1). In the European population, the incidence of UM ranges from under 2 to over 8 per million annually and is similar to the mean value of 4.3 per million for the USA. No significant changes of UM prevalence have been observed in either of the populations in long-term clinical observations (1-4). The incidence rate increases with age and tends to plateau among adults 75 years and older. The mean age at UM diagnosis is 58-61.4 years (±15 years; range 3-100 years) and has increased gradually over the past 40 years (5, 6). Studies report slightly a higher incidence rate among men (2, 3, 5).

Most cases of UM occur in the White population (95-98%) (3, 5-7). In the European population, a latitudinal gradient of incidence was reported: in the south of Europe, the rate was lowest and increased towards the north to reach the highest values in Scotland and Scandinavian countries (2). In the USA, no geographical variations were noted except for in Hawaii where the incidence rate was considerably lower (one per million) (3). The authors of both reports pointed to the protective role of increased skin pigmentation and darker eye color as the cause of the observed correlations.

Despite progress in the management of primary tumor, the mortality rate for UM has remained high (1, 8). Although UM accounts for only 5% of all melanomas, it is responsible for 13% of deaths due to this type of tumor (4, 9). A wide variety of therapeutic options, namely brachytherapy, proton beam irradiation, transpupillary thermotherapy, photocoagulation, local resection, endoresection, and enucleation, is available. The fact still remains, however, that almost half of patients with UM develop metastases, which may be due to practically undetectable neoplasm spread present as early as

at diagnosis of the primary focus (10). UM spreads through the blood, and the liver is the preferred metastatic site (89%), followed by the lungs (29%) and bones (17%) (11). Uveal melanoma-related mortality reaches 31% by 5 years from the diagnosis of UM, and 45%, 49% and 52% by 15, 25 and 35 years, respectively (12). Therefore, it seems that the oncological follow-up should span at least 15 years after completion of primary tumor treatment.

It is common practice that all patients with UM are screened for liver metastasis every 6 or 12 months using imaging techniques or liver function tests, even though adjuvant therapy fails to yield satisfactory therapeutic effect and metastasis can hardly ever be managed by surgery (13-16), liver chemoembolization (17-19) or immunotherapy (20).

While the 5-year relative survival among patients with UM ranges from 77% to 84% (21), the mean survival time following detection of UM metastasis is 3-4 months, with subsequent 1-year survival of 10-15%, and only 1% of patients live longer than 5 years after diagnosis (11, 22). In patients who developed primary metastasis to sites other than the liver, the mean survival time is normally longer and is 19-28 months with statistical 1-year survival of 76% (23-25).

Etiopathogenesis of UM remains unclear. Guanine nucleotide-binding protein subunit alpha-Q (GNAQ) and guanine nucleotide-binding protein subunit alpha-11 (GNA11) gene mutations have been shown to be the first element in the chain of changes that lead to the development of most cases of UM (26, 27), and to be related with the activation of mitogen-activated protein kinase (MAPK) pathway and other intracellular signaling pathways (28, 29). It was demonstrated that the co-existence of *GNAQ* mutation and breast cancer 1-associated protein 1 (*BAP1*) gene mutation is associated with increased metastatic potential of UM. Families with *BAP1* germline mutations are more susceptible to a number of cancer types including choroidal melanoma (30-32), however, *BAP1* mutations are usually somatic, and the prevalence of hereditary UM is lower than 1% (33). The role of environmental factors in UM pathogenesis is still elusive, although sunlight exposure has been implicated in development of *GNAQ/GNA11* mutation (34-36).

Clinical Prognosticators

The clinical and histopathological features that are predictive of poor prognosis of UM are: older age, ciliary body location, large basal diameter and thickness of tumor, the presence of closed connective tissue loops, epithelioid cell type, high mitotic rate, extraocular tumor extension, brown color of the tumor, presence of subretinal fluid and intraocular hemorrhage (6, 37, 38).

i. Patient age at diagnosis. The work by Shields *et al.*, which investigated 8,033 cases, is the largest study available to date discussing the prognostic significance of age in UM (39). It was shown that 1% of UM is diagnosed in patients below 20 years of age, 53% those aged 21-60 years and 45% in patients over 60 years old. Young patients are relatively often diagnosed with tumors of the iris (21%), while in all age groups, UM is most commonly located in the posterior part of the uvea (71, 91 and 90%, respectively) (39).

Mean basal tumor diameter and thickness, as well as the frequency of extrascleral extension, were shown to increase with age, similarly to the rate of occurrence of metastases and tumor-related mortality (39).

Metastasis at 3, 5, 10 and 20 years was detected in patients under 20 years old in 1.7%, 8.8%, 8.8% and 20.2% of cases, respectively, in the group aged 21-60 years in 6.2%, 12.2%, 23.0% and 34.2% of cases, and in those over 60 years old in 11.1%, 18.7%, 27.7% and 38.8% of cases (39).

The corresponding 3-, 5-, 10- and 20-year tumor-related mortality rates were 0%, 2.2%, 5.1% and 17.0%, 3.2%, 6.2%, 11.0% and 16.6%, and 6.5%, 11.0%, 15.9% and 20.1%, respectively (39).

ii. Tumor location. Although UM may be potentially diagnosed on clinical examination with almost 100% accuracy (34), in many patients, the tumor is missed or misdiagnosed (40). A lack of subjective symptoms in nearly one-third of patients, comorbid eye disorders, or diseases which hinder diagnosis, as well as incorrect technique (*e.g.* examination without mydriasis) may be the root of some of the problems. Tumor location may also make diagnosis more difficult: 3.5% of UM occurs in the iris, 6.1% in the ciliary body, and 90.3% in the posterior part of the uvea (6).

UM located in the ciliary body initially does not result in visual impairment and often remains undiagnosed until it is large enough to distort the iris or emerge from beneath its edge and induce other structural and functional changes within the eyeball. Sometimes it is diagnosed only after extraocular spread is observed. Even though ciliary body location of UM is an unfavorable prognostic factor irrespective of its size or cellular type (41), it was demonstrated that UM involving the ciliary body is more likely to comprise epithelioid cells and have a greater diameter than that confined to the posterior part of the uvea (42). It is assumed that an anatomical location that impedes early diagnosis, as well as high mobility of the ciliary body due to the contractions of the ciliary muscle, the numerous vessels and a likely occurrence of extravascular matrix patterns of poor prognosis in this area may be associated with higher metastatic potential of UM (42, 43). Delayed treatment often necessitates a more radical form of treatment *i.e.* enucleation of the eye (40).

UM that originates from the iris is associated with better prognosis, which may stem from the fact that such tumors are more easily identified and, as a result, treatment is applied

much sooner (41). Clinical features that affect the metastatic potential of UM of the iris are age at diagnosis, infiltration of the anterior chamber angle, extraocular extension, elevated intraocular pressure and prior surgeries (44).

Tumors of the posterior part of the uvea, which directly or indirectly (*e.g.* due to concomitant retinal detachment) affect the macula, may induce blurry and distorted vision. It seems that these symptoms should contribute to cancer being diagnosed earlier than in the case of ciliary location. However, in the study by Shields *et al.*, a relatively small difference was reported as regards patient age at diagnosis (6). For UM of the iris, ciliary body and posterior part of the uvea, it was reported as 50, 59 and 58 years, respectively.

At diagnosis, the mean thickness of tumor located in the iris was 2.7 mm, 6.6 mm in the ciliary body, 5.5 mm in the posterior part of the uvea, and the mean largest basal diameter was 6.5 mm, 11.7 mm, and 11.3 mm, respectively. At 3 years, metastases were observed in 0.5%, 12%, 8% of cases, respectively, at 5 years in 4.1%, 19%, 15%, and at 10 years in 6.9%, 33%, 25% (6).

5-Year mortality for UM of the ciliary body was reported to be 22-53% and for that of the posterior part of the uvea 14%, and 10-year mortality for iris UM was 5-6% (41).

iii. Tumor size. Meta-analysis of Diener-West *et al.* attempted to provide systematic results of eight studies on mortality rates following enucleation for UM (45). For small (<3 mm-thick and <10 mm in basal diameter), medium (3 to 8 mm-thick and <15 mm in basal diameter) and large (>8 mm-thick and >15 mm in basal diameter) tumors, 5-year overall mortality was 16%, 32% and 53%, respectively.

Shields *et al.* adopted tumor thickness as the criterion of tumor size, as they decided that the acquisition of this dimension by ultrasonography ensures higher precision than the measurement of basal tumor diameter (6). They concluded that small tumors (0 to 3.0 mm-thick) metastasize at 5, 10 and 20 years in 6%, 12% and 20% of cases respectively, medium (3.1 to 8.0 mm-thick) tumors in 14%, 26% and 37%, and large (>8.0 mm-thick) tumors in 35%, 49% and 67%. Every 1 mm increment in thickness of primary UM equated to a 5% higher probability of metastasis development and *e.g.* for a 4 to 5 mm-thick tumor, the probability was approximately 25%, while for one 7 to 8 mm-thick, it increased to 40% (6).

In another study, Shields *et al.* compared the prognosis for small tumors (<3 mm-thick) of diffuse (thickness/base ratio ≤20%) and nondiffuse (thickness/base ratio >20%) types (46); 17% of tumors were diffuse. UM-related metastasis was detected in 8% and 4% of cases, respectively, at 5 years, 16% and 10% at 10 years, and 19% and 16% at 15 years. The corresponding UM-related mortality rates were 6% and 2%, 11% and 4%, and 16% and 6%, respectively. This considerably worse prognosis for patients with diffuse tumors was even more prominent when tumors up to 2 mm-thick were analyzed.

Tumor basal diameter and its thickness have been reported to be strongly correlated with UM prognosis by numerous authors (38, 47, 48).

iv. Methods of treatment. The aim of the multicenter Collaborative Ocular Melanoma Study (COMS) was to solve the problem of selecting the optimum method of treatment for primary UM (34). The three-arm study evaluated the effectiveness of management of small, medium and large tumors (1.5 to 2.4 mm-thick and 5-16 mm in diameter, 2.5 to 10 mm-thick and ≤16 mm in diameter, >10 mm-thick and >16 mm in diameter, respectively).

In the case of large tumors (1,003 patients), the effectiveness of enucleation was compared to enucleation with preoperative radiation therapy. 5-Year survival was not statistically different between the groups and 5-year tumor-related mortality was 28% and 26%, respectively (34).

For medium tumors (1,317 patients), enucleation was compared with brachytherapy using iodine-125. The mortality rate in both groups was almost identical. It should be noted, however, that ciliary body location tumors and tumors located near the optic nerve disc which are inaccessible for brachytherapy were excluded from the COMS (34).

A group of 204 patients diagnosed with a small UM was entered into a registry and managed with watchful waiting. 5-Year and 8-year cancer-related death was 1% and 3.7%, respectively (34).

Even though COMS received criticism due to the study criteria applied, the data collected brought about a shift in treatment modalities for small and medium tumors towards vision-preserving therapies.

Similar survival times of patients with primary UM treated by different methods (34, 41) prove that improving diagnosis and management of metastasis is vital for prognosis.

Histopathological Prognostic Factors

i. Cell type. The first classification of malignant melanocytic eye tumors was proposed in 1931 by Callender (49), and then modified by McLean *et al.* (50).

The main criterion of division into histologic types of UM is the morphological subtype of cells as epithelioid or spindle cell in the tumor. Epithelioid cells have abundant acidophilic cytoplasm, large round or oval nuclei, a high nuclear-to-cytoplasmic ratio and a high number of mitotic figures. They are large, polymorphous and have a tendency towards discohesion. Their presence in the tumor is strictly related with considerably higher UM metastasis development probability and higher mortality rate (38, 51).

Spindle cells are elongated with large nuclei and scant cytoplasm (low nuclear -to-cytoplasmic ratio). They are

uniformly and densely arranged and may form palisades. There are very few cells with prominent nucleoli, and hardly any mitotic figures are observed.

The epithelioid cell type comprises approximately 3-5% of all UM and is associated with the least favorable prognosis. The 15-year mortality rate among patients diagnosed with epithelioid cell type UM is 75% (41).

Spindle cell type accounts for approximately 40% of all UM. The 15-year mortality rate is 20% (41).

Up to 50% of all UM are the most frequent mixed type. The 15-year mortality rate is approximately 60% but considerable differences are observed depending on the percentage of epithelioid and spindle cells (41).

ii. Mitotic activity. McLean *et al.* demonstrated a strong association between the number of mitoses observed per 40 high-power fields (HPF) and prognosis in patients with UM (51). Tumors of low mitotic activity (0-1/40 HPF) were associated with 6-year mortality rate of 15-23%, those with medium activity (2-8/40 HPF) 40-47%, and those with high activity (9-48/40 HPF) 56%. In a study by Damato *et al.*, there was a statistically significant correlation between tumors with mitotic activity higher than 4/40 HPF and the development of metastasis and metastasis-related mortality (38). Angi *et al.* demonstrated that the conventional mitotic activity count that involves counting mitotic figures in a routine hematoxylin and eosin staining depends on the experience of the person performing the count and carries an error of underestimation (52). They demonstrated that the use of phospho-histone H3 (Ser10) mitotic marker results in a higher number of mitoses being recognized in the analyzed material, increasing the reproducibility of counting and makes it more independent of the examiner's experience.

iii. Tumor-infiltrating lymphocytes (TILs) - lymphocytic inflammatory infiltration. Globally, inflammatory infiltration in UM, which involves an increased number of lymphocytes and macrophages and human leukocyte antigen (HLA) I and HLA II expression, is associated with worse prognosis (53).

Lymphocytic infiltration is observed in 17% of cases of UM (54), more frequently in ciliary body UM than in tumors confined to the posterior part of the uvea (42). TILs in UM are mainly suppressor/cytotoxic T-lymphocytes ($CD8^+$), to a lesser degree T-helper lymphocytes ($CD4^+$), as well as regulatory T-lymphocytes [$CD3^+$ forkhead box P3 $(FOXP3)^+$] (55, 56).

Lang *et al.* showed that brisk and non-brisk lymphocytic infiltration was associated with mixed and epithelioid cell UM (93.3% and 78.4%, respectively) (54). However, no statistically significant difference in mortality was demonstrated in their study in relation to TILs presence or absence in UM. In a study by de la Cruz *et al.* brisk TILs (defined as the presence of 100 or more lymphocytes per 20 HPF) was observed in 12.4% of UM (57). A group of 125 tumors with brisk TILs was compared with a control group with less intense inflammatory infiltration and it was shown that TILs are a significant factor of adverse prognosis in patients with UM. The 15-year survival rate in the group of patients with low lymphocytic infiltration is up to 69.6% and falls to 36.7% in the group with high infiltration. The authors suggested that the presence of TILs results from the general response of the immunological system to melanoma cells in the circulatory system, which act as specific circulating tumor cells (CTCs) that are a potential source of metastasis. In this approach, TILs identified in the primary UM should be considered indirect evidence of systemic cancer spread (57).

Better prognosis observed with down-regulation of HLA class I on UM cells is explained by their higher susceptibility to natural killer lymphocytes (53).

The influence of T-regulatory lymphocytes on the course of UM and prognosis remains unclear. There are some hypotheses that they may be involved in the induction of immunosuppressive mechanisms that block the proper response to the tumor (53).

Tumor-associated macrophages (TAMs) of varying grades also occur in UM with infiltration being low in 17%, moderate in 51%, and pronounced in 32% (58). The percentage of macrophages in lymphocytic infiltration is correlated with metastasis-related mortality and female sex, largest basal diameter of the tumor, epithelioid cell type, strong pigmentation and microvascular density (58). Most of the TAMs that are found in UM belong to the M2 subgroup which supports tissue remodeling and angiogenesis (59).

iv. Extravascular matrix pattern. Folberg *et al.* described nine morphological patterns of UM extravascular matrix (60). They found that the patterns are created by the tumor vessels, which gave rise to controversies (61, 62). Irrespective of the ongoing research into the origin of the connective tissue septal networks (containing blood vessels) between the complexes of tumor cells observed in periodic acid-Schiff staining, Folberg *et al.* demonstrated that their arrangement affects prognosis (60). They described two patterns, namely loops, and networks, whose common feature is the presence of at least one closed loop. Then they compared the patterns that did not contain a closed loop with the ones that did. During the follow-up, UM ended in the death of two patients in the first group (19 tumors) and of 18 patients in the second group (21 tumors), respectively. Mäkitie *et al.* demonstrated that the extravascular matrix pattern may be determined in 80% of UM cases acquired as a result of enucleation (63). The loop pattern was identified in 60% of cases and the network pattern in 35%. Ten-year UM-related survival was statistically worse for those with network pattern tumors, compared with those loops without networks and those with no loops (0.41 *versus* 0.53 *versus* 0.83; $p<0.0001$), and the prognosis for tumors

with a network pattern and those with loops without networks did not differ significantly.

Rummelt *et al*. stated the strong association of ciliary body UM with death from metastatic melanoma (p=0.0006), along with the following extravascular matrix patterns: parallel vessels (p=0.0043), parallel with cross-linking (p=0.0001), arcs (p=0.0028), arcs with branching, loops and network (p=0.0001 each). The aggressive behavior of ciliary body melanomas appears to be related to the tendency for vascular networks to develop in this location. Regardless of location, ciliary body or choroid, the presence of vascular networks shortens survival (42).

Connective tissue septa that form extravascular matrix patterns associated with worse prognosis were found to contain a higher number of blood vessels (62). This means non-invasive methods of assessment vascularization for UM prognosis might be applied (64, 65). Characteristic extravascular matrix patterns are also reported in UM metastases, irrespective of their location (66). Further studies concerning the origin and role of tissues that form extravascular matrix patterns may help improve UM therapy.

v. Degree of pigmentation. McLean *et al*. showed that the degree of tumor pigmentation has no prognostic value for patients with tumors containing epithelioid cells (51). On the other hand, prognostic value assigned to the amount of pigment in spindle-cell tumors may to a large extent be attributed to artifacts produced in the course of preparation of histopathological specimen. Accurate definition of the prognostic significance of pigmentation warrants further histopathological and statistical analysis.

vi. Extrascleral extension. Extrascleral extension may occur *via* aqueous channels (29.8%), ciliary arteries (27.4%), vortex veins (18.5%), ciliary nerves (8.9%) or the optic nerve (0.8%). In 10.4% of patients, the tumor spreads to the extraocular space simultaneously *via* a variety of routes.

Extrascleral extension is strongly correlated with involvement of the anterior chamber angle, basal diameter of the tumor, the presence of epithelioid cells, closed connective tissue loops and monosomy 3. Interestingly, extraocular extension along aqueous drainage channels, which is the most frequent route, correlated positively with anterior chamber angle involvement and inversely with basal tumor diameter and tumor thickness.

Metastasis-related mortality is higher irrespective of the route of extrascleral spread route and was not found to correlate with the size of extraocular tumor. It seems that extrascleral tumor extension may be considered incidental to tumor malignancy, and in the case of UM of the posterior part of the uvea, also to its stage of advancement rather than being an independent cause of development of distant metastases (37).

In a study by Schmittel *et al*., 5-year metastasis-free survival in patients with extraocular extension of UM was 28% *versus* 80.6% among patients without extraocular tumor growth (48). The mean time to metastasis development in patients with extraocular tumor growth was 35 months. The 5-year survival was 50% among patients with extraocular tumor growth and 83.3% among those without.

Cytogenetic abnormalities as potential prognosticators

The correlation between UM and changes within chromosomes 1, 3, 6 and 8 was noted a long time ago. The most frequent aberrations include the loss in the short arm of chromosome 1 (27%); losses in the short arm (45%), and long arm (49%) of chromosome 3; loss in the long arm (39%), and gain in the short arm (39%) of chromosome 6; loss in the short arm (20%), and gain in the long arm 8 (69%) and the short arm (20%) of chromosome 8 (67-69).

Hoglund *et al*. confirmed the existence of two independent cytogenetic pathways in UM that are of key importance for UM progression (68). The early alteration of one of them is the occurrence of monosomy 3, and subsequent 8q+, 8p- and 1p- aberrations. In the second one, gain of the short arm of chromosome 6 and subsequent 6q-, 8q+ imbalances occur. Co-occurrence of monosomy 3 and 6p imbalance is reported in only 4% of UM, while in 18% of primary tumors, the proper number of chromosomes 3 and 6p was observed (69). Furthermore, similar cytogenetic aberrations were observed in cutaneous melanoma. This is intriguing considering the many discrepancies identified clinically in the course of melanoma in both locations (68). Taking into account clinical data, it was shown that the subgroup of patients with monosomy 3 and normal short arm of chromosome 6 was associated with the highest probability of development of metastatic foci. Indirect risk was identified for the subgroup with disomy 3 and 6p+, and the lowest for the subgroup without aberrations in 3 and 6p (69).

Monosomy 3 is detected in approximately 50% of primary UM. It is an independent unfavorable prognostic factor of shorter survival and a factor of higher risk of developing organ metastasis (more prominent than any other clinical factor) (70). In a study by Prescher *et al*., during the follow-up period UM metastasis was detected in 57% patients with confirmed monosomy 3 in the primary tumor (70). The mean survival in this subgroup was less than 6 months from the diagnosis of metastasis. The authors also suggested concomitant involvement of several genes located on chromosome 3 in the changes occurring in the course of UM progression and the secondary development of distant metastases. Suppressor genes, whose inactivation conditions the development of UM, may be located both on 3p and 3q, which might explain a correlation with the loss of the entire

(normal) chromosome (70, 71) that is more frequent here than in other cancer types.

The selection of examination technique is an important issue in the search for monosomy 3. Isodysomy 3 may occur in the UM progression as a result of the loss of one chromosome 3, and the subsequent duplication of the faulty chromosome (72). Some of the methods of karyotype evaluation only allow detection of "standard" monosomy, which leads to the underestimation of the number of faulty cells. Techniques based on the analysis of the number of chromosomes, such as fluorescence *in situ* hybridization and comparative genomic hybridization, have lower sensitivity and specificity in the prognosis of UM metastasis than methods allowing for the detection of the loss of heterozygosity of chromosome 3, such as single nucleotide polymorphism (73, 74).

It must be stressed that although a strong correlation between monosomy 3 and prognosis in UM was confirmed, this factor cannot be the only prognostic criterion. Clinical assessment is of key importance for the precise prognostic stratification of patients with UM, with special attention paid to the basal tumor diameter and identification of the histopathological type of UM. Genetic typing should also allow for other mutations and aberrations specific for UM (38, 74).

8q+ Chromosomal aberration is the one most frequently observed in UM. Therefore, the order of occurrence of monosomy 3 and 8q+ in the course of UM, as well as the role of either of these as the tumor-initiating aberration, are still controversial (69, 75). The number of copies of the long arm of chromosome 8 may increase as a result of its gain (8q+/8pnl) and as a result of trisomy 8 (8q+/8p+) or isochromosome 8 (8q+/8p-) that in UM occurs almost exclusively with monosomy 3 (69). Irrespective of the chronology of changes, we know that 8q+ is more frequently found in patients with monosomy 3, which worsens prognosis depending on the number of copies of the long arm of chromosome 8 (69, 75). Monosomy 3 and an increased number of chromosome 8 are strongly correlated with the involvement of ciliary body, basal tumor diameter, epithelioid cell UM, the presence of closed loops, and high mitotic rate. Furthermore, monosomy of chromosome 3 is strictly correlated with the presence of extraocular spread (38). Damato *et al.* determined that 5-year UM-related mortality was 6% with no aberrations in chromosomes 3 or 8, 31% with gain of chromosome 8, 40% with monosomy 3, and 66% with co-existent monosomy 3 and chromosome 8 gain (38). However, the study was only focused on tumors originating in the posterior part of the uvea.

In UM, changes occur in the genes that are related with centrosomal function, regulation of the cell cycle and DNA damage repair *i.e.* the key processes in maintenance of genomic integrity. Ataxia telangiectasia and Rad3 related (*ATR*) and phosphatase and tensin homolog (*PTEN*) gene expression is decreased in UM. Mutations of *ATR* gene located on chromosome 3q22 are responsible for microsatellite instability. Mutations of *PTEN* tumor-suppressor gene lead to the loss of genomic integrity *via* a number of ways. The occurrence of *ATR* and *PTEN* gene damage may partly explain the gradual increase of aneuploidy in melanoma development (69). However, Ehlers *et al.* showed that poor prognosis in UM is related with early chromosomal alterations rather than with the subsequent increase in their number (69).

The use of gene-expression profiling by Onken *et al.* (76) allowed for identification of 62 discriminating genes in UM. They were used to divide the analyzed tumors into two classes. Class 2 tumors had a lower number of genes with their loci on chromosome 3 and a higher representation of genes of the long arm of chromosome 8, which was in agreement with previous studies on chromosomal aberrations in UM. To determine the prognostic value of the classifier in UM metastasis, 50 cases were analyzed. The 92-month survival probability was 95% in class 1 and 31% in class 2. The prognostic accuracy of molecular classifier superseded all clinical and histopathological prognostic factors. It should be noted that half of the tumors were evaluated based on the sample that corresponded to the size of the material acquired at biopsy (76). Class 1 was further divided into subclass 1a and 1b that differed as regards prognosis, with 5-year cancer-related mortality of 2% and 21% for class 1a and 1b, respectively, and 72% for class 2 (77). Class 2, associated with a high risk of developing metastasis, was found to be correlated with frequent occurrence of *BAP1* gene mutation at the 3p21.3 region. Harbour *et al.* identified *BAP1* mutations in 26 out of 31 (84%) class 2 UM and in only one out of 26 class 1 UM (78). Class 2 was associated with the largest basal tumor diameter, poor cellular differentiation and epithelioid cell tumor, involvement of ciliary body, the presence of extraocular spread, and closed loops, as well as the loss of heterozygosity of chromosome 3, increased aneuploidy, higher mitotic rate and up-regulation of Ki-67 (33). Continuing their studies into molecular classifier of UM based on gene expression profiling, Onken *et al.* proposed that the number of analyzed genes be reduced to 12 discriminating genes and three control genes (79). The use of polymerase chain reaction has made the evaluation largely independent from the method of obtaining the material for testing. Additionally, the authors showed that heterogeneity of tumor tissue does not have a significant impact on the result of tests performed on the material obtained using fine-needle aspiration biopsy. Modifications of this method help to optimize it for routine clinical applications and for eye- and vision-sparing therapies.

Estimating prognosis in clinical practice, particularly in individual patients, requires prior validation of research methods (80). The Collaborative Ocular Oncology Group, which analyzed results from 12 independent research centers,

showed in its first report that gene-expression profiling supersedes the evaluation of chromosome 3 and TNM classification to offer more accurate prognosis as regards the metastatic potential of UM (81). Gene-expression profiling allowed UM molecular class to be determined in 97.2% of cases, of which 61.9% were class 1 and 38.1% class 2. Throughout the follow-up period (18 months on average), UM metastases were detected in 1.1% of cases in class 1 and 25.9% in class 2 (81). This allowed a commercially available test to be introduced into the market (DecisionDx-UM; Castle Biosciences, Incorporated). It is now used in several centers (82, 83).

The excellent progress made over the past decade in molecular biology of UM will enable for further development of new prognostic tests that will stratify patients into prognostic subgroups that differ significantly as regards long-term prognosis and risk of distant metastases with even higher precision and accuracy. Moreover, advances in molecular biology may help discover new signaling pathways involved in UM development, which may pave the way towards new target drugs for personalized therapy.

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Clinical Significance of Nucleoli Cytomorphology Assessment in Patients With Uveal Melanoma

TOMASZ BERUS[1], ANNA MARKIEWICZ[2], PRZEMYSŁAW BIECEK[3], JOLANTA ORŁOWSKA-HEITZMAN[4], AGNIESZKA HAŁOŃ[5], BOŻENA ROMANOWSKA-DIXON[2] and PIOTR DONIZY[5]

[1]*Department of Ophthalmology, 4th Military Clinical Hospital with Polyclinic, Wroclaw, Poland;*
[2]*Department of Ophthalmology and Ocular Oncology, Jagiellonian University Medical College, Krakow, Poland;*
[3]*Faculty of Mathematics, Informatics, and Mechanics, University of Warsaw, Warsaw, Poland;*
[4]*Department of Pathomorphology, Jagiellonian University Medical College, Krakow, Poland;*
[5]*Department of Pathomorphology and Oncological Cytology, Wroclaw Medical University, Wroclaw, Poland*

Abstract. *Aim: To assess the prognostic significance of nucleolar morphological parameters in a large cohort of patients with uveal melanoma. Patients and Methods: The presence, size and number of nucleoli of cancer cells were assessed in haematoxylin and eosin (HE)-stained slides of 164 formalin-fixed paraffin-embedded primary uveal melanoma tissue specimens. The results were correlated with clinicopathological features and patient survival. Results: The presence of macronucleoli and multiple nucleoli significantly correlated with the epithelioid type of uveal melanoma, high mitotic rate, and marked pleomorphism. There was a positive correlation between the presence of macronucleoli as well as the number of nucleoli and the largest tumour basal diameter. The increased nucleolus count in tumour cells positively correlated with primary tumour (pT) staging. The presence of both prominent and multiple nucleoli was associated with significantly reduced overall and disease-free survival. Conclusion: Histological assessment of nucleolar morphology in routine HE staining would be a helpful low-cost method to obtain reliable prognostic information.*

Nucleoli are nuclear ultrastructures assembled during telophase out of decondensed chromosomal segments containing the genes for ribosomal RNA (rRNA), the nucleolus organizer regions (NOR). They exert their function during interphase and disappear alongside other nuclear structures during prophase as the chromatin condenses again prior to another cell-division cycle (1, 2).

Correspondence to: Tomasz Berus, MD, Department of Ophthalmology, 4th Military Clinical Hospital with Polyclinic, Weigla 5, 50-981 Wroclaw, Poland. E-mail: tberus@op.pl

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The nucleolus is the site of rRNA transformation from the transcription stage to pre-ribosomal particle formation (3). Therefore, it is referred to as the ribosome factory, responsible for protein synthesis. Ribosome biogenesis is one of the most important and complex metabolic processes, and utilises up to 80% of a cell's energy resources (4, 5). Its hyperactivation, which may be triggered by oncogene activation or loss of tumour-suppressor genes, is crucial for carcinogenesis and cancer progression. This is true both for mutations directly affecting ribosomal structure and in the widely understood dysregulation of ribosome biogenesis. Down-regulation of ribosome production leads to cell-cycle inhibition and induction of apoptosis, *via* both p53-dependent and -independent pathways, which can be exploited as a potential therapeutic strategy (6-8).

The nucleoli are very high-density structures due to their high content of functional proteins, of which more than 4,500 have been identified to date (9). Alongside other cofactors, these proteins regulate ribosome biogenesis and a number of signalling pathways involved in cell response to stress and promoting survival. There are a number of factors to affect ribosome biogenesis at many stages, of which rRNA synthesis and translation initiation are the key ones. These include protein kinase B; phosphatidylinositol 3-kinase; RAS; MYC; upstream-binding factor; DNA topoisomerase I; fibrillarin; alternative reading frame proteins; argyrophylic proteins, including nucleolin and nucleophosmin; small nucleolar RNAs; as well as ribosomal proteins, both small and large ribosomal subunits (10-18).

Metabolic activity in the nucleoli during intense cell proliferation affects their cytomorphological features (*e.g.* size and number in a cell) (1). Any changes to it, which can be identified in a routine histological assessment, may be of prognostic significance in patients with cancer. The aim of this study was to assess the relationship between the cytomorphological characteristics of nucleoli (presence, size

and number) and detailed clinicopathological parameters in patients with uveal melanoma.

Patients and Methods

Patients. The study group consisted of 164 patients diagnosed in 2002-2011 with uveal melanoma treated by primary enucleation at the Department of Ophthalmology and Ocular Oncology, Medical College, Jagiellonian University in Krakow, Poland. Patients were enrolled in the study based on the availability of their medical records and tissue specimens, which included paraffin blocks and histological slides. Comprehensive clinical data were retrieved from the archived medical records, and details of diagnostic and therapeutic procedures performed were sourced from the Ocular Oncology Outpatient Clinic, University Hospital, Krakow, Poland. The study was reviewed and approved by the Ethical Committees of the Jagiellonian University, Krakow, Poland (decision no.: 122.6120.58.216), and the Wroclaw Medical University, Wroclaw, Poland (decision no.: KB-500/2017).

Records were reviewed for clinical and pathological data including age, sex, affected eye, largest basal diameter and height of the tumour, tumour staging [primary tumour (pT) and American Joint Committee on Cancer (AJCC) prognostic stage], tumour location relative to the equator, ciliary body involvement, tumour pigmentation and shape, concomitant glaucoma/retinal detachment, histological subtype, scleral/optic nerve infiltration, as well as tumour necrosis. Additionally, detailed histological parameters, such as mitotic rate, presence of tumour-infiltrating lymphocytes, nuclear pseudoinclusions, intranuclear grooves, multinucleated giant cells and haemorrhage, as well as level of tumour cell pigmentation were considered.

The largest basal diameter and height of the tumour were described in line with the AJCC guidelines (19) and staged according to the Collaborative Ocular Melanoma Study (COMS) (20).

Nucleolar morphology evaluation. Nucleolar morphology was evaluated in 164 haematoxylin and eosin (HE)-stained sections of primary uveal melanomas. Three parameters were ascertained and assessed in this study: (i) Nucleolus presence, (ii) nucleolus size, and (iii) number of nucleoli. The nucleolus presence was determined based on a global assessment of melanoma cells in a single HE-stained slide and graded according to the following algorithm: 0: Lack of nucleoli, 1: a small percentage of cells with nucleoli (≤20% of melanoma cells in the single HE-stained slide of primary uveal melanoma), 2: a high percentage of cells containing nucleoli (>20% of melanoma cells with visible nucleoli). Nucleolar size in melanoma cells was graded according to the following algorithm: 0: Lack of nucleoli, 1: nucleoli present but inconspicuous, 2: prominent nucleoli (macronucleoli) present. The number of nucleoli in melanoma cells was graded according to the following algorithm: 0: Lack of nucleoli, 1: single micro- or macronucleolus in the nucleus, 2: two or more nucleoli per nucleus.

Statistical analysis. Statistical analysis was performed using the R language (available online: https://www.r-project.org/). For the purposes of the analysis, the study cohort was divided into subsets based on (i) Nucleolus presence, *i.e.* lack of nucleoli in melanoma cells or a small percentage of cells containing nucleoli *versus* high >20% of melanoma cells with visible nucleoli; (ii) nucleolus size, *i.e.* lack of nucleoli or inconspicuous nucleoli *versus* presence of prominent nucleoli; and (iii) number of nucleoli, *i.e.* lack or a single micro- or macronucleolus *versus* two or more nucleoli per nucleus of an uveal melanoma cell (Figure 1).

In order to determine the overall and disease-free survival, Kaplan–Meier curves and the log-rank test were used; all analyses were carried out using the survival package for R. In order to determine the correlations between the dichotomised cytomorphological nucleolar parameters and continuous variables, the Wilcoxon two-sample test was used. The correlations between cytomorphological parameters of nucleoli and binary variables were determined using Fisher's exact test, while the correlations with other categorical variables were determined using the chi-square test. p-Values below 0.05 was considered significant for all comparisons.

Results

Correlations with clinical parameters. There was a positive correlation between largest basal diameter of the tumour and both nucleolus number and the presence of macronucleoli, which remained significant regardless of whether the AJCC (p=0.042 and p=0.019) or COMS (p=0.0089 and p<0.001) criteria were applied. There was no correlation between tumour height and number or size of nucleoli.

An increased number of nucleoli in tumour cells was positively correlated with pT staging determined as per the College of American Pathologists (CAP) protocol (19) (p=0.013). There was also a trend towards an association between nucleolus size and pT, which was on the verge of significance (p=0.053). There was a positive correlation between tumour staging and the presence of macronucleoli, as well as multiple nucleoli (p=0.041 and p=0.013).

There was a positive correlation of peripheral tumour location and nucleolus number and size (p=0.029 and p=0.0024). Similarly, there was a significant correlation between the presence of prominent nucleoli and ciliary body involvement (p=0.0069).

Furthermore, the presence of macronucleoli correlated positively with clinically assessed darker tumour pigmentation (p<0.001).

Table I also includes a division of cases into those with and without nucleoli. Correlations were determined between the presence of nucleoli and the evaluated clinical parameters. We only found a significant correlation between tumour height (as per COMS) and the presence of nucleoli (p=0.023).

There was no correlation between the presence, size or number of nucleoli and other evaluated clinical parameters, such as sex, age upon diagnosis, primary tumour shape, as well as concomitant retinal detachment or glaucoma.

Correlations with histological parameters. The presence of macronucleoli and multiple nucleoli significantly correlated with the epithelioid type of uveal melanoma (p<0.001 and p=0.013), high mitotic rate (p=0.0039 and p=0.0041), and marked pleomorphism (p<0.001 for both correlations), as well as the presence of multinucleated giant cells (p<0.001

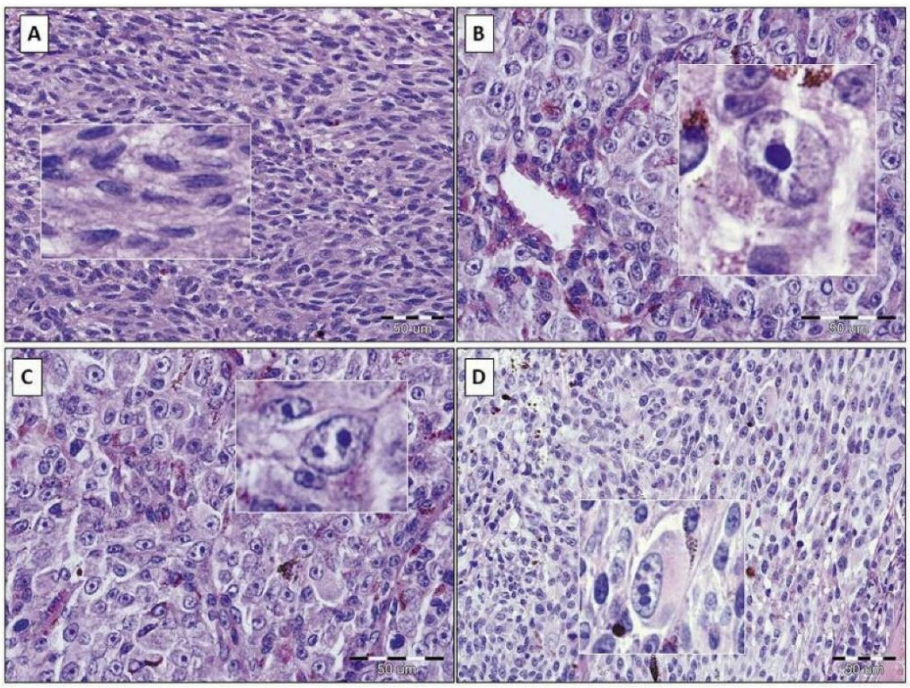

Figure 1. *Cytomorphology of nucleoli in uveal melanoma cells is revealed by haematoxylin and eosin staining. A: Lack of nucleoli in neoplastic cells (inset, ×400). B: High percentage of melanoma cells with macronucleoli (inset, ×600). C: High representation of cancer cells with distinct nucleoli with the feature of binucleolization (inset, ×600). D: Polynucleolization of uveal melanoma cells (inset, ×600). Bar= 50 μm.*

for both correlations), nuclear pseudoinclusions ($p<0.001$ in both cases) and the absence of intranuclear grooves ($p<0.001$ and $p=0.0019$). Additionally, there was a positive correlation between the pigment content in tumour cells and the presence of larger ($p=0.021$) or multiple nucleoli (p=0.045) (Table II).

The effect of nucleolar cytomorphology on long-term survival – Kaplan–Meier analysis. In Kaplan–Meier analysis, the presence of both prominent and multiple nucleoli (two or more per nucleus) was associated with significantly reduced overall and disease-free survival (Figure 2).

Discussion

The nucleoli are formed around single active NORs, which, in humans, are located on the short arms of acrocentric chromosomes 13, 14, 15, 21 and 22. Thus, the number of primary nucleoli per cell does not exceed 10. They fuse, turning into larger, mature nucleoli, containing several NORs and surrounded by perinucleolar heterochromatin. Further dynamic changes in nucleolar spatial organisation are related to multi-factorial regulation of ribosome biogenesis (21). Abnormal nucleolar reorganisation is one of the key features of neoplastic cells (22).

There is a close link between the rate of cell proliferation and the size of nucleoli (23). Up-regulation of rDNA gene transcription, rRNA processing and transport result in accelerated ribosome biosynthesis. High expression of nucleolar proteins, including upstream binding factor, DNA topoisomerase 1 and fibrillarin, is a necessary prerequisite for rapid ribosome assembly in the amount needed for cell division. On the other hand, the loss of RNA polymerase I activity inhibition by retinoblastoma protein (pRb) (due to its high phosphorylation) and p53 (due to mutation), have similar consequences. Both up-regulation of proliferation-stimulating factors and functional inactivation of proliferation-inhibiting factors are associated with the presence of prominent nucleoli (24). Furthermore, the above mechanisms are also responsible for uncontrolled tumour growth (25). The total nucleolar area per cell is a morphological parameter reflecting the rapidity of tumour cell proliferation (23). Our results are consistent with the above molecular findings, since the presence of macronucleoli and their high number per cell are typical of aggressive uveal melanoma phenotype.

Table I. *Summary statistics for relation between characteristics of nucleoli in uveal melanoma cells according to clinical parameters.*

Clinical parameter	Characteristics of nucleoli								
	Presence			Size			Number		
	Low	High	*p*-Value	Low	High	*p*-Value	Low	High	*p*-Value
Age in years (18-86)[a]									
Median (range)	56 (52-62)	60 (52-67)	0.46	59 (51-66)	62 (57-67)	0.067	60 (52-66)	61 (55-67)	0.75
Gender, n (%)[b]									
Female	3 (50%)	81 (51%)	>0.99	50 (51%)	34 (52%)	0.87	67 (54%)	17 (41%)	0.16
Male	3 (50%)	77 (49%)		49 (49%)	31 (48%)		56 (46%)	24 (59%)	
Side, n (%)[b]									
Right	4 (67%)	82 (52%)	0.68	46 (46%)	32 (49%)	0.75	65 (53%)	21 (51%)	0.86
Left	2 (33%)	76 (48%)		53 (54%)	33 (51%)		58 (47%)	20 (49%)	
Largest basal tumor diameter (AJCC), n (%)[c]									
>9-12 mm	0 (0%)	13 (8%)	0.23	11 (11%)	2 (3%)	**0.019**	12 (10%)	1 (2%)	**0.042**
>12-15 mm	3 (50%)	24 (15%)		21 (21%)	6 (9%)		24 (20%)	3 (7%)	
>15-18 mm	1 (17%)	40 (25%)		25 (25%)	16 (25%)		32 (26%)	9 (22%)	
>18 mm	2 (33%)	81 (51%)		42 (42%)	41 (63%)		55 (45%)	28 (68%)	
Largest basal tumor diameter (COMS), n (%)[c]									
<10 mm	0 (0%)	2 (1%)	0.40	2 (2%)	0 (0%)	**<0.001**	2 (2%)	0 (0%)	**0.0089**
10 -<16 mm	3 (50%)	44 (28%)		38 (38%)	9 (14%)		42 (34%)	5 (12%)	
≥16 mm	3 (50%)	112 (71%)		59 (60%)	56 (86%)		79 (64%)	36 (88%)	
Greatest tumor height (AJCC), n (%)[c]									
≤3 mm	1 (17%)	0 (0%)	0.095	1 (1%)	0 (0%)	0.88	1 (1%)	0 (0%)	0.61
>3-6 mm	0 (0%)	15 (9%)		10 (10%)	5 (8%)		11 (9%)	4 (10%)	
>6-9 mm	1 (17%)	42 (27%)		27 (27%)	16 (25%)		36 (29%)	7 (17%)	
>9-12 mm	3 (50%)	55 (35%)		36 (36%)	22 (34%)		43 (35%)	15 (37%)	
>12-15 mm	1 (17%)	36 (23%)		20 (20%)	17 (26%)		25 (20%)	12 (29%)	
>15 mm	0 (0%)	10 (6%)		5 (5%)	5 (8%)		7 (6%)	3 (7%)	
Greatest tumor height (by COMS), n (%)[c]									
<3 mm	1 (17%)	0 (0%)	**0.023**	1 (1%)	0 (0%)	0.66	1 (1%)	0 (0%)	0.38
3 mm-<8 mm	0 (0%)	33 (21%)		22 (22%)	11 (17%)		28 (23%)	5 (12%)	
≥8 mm	5 (83%)	125 (79%)		76 (77%)	54 (83%)		94 (76%)	36 (88%)	
Primary tumor (pT), n (%)[c]									
2	1 (17%)	12 (8%)	0.29	10 (10%)	3 (5%)	0.053	12 (10%)	1 (2%)	**0.013**
3	3 (50%)	48 (30%)		36 (36%)	15 (23%)		44 (36%)	7 (17%)	
4	2 (33%)	98 (62%)		53 (54%)	47 (72%)		67 (54%)	33 (80%)	
Stage, n (%)[b]									
II	4 (67%)	50 (32%)	0.092	39 (39%)	15 (23%)	**0.041**	47 (38%)	7 (17%)	**0.013**
III	2 (33%)	108 (68%)		60 (61%)	50 (77%)		76 (62%)	34 (83%)	
Tumour location, n (%)[c]									
In front of the equator	3 (50%)	68 (52%)	0.73	33 (41%)	38 (69%)	**0.0024**	46 (46%)	25 (71%)	**0.029**
Equator	0 (0%)	19 (15%)		12 (15%)	7 (13%)		17 (17%)	2 (6%)	
Behind the equator	3 (50%)	43 (33%)		36 (44%)	10 (18%)		38 (38%)	8 (23%)	
Ciliary body involvement, n (%)[b]									
Not involved	6 (100%)	103 (65%)	0.18	74 (75%)	35 (54%)	**0.0069**	84 (68%)	25 (61%)	0.45
Involved	0 (0%)	55 (35%)		25 (25%)	30 (46%)		39 (32%)	16 (39%)	
Degree of pigmentation[c]									
Amelanotic	2 (33%)	24 (16%)	0.22	22 (24%)	4 (6%)	**<0.001**	23 (19%)	3 (8%)	0.28
Mild	3 (50%)	58 (39%)		41 (45%)	20 (32%)		46 (39%)	15 (42%)	
Intense	1 (17%)	66 (45%)		29 (32%)	38 (61%)		49 (42%)	18 (50%)	
Shape, n (%)[b]									
Dome	3 (50%)	82 (53%)	>0.99	48 (49%)	37 (58%)	0.34	62 (50%)	23 (61%)	0.35
Mushroom	3 (50%)	73 (47%)		49 (51%)	27 (42%)		61 (50%)	15 (39%)	
Retinal detachment[b]									
No	1 (17%)	30 (19%)	>0.99	21 (21%)	10 (15%)	0.42	26 (21%)	5 (12%)	0.25
Coexistence	5 (83%)	128 (81%)		78 (79%)	55 (85%)		97 (79%)	36 (88%)	
Glaucoma, n (%)[b]									
No	4 (80%)	136 (86%)	0.54	85 (87%)	55 (85%)	0.82	104 (85%)	36 (88%)	0.80
Coexistence	1 (20%)	22 (14%)		13 (13%)	10 (15%)		18 (15%)	5 (12%)	

AJCC: American Joint Committee on Cancer; COMS: Collaborative Ocular Melanoma Study. *p*-Values from: [a]Wilcoxon two-sample test, [b]Fisher's exact test, [c]chi-square test. Bold values indicate statistical significance ($p<0.05$).

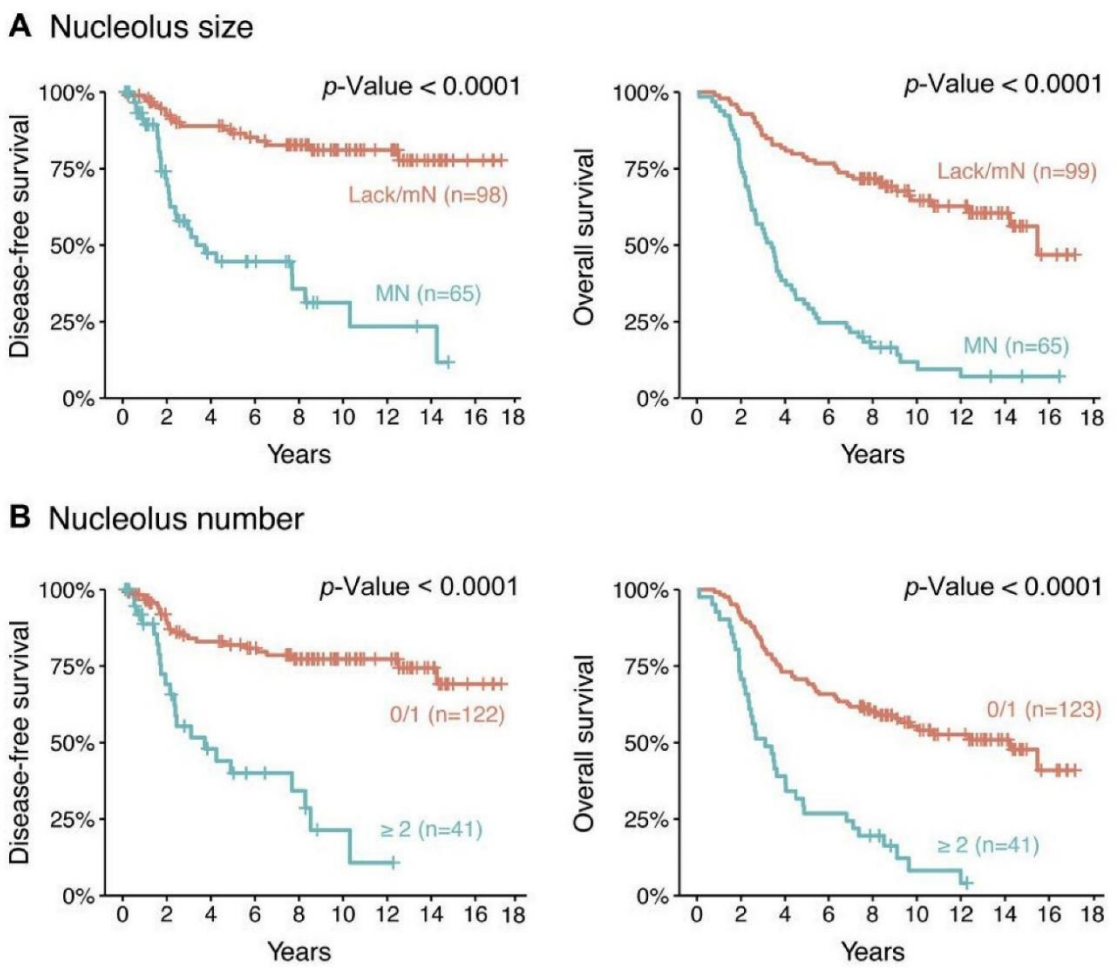

Figure 2. *Kaplan–Meier analysis of the prognostic impact of nucleolar parameters in patients with uveal melanoma. The presence of macronucleoli (MN) and an increased number of nucleoli were associated with significantly shorter disease-free and overall survival. mN: Micronucleoli.*

The number of nucleoli per cell differs between cells, both in the same and in different tissues (26). The mechanisms which control this number have not been fully understood to date. Freed *et al*. identified 139 proteins whose depletion altered the number of nucleoli per nucleus from two or three to only one (5). Most of them simultaneously regulate ribosome synthesis, crucial for rDNA transcription, pre-rRNA processing, or global cellular translation (27). Therefore, these factors which control ribosome synthesis may not only alter the structure of nucleoli but also determine their number.

Abnormal nucleolar cytomorphology was one of the first identified histological features of neoplasms. An increased number of nucleoli and the presence of macronucleoli (prominent nucleoli) are seen in a number of neoplasms, being a negative prognostic factor in some of them (28, 29). This has, for instance, been demonstrated in skin melanoma (30).

Nucleolar assessment has been used, although to a limited extent, in diagnosis of uveal melanoma since 1931, when Callender proposed his diagnostic classification (31). The finding of indistinct or distinct nucleoli in spindle-cell type uveal melanoma prompted tumour classification as type A or B. A modification of the said classification, introduced more than 50 years later (32), was based on a method proposed by Gamel *et al*. (33, 34), whose studies using digital data analysis confirmed the association between 13 nucleolar parameters and prognosis in uveal melanoma. The standard deviation of

Table II. *Summary statistics for relation between characteristics of nucleoli in uveal melanoma cells according to histopathological parameters.*

Histopathological parameter	Characteristics of nucleoli, n (%)								
	Presence			Size			Number		
	Low	High	*p*-Value	Low	High	*p*-Value	Low	High	*p*-Value
Histological subtype[a]									
Spindle cell	3 (50%)	26 (16%)	0.12	28 (28%)	1 (2%)	**<0.001**	26 (21%)	3 (7%)	**0.013**
Mixed cell	3 (50%)	111 (70%)		66 (67%)	48 (74%)		86 (70%)	28 (68%)	
Epithelioid cell	0 (0%)	21 (13%)		5 (5%)	16 (25%)		11 (9%)	10 (24%)	
Mitotic rate [per 40 HPF (8 mm^2)][b]									
0-4	6 (100%)	101 (64%)	0.095	74 (75%)	33 (52%)	**0.0039**	88 (72%)	19 (46%)	**0.0041**
5-31	0 (0%)	56 (36%)		25 (25%)	31 (48%)		34 (28%)	22 (54%)	
Scleral infiltration[b]									
None or intrascleral	6 (100%)	153 (97%)	>0.99	96 (97%)	63 (97%)	>0.99	120 (98%)	39 (95%)	0.60
Full-thickness	0 (0%)	5 (3%)		3 (3%)	2 (3%)		3 (2%)	2 (5%)	
Invasion of optic nerve[a]									
None	4 (67%)	129 (82%)	0.36	77 (78%)	56 (86%)	0.35	95 (77%)	38 (93%)	0.071
Optic nerve head	2 (33%)	26 (16%)		20 (20%)	8 (12%)		25 (20%)	3 (7%)	
Optic nerve	0 (0%)	3 (2%)		2 (2%)	1 (2%)		3 (2%)	0 (0%)	
Necrosis[b]									
None	5 (83%)	131 (87%)	0.57	80 (88%)	56 (86%)	0.81	101 (87%)	35 (88%)	>0.99
Present	1 (17%)	19 (13%)		11 (12%)	9 (14%)		15 (13%)	5 (12%)	
Marked pleomorphism[b]									
None	6 (100%)	141 (89%)	>0.99	98 (99%)	49 (75%)	**<0.001**	120 (98%)	27 (66%)	**<0.001**
Present	0 (0%)	17 (11%)		1 (1%)	16 (25%)		3 (2%)	14 (34%)	
TILs[b]									
None	6 (100%)	139 (88%)	>0.99	90 (91%)	55 (85%)	0.22	110 (89%)	35 (85%)	0.57
Present	0 (0%)	19 (12%)		9 (9%)	10 (15%)		13 (11%)	6 (15%)	
Multinucleated giant cells[b]									
None	6 (100%)	119 (75%)	0.34	94 (95%)	31 (48%)	**<0.001**	104 (85%)	21 (51%)	**<0.001**
Present	0 (0%)	39 (25%)		5 (5%)	34 (52%)		19 (15%)	20 (49%)	
NPIs[b]									
None	5 (83%)	96 (61%)	0.41	73 (74%)	28 (43%)	**<0.001**	86 (70%)	15 (37%)	**<0.001**
Present	1 (17%)	62 (39%)		26 (26%)	37 (57%)		37 (30%)	26 (63%)	
Intranuclear grooves[b]									
None	4 (67%)	125 (79%)	0.61	68 (69%)	61 (94%)	**<0.001**	90 (73%)	39 (95%)	**0.0019**
Present	2 (33%)	33 (21%)		31 (31%)	4 (6%)		33 (27%)	2 (5%)	
Hemorrhage[b]									
None	6 (100%)	124 (78%)	0.35	84 (85%)	46 (71%)	**0.048**	102 (83%)	28 (68%)	0.073
Present	0 (0%)	34 (22%)		15 (15%)	19 (29%)		21 (17%)	13 (32%)	
Pigmentation[a]									
Lack	1 (17%)	13 (8%)	0.27	12 (12%)	2 (3%)	**0.021**	13 (11%)	1 (2%)	**0.045**
Low	4 (67%)	83 (53%)		56 (57%)	31 (48%)		69 (56%)	18 (44%)	
High	1 (17%)	62 (39%)		31 (31%)	32 (49%)		41 (33%)	22 (54%)	

TILs: Tumour-infiltrating lymphocytes; NPIs: nuclear pseudoinclusions. *p*-Values from: [a]chi-square test, [b]Fisher's exact test. Bold values indicate statistical significance ($p<0.05$).

nucleolar area correlated negatively with cancer-specific survival and had the highest prognostic value. Including the largest basal diameter in the correlation analysis additionally increased its prognostic accuracy (35). The method using the mean measurement of the 10 largest nucleoli (MTLN), first reported by Huntington *et al*. (36) and modified by McCurdy *et al*. (37), was proposed as technically simpler, cheaper and faster, while maintaining a similar prognostic value. However, other studies emphasized difficulties in the determination of measurement standards, with a relatively long learning curve and consequently low intraobserver reproducibility. More importantly, MTLN - assessed in either simple or multiple correlation analysis - was not associated with prognosis (38).

The discovery of argyrophilic properties of nucleolin, B23, upstream-binding factor and RNA polymerase I subunits enabled the use of the silver staining method to evaluate nucleoli (39). Tissues with high cellular proliferation usually show a more intense reaction, due to a high content of B23 and nucleolin during the S-G2-phase, with their respective low contents during the G1 phase. (40) Tuccari *et al*. proposed a

standardised method for evaluating silver-stained NOR (AgNOR) in order to determine the prognosis in uveal melanoma (41). As a result of the surface area calculations of AgNOR, the mean AgNOR area (NORA) can be computed. Larger primary tumours and non-spindle-cell uveal melanoma tend to have higher NORA values. They are also associated with a significantly higher risk of cancer-specific death. NORA proved to be an independent prognostic factor for survival (41).

Our findings are consistent with previous reports. We have shown that the presence of both a high number of nucleoli and prominent nucleoli was associated with significantly shorter overall and disease-free survival. What distinguishes our study from others' is the practicality of the method for nucleolar morphology assessment. Based on standard HE staining without the need to use additional equipment, it can be widely adopted in almost every hospital laboratory. On the other hand, the indisputable disadvantage of our method is its subjectivity, due to which it can only be used by experienced pathologists. Nevertheless, neither the MTLN nor NORA methods are completely objective. Furthermore, their complexity and multiple-step procedures make it impossible to adopt the methods in routine practice.

In this context, it may be justified to return to the observations and assumptions made by Gamel *et al*. (33-35) and others (42). Nowadays, 35 years later, digital recording and analysis of cytomorphological parameters and their standard deviations after HE staining should not be particularly difficult. High-resolution cameras, high computer performance and the possibility to use artificial intelligence are now available even to mobile phone users. With the right software, it should possible to simplify, speed up and ensure improved objectivity of the evaluation of nucleolar cytomorphology.

Conclusion

Macronucleoli and multiple nucleoli were significantly correlated with worse clinical outcomes. The histological assessment of nucleolar morphology through routine HE staining would be a helpful low-cost method for obtaining reliable prognostic information.

Conflicts of Interest

The Authors have no conflicts of interest concerning this study.

Authors' Contributions

T.B.: Conceptualization, methodology, investigation, resources, data curation, writing - original draft, visualization; A.M.: investigation, resources, data curation; P.B.: software, formal analysis, visualization; J.O.H.: investigation, resources; A.H.: methodology, resources; B.R.D.: investigation, resources, supervision; P.D.: conceptualization, methodology, investigation, resources, data curation, writing - review & editing, visualization, supervision, project administration, funding acquisition. All Authors have reviewed the article and approved the submitted version.

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ORIGINAL PAPER

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Downregulation of Polo-like kinase-1 (PLK-1) expression is associated with poor clinical outcome in uveal melanoma patients

Tomasz Berus[1], Anna Markiewicz[2], Katarzyna Kobylinska[3], Przemyslaw Biecek[3], Jolanta Orlowska-Heitzman[4], Bozena Romanowska-Dixon[2], Piotr Donizy[5]

[1]Department of Ophthalmology, 4th Military Clinical Hospital with Polyclinic, Wroclaw, Poland

[2]Department of Ophthalmology and Ocular Oncology, the Jagiellonian University, Medical College, Krakow, Poland

[3]Faculty of Mathematics, Informatics and Mechanics University of Warsaw, Warsaw, Poland

[4]Department of Pathomorphology, the Jagiellonian University, Medical College, Krakow, Poland

[5]Department of Pathomorphology and Oncological Cytology, Wroclaw Medical University, Wroclaw, Poland

Abstract

Introduction. Uveal melanoma (UM) is the most common primary eye tumour in adults. Distant metastases are seen in 50% of cases regardless of treatment, which contributes to high mortality rates. Polo-like kinase-1 (PLK-1) is a protein regulator of mitotic entry and cytokinesis. Increased PLK-1 expression has been shown in different tumours, which makes its inhibition a potential treatment target. To date, no study has been published to discuss the prognostic role of PLK-1 expression in patients with uveal melanoma.

Material and methods. We assessed by immunohistochemistry PLK-1 expression in uveal melanoma cells collected in 158 patients treated by primary enucleation. We determined the correlation between PLK-1 levels evaluated by the immunoreactivity scale (IRS) method and detailed clinical as well as histological parameters. Additionally, we determined the association between PLK-1 expression levels and long-term prognosis.

Results. Elevated PLK-1 expression in tumour cells, defined as IRS >2, was observed in 70% (111/158) of cases, whereas low expression or no expression was seen in the remaining 30% (47/158) of patients. There was a significant correlation between low PLK-1 expression and a higher clinical tumour stage (pT, $p = 0.04$) as well as a higher AJCC prognostic stage group ($p = 0.037$). We observed an inverse correlation between PLK-1 expression and tumour cell pigment content ($p = 0.0019$). There was no correlation between PLK-1 expression and other histological parameters such as mitotic rate or histological subtype. The Kaplan-Meier's analysis demonstrated that low PLK-1 expression was associated with significantly reduced overall survival ($p = 0.0058$). A similar trend, albeit not significant, was observed for disease-free survival ($p = 0.088$).

Conclusions. Downregulated PLK-1 expression is a negative prognostic factor in uveal melanoma. It warrants further, multicentre research on prognostic role of PLK-1 expression and possibility of PLK-1 inhibition in uveal melanoma. *(Folia Histochemica et Cytobiologica 2020, Vol. 58, No. 2, 108–116)*

Key words: uveal melanoma; polo-like kinase-1; prognostic factor; IHC

Correspondence address: Tomasz Berus, MD
Department of Ophthalmology
4th Military Clinical Hospital with Polyclinic,
Weigla 5, 50–981 Wroclaw, Poland
e-mail: tberus@op.pl

Introduction

Uveal melanoma (UM) is the most common primary eye tumour in adults. The incidence in the general population is below 10 cases per million population per year [1]. We have previously discussed epidemi-

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ology and prognostic factors in uveal melanoma in a comprehensive review [2]. Depending on the clinical course of disease, chances for vision preservation and patient expectations, primary tumours can be effectively treated with brachytherapy, proton beam irradiation, transpupillary thermotherapy, local resection, endoresection, or enucleation. Regardless of the selected treatment modality, almost 50% of affected patients develop distant metastases, which contributes to very high mortality rates [2]. Conventional chemotherapy, isolated hepatic perfusion, immunoembolisation, surgery and checkpoint inhibitors have very limited efficacy in metastatic UM with the median overall survival (OS) of 1.07 years (range: 0.59–2.50 years) across all treatment modalities [3].

Polo-like kinase-1 (PLK-1) is a serine/threonine-protein kinase consisting of a highly conservative N-terminal kinase domain (KD) of 252 amino-acids and a C-terminal Polo-box domain (PBD), that is, two conserved polo-box regions of 30 amino-acids connected via a short linker. An interaction with peptides phosphorylated by other kinases involved in the cell cycle changes the PBD conformation. Acting like a clip, it docks PLK-1 at its accurately selected target site during the appropriate stage of cell division [4, 5], whereby PLK-1 becomes a master regulator of mitosis and cytokinesis [6].

PLK-1 has been implicated in Cdk1-cyclin B activation at mitotic entry, centrosome maturation, bipolar spindle formation, activation of anaphase promoting complex/cyclosome (APC/C), accumulation of spindle assembly checkpoint (SAC) proteins at kinetochores, sister chromatid separation, as well as cytokinesis [7–9]. Furthermore, PLK-1 has recently been shown to play a role in microtubule dynamics, DNA replication, chromosome dynamics, p53 regulation, and recovery from the G2 DNA-damage checkpoint [10].

PLK-1 overexpression has been demonstrated in a number of human tumours, where it often correlates with increased cellular proliferation and poor prognosis [11–18], *e.g.* in skin melanoma [19, 20]. Therefore, it is currently considered a prooncogenic factor, which exerts its effect by affecting cell cycle checkpoints and causing genetic instability. As such, it is the target of cancer therapies [21], which seems potentially plausible also in UM [22].

The aim of this study was to assess the PLK-1 expression in UM as well as its correlation with detailed clinical and pathological parameters, and long-term survival.

Material and methods

Patients. The study group consisted of 158 patients with uveal melanoma treated by primary enucleation at the Department of Ophthalmology and Ocular Oncology, Medical College, Jagiellonian University in Krakow, Poland, diagnosed in 2002–2011. Patients were enrolled in the study based on the availability of their medical records and tissue specimens, which included paraffin blocks and histological slides. Comprehensive clinical data was retrieved from the archived medical records, and details of diagnostic and therapeutic procedures performed were sourced out from the Ocular Oncology Outpatient Clinic, University Hospital, Krakow, Poland. The study was reviewed and approved by the ethical committees of the Jagiellonian University, Krakow, Poland (decision no. 122.6120.58.216), and the Wroclaw Medical University, Wroclaw, Poland (decision no. KB-500/2017).

Records were reviewed for clinical and pathological data including age, sex, affected eye, largest basal diameter and thickness of the tumour, tumour staging (pT and AJCC prognostic stage group), tumour location relative to the equator, ciliary body involvement, clinical tumour pigmentation and shape, concomitant glaucoma and/or retinal detachment, histological subtype, scleral and/or optic nerve infiltration, as well as tumour necrosis. Additionally, detailed histological parameters, such as mitotic rate, presence of tumour-infiltrating lymphocytes (TILs), nuclear pseudoinclusions (NPIs), intranuclear grooves, multinucleated giant cells and haemorrhage, as well as tumour cell pigmentation level were considered. The largest basal diameter and thickness of the tumour were described in line with the guidelines of the American Joint Committee on Cancer (AJCC) [23].

Immunohistochemistry. Paraffin blocks with tissues of 158 primary uveal melanomas were cut with a microtome to prepare 4 μm-thick sections which were subsequently mounted on sialinized slides (Agilent DAKO, Santa Clara, CA, USA). The slides then underwent automated dewaxing, rehydration and heat-induced epitope retrieval with EnVision Target Retrieval Solution (Agilent DAKO) for 30 min at 97°C in PT Link Pre-Treatment Module for Tissue Specimens (DAKO). Automated immunohistochemical staining with anti-PLK-1 (rabbit monoclonal antibody, 208G4; #4513; dilution 1:100; Cell Signalling Technology, Danvers, MA, USA) was performed in Autostainer Link 48 (DAKO) and Liquid Permanent Red (Agilent DAKO) was utilized as a detection system. Human colorectal adenocarcinoma was stained as positive control. Negative controls were processed using FLEX Rabbit Negative Control, Ready-to-Use (Agilent DAKO) in place of the primary antibody.

Evaluation of PLK-1 expression. The expression of PLK-1 in UM cells (Fig. 1) was determined using the semi-quantitative method. The two IHC reaction parameters used were the percentage of cells with a positive cytoplasmic reaction (the percentage of reactive tissue) and the intensity of cytoplasmic PLK-1 reaction. The Remmele and Stegner semiquantitative immunoreactive score (IRS) was used to compute

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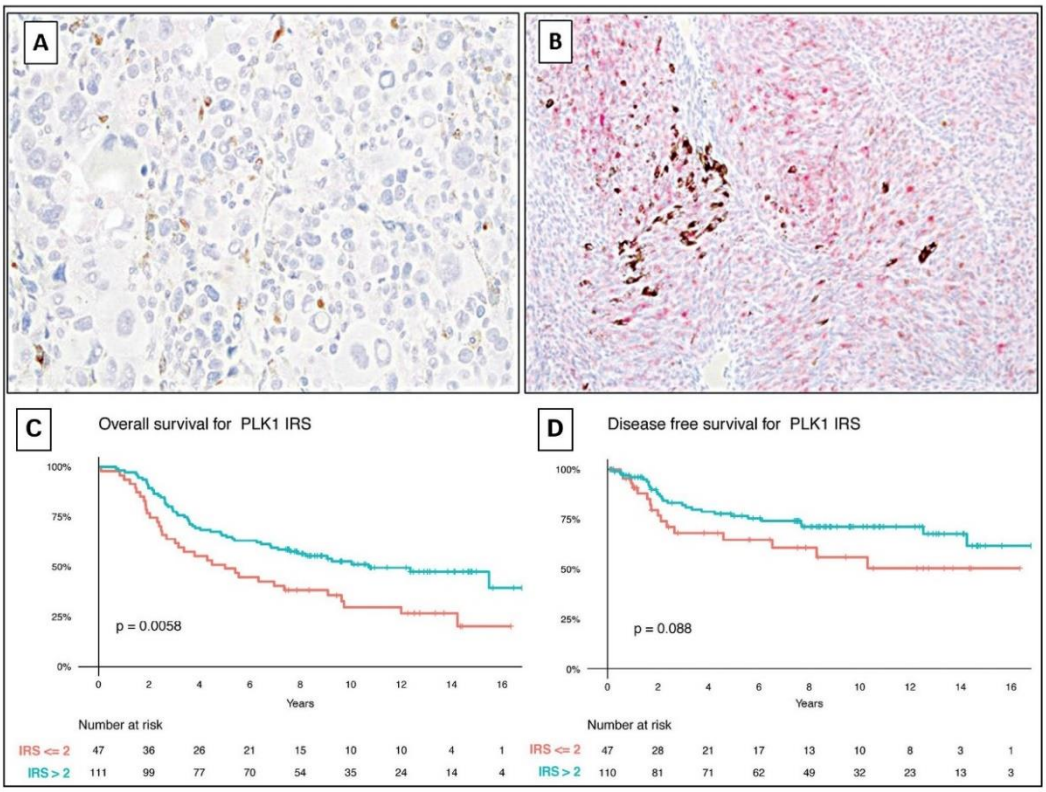

Figure 1. PLK-1 expression in uveal melanoma. **A.** Lack of PLK-1 immunoreactivity in neoplastic cells (400×). **B.** Enhanced expression of PLK-1 in uveal melanoma cells (200×). **C.** Kaplan-Meier analysis of the prognostic impact of PLK-1 expression in uveal melanoma patients. Downregulation of PLK-1 expression was significantly correlated with reduced overall survival ($p = 0.0058$). **D.** A similar trend as in (C), albeit not significant, was observed for disease-free survival ($p = 0.088$) (D).

the above parameters [24]. In the IRS, the percentage of reactive cells scores 0–4 points and staining intensity scores 0–3 points. The ultimate IRS is a product of multiplication of the above parameters, ranging between 0 and 12 points.

Tumoural pigmentation was assessed using a three-step scale: 0 – lack of melanin or melanin was present in < 10% of melanoma cells; 1 (low): melanin was present in 11–50% of melanoma cells; 2 (high): melanin was present in 51–100% melanoma cells.

Statistical analysis. Statistical analysis was performed using the R language [25] and the survminer tool [26]. For the purposes of correlation analysis, we assumed a dichotomous division of PLK-1 expression into low and high corresponding to semiquantitative IRS of ≤ 2 and > 2, respectively. In order to determine the overall survival (OS) and disease-free survival (DFS), Kaplan-Meier curves and the log-rank test were used; all analyses were carried out using the survival package for R [25, 26]. In order to determine the correlations between the PLK-1 expression and continuous variables, the Wilcoxon two-sample test was used. The correlations between PLK-1 expression and binary variables were determined using the Fisher's exact test while the correlations with other categorical variables were determined using the chi-square test. The p value below 0.05 was considered significant for all comparisons.

Results

PLK-1 immunoreactivity in uveal melanoma cells

High PLK-1 expression, defined as IRS > 2, was observed in 70% (111/158) of specimens, whereas low expression or no expression was seen in the remaining 30% (47/158) of specimens, including undetectable PLK-1 expression in 3.8% (6/158) of specimens (Fig. 1A–B). The mean IRS for PLK-1 expression in tumour cells was 4 (median: 4).

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Correlations of PLK-1 expression with clinical parameters

There was a significant inverse correlation between PLK-1 expression and the basal tumour diameter ($p = 0.044$). Similarly, there was a significant correlation between low PLK-1 expression and higher clinical tumour stage (pT, $p = 0.040$) as well as AJCC prognostic stage group ($p = 0.037$). Interestingly, high PLK-1 expression was associated with more advanced age of patients ($p = 0.0019$), whereas low PLK-1 expression was associated with a higher incidence of retinal detachment secondary to UM ($p = 0.0076$) (Table 1).

Correlations of PLK-1 expression with histological parameters

There was an inverse correlation between PLK-1 expression and tumour cell pigment content ($p = 0.0019$) and a positive correlation between PLK-1 expression and the presence of nuclear grooves ($p = 0.017$). On the other hand, low PLK-1 expression significantly correlated with the presence of nuclear pseudoinclusions (NPIs) ($p = 0.0071$). There was no significant correlation between PLK-1 expression and other histological parameters such as mitotic rate or histological subtype (Table 2).

The effect of PLK-1 expression on long-term survival

The Kaplan-Meier's analysis demonstrated that low PLK-1 expression was associated with significantly reduced overall survival ($p = 0.0058$). A similar trend, albeit not significant, was observed for disease-free survival ($p = 0.088$) (Fig. 1C–D).

Discussion

PLK-1 is a protein with important roles in the regulation of the cell cycle. It is physiologically strongly expressed in tissues undergoing intensive proliferation, such as testes, thymus, and spleen, or during proliferative events such as in developing embryos *etc*. [27]. Hence, the question follows whether high PLK-1 expression in tumour cells is associated with oncogenesis or intense cell proliferation. Over 25 years of PLK-1-related research, a number of papers have been published to characterise its mechanism of action, both in the cell cycle and in cellular response to DNA damage [28–30].

PLK-1 and the p53 tumour suppressor protein are closely related in an inhibitory feedback loop, which is the fundamental mechanism whereby PLK-1 participates in oncogenesis [28]. High PLK-1 expression leading to cell cycle acceleration was demonstrated in tumour cells lacking functional p53. However, overexpression of PLK-1 inhibits the effect of p53. As a result, the cell is incapable of apoptosis in response to DNA damage and continues to function with increasing genomic instability and aneuploidy [29, 31–36]. PLK-1 depletion breaks the vicious circle restoring the p53 function. Importantly, it also triggers tumour cell apoptosis whilst preserving normal cells [37–39]. Apart from interaction with p53, PLK-1 may regulate tumorigenesis by modulating Myc stability [40, 41] and affecting PTEN [42] as well as other tumour suppressors [43].

This provides the theoretical basis for the research of PLK-1 inhibitors, which block kinase domain or PBD [4]. One of them, volasertib, was granted a Breakthrough Therapy designation by the FDA [44] and reached Phase III of clinical trials in patients aged 65 years and above with previously untreated acute myeloid leukaemia [45, 46]. Nevertheless, despite expectations based on preclinical study findings, no significant clinical success of PLK-1 inhibitors has been reported to date [47]. The search for more selective inhibitors is ongoing, as kinases, including those of the PLK family, can often exert opposing effects on tumour development [27, 47]. Using PLK-1 inhibitors in combination therapy as agents reducing cancer resistance to other therapies, seems promising at the moment [46, 47].

As pharmaceutical companies and researchers have been trying to find a therapeutic use of PLK-1 inhibitors, the kinase has also sparked significant controversies [48]. While PLK-1 overexpression is linked to uncontrolled cell proliferation and impaired response to DNA damage, its low expression impairs cell cycle processes, such as spindle assembly or centrosome maturation, leading to tumour progression [30]. Recent studies in mice not only confirmed these findings, but also demonstrated the potential of PLK-1 as a tumour suppressor [49–52]. This inhibition effect is possible in interaction with specific oncogenes (such as K-Ras, Her2 or APC^{min}) and may be caused by up- or down-regulation of PLK-1 expression [43], both of which can induce genetic instability and aneuploidy. Hence, the outcomes are likely determined by other factors rather than a stand-alone PLK-1 expression level, such as oncogenesis, tumour progression or potential protective/repair mechanisms.

De Cárcer [43] analysed data from the Cancer Genome Atlas (TGCA) [53] and the Kaplan Meier Plotter database [54, 55], demonstrating that PLK-1 overexpression may lead to different outcomes depending on tumour type. For example, it was linked to shorter overall survival (OS) in patients with lung, bladder, and kidney clear cell carcinoma, whereas in patients with thymoma, lung squamous cell carcino-

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Table 1. Summary statistics for relation between expression of PLK-1 in uveal melanoma cells and clinical parameters

Clinical parameters	PLK-1 IRS		
	Low ≤ 2 (No. 47)	High > 2 (No. 111)	*p* value
Age in years (18–86)[a]	63 (58–72)	59 (51–64)	**0.0019**
Gender[c]			1.0
Female	24 (51%)	58 (52%)	
Male	23 (49%)	53 (48%)	
Side[c]			0.86
Right	22 (47%)	54 (49%)	
Left	25 (53%)	57 (51%)	
Largest basal tumour diameter (by AJCC)[b]			**0.044**
> 9–12 mm	2 (4%)	11 (10%)	
> 12–15 mm	3 (6%)	24 (22%)	
> 15–18 mm	13 (28%)	26 (23%)	
> 18 mm	29 (62%)	50 (45%)	
Greatest tumour height (by AJCC)[b]			0.75
≤ 3 mm	0 (0%)	1 (1%)	
> 3–6 mm	2 (4%)	12 (11%)	
> 6–9 mm	13 (28%)	28 (25%)	
> 9–12 mm	16 (34%)	40 (36%)	
> 12–15 mm	12 (26%)	24 (22%)	
> 15 mm	4 (9%)	6 (5%)	
Primary tumour (pT)[b]			**0.040**
2	1 (2%)	12 (11%)	
3	11 (23%)	39 (35%)	
4	35 (74%)	60 (54%)	
Stage[b]			**0.037**
IIA	0 (0%)	10 (9%)	
IIB	10 (21%)	33 (30%)	
IIIA	15 (32%)	37 (33%)	
IIIB	16 (34%)	26 (23%)	
IIIC	6 (13%)	5 (5%)	
Localization[b]			0.53
In front of the equator	39 (55%)	32 (49%)	
Equator	11 (15%)	8 (12%)	
Behind the equator	21 (30%)	25 (38%)	
Ciliary body involvement[c]			0.41
Ciliary body not involved	53 (63%)	56 (70%)	
Ciliary body involved	31 (37%)	24 (30%)	
Degree of pigmentation[b]			0.21
Amelanotic	4 (10%)	22 (21%)	
Mild pigmentation	16 (38%)	42 (39%)	
Intense pigmentation	22 (52%)	43 (40%)	
Shape[c]			0.73
Dome shape	23 (50%)	60 (55%)	
Mushroom shape	23 (50%)	50 (45%)	
Retinal detachment[c]			**0.0076**
No RD	3 (6%)	28 (25%)	
Coexistence of RD	44 (94%)	83 (75%)	
Glaucoma[c]			0.46
No glaucoma	39 (83%)	96 (87%)	
Coexistence of glaucoma	8 (17%)	14 (13%)	

[a]*p* value of Wilcoxon two sample test; [b]*p* value of chi^2 test; [c]*p* value of Fisher's exact test. Statistically significant results ($P < 0.05$) are shown in bold text.

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Table 2. Summary statistics for relation between expression of PLK-1 in uveal melanoma cells and histopathological parameters

Histopathological parameters	PLK-1 IRS		
	Low ≤ 2 (No. 47)	High > 2 (No. 111)	*p* value
Histologic subtype[a]			0.46
Spindle cell melanoma	6 (13%)	23 (21%)	
Mixed cell melanoma	34 (72%)	75 (68%)	
Epithelioid cell melanoma	7 (15%)	13 (12%)	
Mitotic rate[b]			0.47
0–4	32 (70%)	70 (63%)	
5–31	14 (30%)	41 (37%)	
Scleral infiltration[b]			0.16
None or intrascleral infiltration	44 (94%)	109 (98%)	
Full-thickness infiltration	3 (6%)	2 (2%)	
Invasion of the optic nerve[a]			0.59
No invasion	38 (81%)	91 (82%)	
Optic nerve head invasion	9 (19%)	17 (15%)	
Optic nerve invasion	0 (0%)	3 (3%)	
Necrosis[b]			0.60
No necrosis	39 (85%)	92 (88%)	
Necrosis present	7 (15%)	12 (12%)	
Marked pleomorphism[b]			0.57
No marked pleomorphism	41 (87%)	101 (91%)	
Marked pleomorphism present	6 (13%)	10 (9%)	
TILs[b]			0.44
No TILs	43 (91%)	96 (86%)	
TILs present	4 (9%)	15 (14%)	
Multinucleated giant cells[b]			0.54
No multinucleated giant cells	34 (72%)	86 (77%)	
Multinucleated giant cells present	13 (28%)	25 (23%)	
NPIs[b]			**0.0071**
No NPIs	21 (45%)	76 (68%)	
NPIs present	26 (55%)	35 (32%)	
Intranuclear grooves[b]			**0.017**
No intranuclear grooves	43 (91%)	82 (74%)	
Intranuclear grooves present	4 (9%)	29 (26%)	
Haemorrhage[b]			0.082
No haemorrhage	33 (70%)	93 (84%)	
Haemorrhage present	14 (30%)	18 (16%)	
Pigmentation[a]			**0.0019**
Lack of melanin	2 (4%)	12 (11%)	
Low pigmentation	18 (38%)	68 (61%)	
High pigmentation	27 (57%)	31 (28%)	

[a]*p* value of chi² test; [b]*p* value of Fisher's exact test. Statistically significant results ($P < 0.05$) are shown in bold font.

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ma or rectal adenocarcinoma, higher PLK-1 levels seemed to be associated with significantly longer OS [43]. Interestingly, PLK-1 overexpression did not affect survival prognosis in patients with ovarian cancer, stomach adenocarcinoma and cervical squamous cell carcinoma [43]. Nevertheless, the effect of PLK-1 expression on long-term follow-up in patients with uveal melanoma was not assessed in that study.

In our research, contrary to most mentioned above reports, indicating PLK-1 as a prognostic factor for poor prognosis, we observed high PLK-1 expression in smaller UM tumours and in patients with lower clinical tumour stage (pT and AJCC). Furthermore, the Kaplan-Meier survival analysis demonstrated that high PLK-1 expression was associated with significantly shorter overall survival, with a similar trend in disease-free survival.

PLK-1 is one of the 50 most overexpressed genes of primary cutaneous melanoma (CM) and its metastases as compared with melanocytic nevi [56]. The expression of PLK-1 is dynamically regulated during CM cell cycle and is vital for cell survival. The level of PLK-1 varies with tumour thickness and has prognostic value for CM. High PLK-1 expression was significantly correlated with unfavourable clinical outcome [20]. Also for thin melanomas (< 0.75 mm), which should have an excellent prognosis, high expression of PLK-1 is a reliable marker for identifying patients at high risk of metastasis [19]. Kinetochore complex component (NDC80), a downstream effector in the PLK-1 signalling pathways, involved in the occurrence of many tumours and highly expressed in a variety of cancer types, is also associated with poor overall survival in metastatic CM [57, 58]. Therefore, determining PLK-1 expression, in addition to the Breslow thickness, can help identify patients with aggressive tumours.

Specific inhibition of PLK-1 using the commercially available inhibitor BI 2536 leads to a dose- and time-dependent decrease in CM cell viability and induction of apoptosis [56]. Moreover it shows an additive effect with simultaneous inhibition of the mitogen-activated protein kinase (MAPK) signalling pathway or inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK). Therefore, combination of MAPK/MEK and PLK-1 inhibition could be a potentially attractive therapeutic strategy in CM [56, 59–61].

Unfortunately, many differences between CM and UM mean that other therapeutic strategies need to be sought in uveal melanoma. One of proposed explanations is ocular immune privilege, which may likely alter signalling pathways in UM compared to skin melanoma [62]. The studies assessing biological drugs in UM have not shown good results to date [62]. Although PLK-1 inhibitors appear promising in oncology, and PLK-1 has been identified as one of UM-specific therapeutic targets [22], our results support the need for multicentre studies on prognostic significance of PLK-1 expression in uveal melanoma and in vitro studies to determine the effect of inducing or inhibiting PLK-1 expression in UM cells.

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7. Podsumowanie i wnioski

Przedstawione w pracy doktorskiej badania miały na celu poszukiwanie nowych czynników prognostycznych w czerniaku błony naczyniowej oka.

Pierwszym artykułem stanowiącym element rozprawy doktorskiej jest praca przeglądowa, która pozwoliła na usystematyzowanie dostępnych danych na temat parametrów mających znaczenie rokownicze w tym typie nowotworu. Na tej podstawie dokonano wyboru dalszego kierunku badań i, po ich wykonaniu, przedstawiono wartość prognostyczną parametrów cytomorfologicznych jąderek jąder komórkowych oraz ekspresji kinazy serynowo-treoninowej PLK1 w UM.

Druga publikacja wchodząca w skład rozprawy doktorskiej obejmuje zagadnienia związane z wartością rokowniczą cech cytomorfologicznych jąderek jąder komórkowych. Wyboru ocenianych parametrów dokonano mając na uwadze praktyczną stronę badania podczas oceny preparatów w codziennej praktyce ośrodka leczącego pacjentów z UM. Ideą było sprawdzenie, czy uwzględnienie relatywnie prostych cech – obecności, wielkości i liczebności jąderek – w preparatach histopatologicznych barwionych standardową metodą z zastosowaniem hematoksyliny i eozyny, wniesie istotne dane do oceny rokowania w UM. Uzyskane wyniki pozwoliły na stwierdzenie, że co prawda sama obecność jąderek ma niewielkie znaczenie w tym kontekście, natomiast ich większy rozmiar i większa liczba już tak. Parametry te korelowały ze znanymi wcześniej, istotnymi dla przebiegu UM czynnikami jak: większa średnica podstawy guza (oba parametry), wyższy kliniczny stopień zaawansowania (stage) (oba parametry), wyższy stopień zaawansowania guza pierwotnego (pT) (większa liczba jąderek; dla makrojąderek obserwowano podobny trend, nie osiągający jednak poziomu istotności statystycznej), zajęcie ciała rzęskowego (makrąjąderka). W zakresie korelacji z parametrami histopatologicznymi stwierdzono, że obecność makrojąderek i mnogich jąderek związana jest istotnie z podtypem nabłonkowatokomórkowym guza pierwotnego, wysokim wskaźnikiem mitotycznym, pleomorfizmem, występowaniem wielojądrowych komórek olbrzymich oraz pseudoinkluzji wewnątrzjądrowych (NPIs), a także brakiem bruzd wewnątrzjądrowych. Większa zawartość barwnika w komórkach nowotworowych, którego znaczenie dla przebiegu UM podlega obecnie weryfikacji w licznych pracach, w powyższym badaniu była również związana w sposób istotny statystycznie z obecnością większych

i mnogich jąderek. Zarówno występowanie makrojąderek, jak i mnogich jąderek wiązały się z istotnie krótszym przeżyciem wolnym od choroby oraz przeżyciem całkowitym w czerniaku błony naczyniowej oka.

Trzecia publikacja składająca się na rozprawę doktorską jest związana z określeniem prognostycznego znaczenia ekspresji białka PLK1. Z dostępnej literatury wynika, że w większości nowotworów, w których wykazano znaczenie prognostyczne ekspresji PLK1, zwiększona jej zawartość w komórkach nowotworowych związana była z gorszym rokowaniem. Jest to tym bardziej oczekiwane, że PLK1 jest kinazą ściśle związaną z proliferacją komórek. Tymczasem w naszym badaniu wykazaliśmy, że w przypadku UM to niska ekspresja PLK1 związana jest z niektórymi znanymi negatywnymi czynnikami rokowniczymi (większa średnica podstawy guza pierwotnego, wyższy stopień zaawansowania klinicznego – pT i stage), a także istotnie krótszym przeżyciem całkowitym (w relacji z przeżyciem wolnym od choroby obserwowaliśmy podobny trend, ale nie uzyskujący poziomu istotności statystycznej). Wyniki te są zgodne z dostępnymi w literaturze pracami analitycznymi prowadzonymi z wykorzystaniem baz tkankowych licznych nowotworów (the Cancer Genome Atlas (TGCA), the Kaplan Meier ploter), które wskazują, że w większości z nich złe rokowanie wiąże się z nadekspresją PLK1, ale są i takie, jak np. gruczolakorak odbytnicy, grasiczak czy rak płaskonabłonkowy płuc, w których jest odwrotnie. Niewątpliwie uzyskane dane, uzasadniają dalsze prace nad rolą PLK1 w przebiegu UM i ostrożność w badaniach nad potencjalnym zastosowaniem inhibitorów PLK1 w leczeniu czerniaka błony naczyniowej oka.

Wnioski:

1. Obecność makrojąderek i mnogich jąderek w jądrach komórek to negatywne czynniki prognostyczne, których obecność jest istotnie skorelowana z gorszym rokowaniem pacjentów z czerniakiem błony naczyniowej oka.
2. Ocena morfologii jąderek w rutynowym barwieniu hematoksyliną i eozyną może być tanią i relatywnie prostą metodą uzyskania wiarygodnych informacji prognostycznych w UM.

3. Niska ekspresja PLK1 w komórkach nowotworowych guza pierwotnego UM jest związana z gorszą prognozą długoterminową pacjentów z czerniakiem gałki ocznej (wykazano istotnie krótszy czas przeżycia całkowitego u pacjentów z obniżoną ekspresją PLK1 w komórkach nowotworowych).

8. Załączniki

- Oświadczenia współautorów publikacji stanowiących podstawę pracy doktorskiej
- Opinia Komisji Bioetycznej
- Dorobek naukowy

Wrocław, 15.07.2020 r.

prof. dr hab. n. med. Agnieszka Hałoń
Katedra oraz Zakład Patomorfologii i Cytologii Onkologicznej
Uniwersytet Medyczny we Wrocławiu

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mój wkład polegał na współtworzeniu koncepcji artykułu, krytycznej recenzji i poprawkach manuskryptu.

prof. dr hab. n. med.
Agnieszka Hałoń
specjalista patomorfolog

Kraków, 15.07.2020 r.

dr n. med. Anna Markiewicz
Katedra i Klinika Okulistyki i Onkologii Okulistycznej
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Zakład Patomorfologii Klinicznej i Doświadczalnej
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Jolanta Orłowska-Heitz

Kraków, 15.07.2020 r.

prof. dr hab. n. med. Bożena Romanowska-Dixon
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Prof. dr hab. med. Bożena Romanowska-Dixon

Wrocław, 15.07.2020 r.

dr hab. n. med. Piotr Donizy
Katedra oraz Zakład Patomorfologii i Cytologii Onkologicznej
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dr hab. Przemysław Biecek
Wydział Matematyki, Informatyki i Mechaniki
Uniwersytet Warszawski

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dr hab. n. med. Piotr Donizy
specjalista patomorfolog
e-mail: pdonizy@usk.wroc.pl

Kraków, 15.07.2020 r.

dr n. med. Anna Markiewicz
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Katarzyna Kobylińska
Wydział Matematyki, Informatyki i Mechaniki
Uniwersytet Warszawski

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Przemysław Biecek

Kraków, 15.07.2020 r.

lek. Jolanta Orłowska-Heitzman
Zakład Patomorfologii Klinicznej i Doświadczalnej
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dr hab. n. med. Piotr Donizy
specjalista patomorfolog
PWZ 2086200 [illegible] 7.845.
e-mail: pdonizy@usk.wroc.pl

KOMISJA BIOETYCZNA
przy
Uniwersytecie Medycznym
we Wrocławiu
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OPINIA KOMISJI BIOETYCZNEJ Nr KB – 125/2018

Komisja Bioetyczna przy Uniwersytecie Medycznym we Wrocławiu, powołana zarządzeniem Rektora Uniwersytetu Medycznego we Wrocławiu nr 133/XV R/2017 z dnia 21 grudnia 2017 r. oraz działająca w trybie przewidzianym rozporządzeniem Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. (Dz.U. nr 47, poz. 480) na podstawie ustawy o zawodzie lekarza z dnia 5 grudnia 1996 r. (Dz.U. nr 28 z 1997 r. poz. 152 z późniejszymi zmianami) w składzie:

dr hab. Jacek Daroszewski (endokrynologia, diabetologia)
prof. dr hab. Krzysztof Grabowski (chirurgia)
dr Henryk Kaczkowski (chirurgia szczękowa, chirurgia stomatologiczna)
mgr Irena Knabel-Krzyszowska (farmacja)
prof. dr hab. Jerzy Liebhart (choroby wewnętrzne, alergologia)
ks. dr hab. Piotr Mrzygłód (duchowny)
mgr Luiza Műller (prawo)
dr hab. Sławomir Sidorowicz (psychiatria)
dr hab. Leszek Szenborn (pediatria, choroby zakaźne)
Danuta Tarkowska (pielęgniarstwo)
prof. dr hab. Anna Wiela-Hojeńska (farmakologia kliniczna)
dr hab. Andrzej Wojnar (histopatologia, dermatologia) przedstawiciel Dolnośląskiej Izby Lekarskiej)
dr hab. Jacek Zieliński (filozofia)

pod przewodnictwem
prof. dr hab. Jana Kornafela (ginekologia i położnictwo, onkologia)

Przestrzegając w działalności zasad Good Clinical Practice oraz zasad Deklaracji Helsińskiej, po zapoznaniu się z projektem badawczym pt.

pod przewodnictwem
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Przestrzegając w działalności zasad Good Clinical Practice oraz zasad Deklaracji Helsińskiej, po zapoznaniu się z projektem badawczym pt.

„Nowe histomorfologiczne i immunohistochemiczne czynniki prognostyczne w czerniaku gałki ocznej"

zgłoszonym przez **lek. Tomasza Berusa** zatrudnionego w Klinicznym Oddziale Okulistycznym 4 Wojskowego Szpitala Wojskowego z Polikliniką we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła wyrazić zgodę na przeprowadzenie badania w Katedrze i zakładzie Patomorfologii i Cytologii Onkologicznej UMed. pod nadzorem prof. dr hab. Agnieszki Halon – promotora i dr. Piotra Donizego – promotora pomocniczego **pod warunkiem zachowania anonimowości uzyskanych danych**.

Uwaga: Badanie to zostało objęte ubezpieczeniem odpowiedzialności cywilnej Uniwersytetu Medycznego we Wrocławiu z tytułu prowadzonej działalności:

Pouczenie: W ciągu 14 dni od otrzymania decyzji wnioskodawcy przysługuje prawo odwołania do Komisji Odwoławczej za pośrednictwem Komisji Bioetycznej UM we Wrocławiu

Opinia powyższa dotyczy: projektu badawczego będącego podstawą rozprawy doktorskiej

Wrocław, dnia 6 marca 2018 r.

BW

Uniwersytet Medyczny we Wrocławiu
KOMISJA BIOETYCZNA
przewodniczący
prof. dr hab. Jan Kornafel

Całkowity Impact Factor: 6.355
Całkowita liczba punktów MNiSW/KBN: 260.000

Tomasz Berus, Anna Turno-Kręcicka, Ewa Kwiatkowska.: Solar retinopathy.
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Pkt. MNiSW/KBN: 10.000

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Ewa Kwiatkowska, Anna Turno-Kręcicka, Tomasz Berus.: Cewkowo-śródmiąższowe zapalenie nerek i błony naczyniowej – opis przypadku [Tubulointerstitial nephritis and uveitis – case report]
Klin Oczna. 2016 Vol.118 no.2; s.147-150
Pkt. MNiSW/KBN: 10.000

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IF: 1.642; Pkt. MNiSW/KBN: 70.000

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Anticancer Res. 2017 Vol.37 no.12; s.6541-6549
IF: 1.865; Pkt. MNiSW/KBN: 20.000

Tomasz Berus, Anna Markiewicz, Przemysław Biecek, Jolanta Orłowska-Heitzman, Agnieszka Hałoń, Bożena Romanowska-Dixon, Piotr Donizy.: Clinical Significance of Nucleoli Cytomorphology Assessment in Patients With Uveal Melanoma
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Folia Histochem Cytobiol. 2020 Vol.58 no.2; s.108-116
IF: 0.854; Pkt. MNiSW/KBN: 70.000