UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

PRACA DOKTORSKA

***lek. Aneta Popiel-Kopaczyk***

# *Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych*

Promotor: prof. dr hab. Piotr Dzięgiel

Promotor pomocniczy: dr n. med. Christopher Kobierzycki

Wrocław 2023

NARODOWE CENTRUM NAUKI

Praca powstała w wyniku realizacji projektu badawczego o numerze STM.A110.17.024 finansowanego ze środków Uniwersytetu Medycznego we Wrocławiu oraz grantu naukowego PRELUDIUM o numerze 2018/29/N/NZ5/01911 finansowanego ze środków Narodowego Centrum Nauki.

„Jeśli widzę dalej to tylko dlatego, że stoję na ramionach olbrzymów”

Isaac Newton

*Pragnę złożyć serdeczne podziękowania Panu Prof. dr hab. Piotrowi Dzięgielowi*
*za niezachwianą wiarę we mnie, wsparcie oraz bycie moim mentorem.*

*Dziękuję Christopherowi Kobierzyckiemu za bycie nieocenionym promotorem pomocniczym.*
*Dziękuję Ci za merytoryczną pomoc, poświęcony czas przy redagowaniu prac*
*oraz cenne wskazówki.*

*Koleżankom i Kolegom z Zakładu Histologii i Embriologii*
*dziękuję za wsparcie i okazaną pomoc.*

*Dziękuję Rodzicom za wiarę w moje możliwości.*

*Specjalne podziękowania pragnę złożyć mojemu wspaniałemu mężowi.*

*Dziękuję Ci za Twoje nieustające zaangażowanie, wsparcie, motywację, uwagi oraz pomoc.*
*Nie udałoby mi się dokończyć tego przedsięwzięcia bez Ciebie.*

*Pracę tę dedykuję moim dzieciom Klarze oraz Janowi.*

# 1. SPIS TREŚCI

# 2. Wykaz publikacji stanowiących pracę doktorską

Poniżej przedstawiłam spis publikacji będących podstawą mojej pracy doktorskiej pt.: „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych". Sumaryczny **IF** cyklu zgodny z rokiem publikacji jest równy **16,767** a **punktacja MNiSW** wynosi **300 punktów**.

I. **Aneta Popiel***, Christopher Kobierzycki, Piotr Dzięgiel The role of Testin in Human Cancers.
*Pathology Oncology Research. 2019*; 25(4):1279-1284.
**IF:2,826; Pkt. MNiSW: 70**

II. **Aneta Popiel***, Aleksandra Piotrowska, Patrycja Sputa-Grzegrzółka, Beata Smolarz, Hanna Romanowicz, Piotr Dzięgiel, Marzenna Podhorska-Okołów, Christopher Kobierzycki. Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia.
*Biomedicines. 2021* Aug 13; 9(8):1010.
**IF: 4,757; Pkt. MNiSW: 20**

III. **Aneta Popiel-Kopaczyk,** Jędrzej Grzegrzółka, Aleksandra Piotrowska, Mateusz Olbromski, Beata Smolarz, Hanna Romanowicz, Agnieszka Rusak, Monika Mrozowska, Piotr Dzięgiel, Marzenna Podhorska-Okołów, Christopher Kobierzycki. The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics.
*Current Issues in Molecular Biology. 2023*; 45(1):490-500.
**IF: 2,976; Pkt. MNiSW: 70**

IV. **Aneta Popiel-Kopaczyk,** Aleksandra Piotrowska, Patrycja Sputa-Grzegrzółka, Beata Smolarz, Hanna Romanowicz, Piotr Dzięgiel, Marzenna Podhorska-Okołów, Christopher Kobierzycki. The Immunohistochemical Expression of Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer. *International Journal of Molecular Sciences. 2023*, 24, (9): 8063.
**IF: 6,208; Pkt. MNiSW: 140**

* Z dniem 15.10.2022 r. zmianie uległo moje nazwisko z Popiel na Popiel-Kopaczyk.

# 3. Wprowadzenie

Choroby nowotworowe zarówno w Polsce jak i na świecie stanowią jedną z podstawowych przyczyn zgonów. Na raka szyjki macicy zapadają głównie kobiety w wieku 35-44 lat [1]. Objawy kliniczne są niespecyficzne co znacząco utrudnia wczesną diagnostykę, a progresja nowotworu często jest szybka. Jego rozwój poprzedzony jest wystąpieniem śródnabłonkowej neoplazji szyjki macicy (ang. *Cervical Intraepithelial Neoplasia,* CIN). Zmiany przedinwazyjne zachodzą w zakresie strefy przejściowej tarczy i kanału szyjki macicy, wewnątrz nabłonka, nie przekraczając błony podstawnej. W zależności od nasilenia zaawansowania zmian wyróżniamy 3 stopnie śródnabłonkowej neoplazji: CIN1 (określana jako zmiany LSIL – ang. *Low-Grade Squamous Intraepithelial Lesion*), CIN2 oraz CIN3 (określane łącznie jako zmiany HSIL – ang. *High-Grade Squamous Intraepithelial Lesion*) [2]. Ryzyko rozwoju inwazyjnego raka szyjki macicy ze zmian HSIL waha się od 10-40% [3-5]. Według informacji Krajowego Rejestru Nowotworów Polska ma jeden z najniższych w Europie wskaźników 5-letnich przeżyć w raku szyjki macicy wynoszący 48,3% przy średniej europejskiej 62,1%. Czynniki, które mają wpływ na wyleczalność raka szyjki macicy to przede wszystkim: stopień zaawansowania klinicznego w chwili rozpoznania, stopień zróżnicowania histologicznego, głębokość naciekania oraz obecność przerzutów lokalnych i odległych [6-8].

Testyna jest białkiem obecnym w niemal wszystkich prawidłowych tkankach człowieka a jej ekspresja w komórce obserwowana jest zarówno w cytoplaźmie jak i błonie komórkowej, wzdłuż włókien aktyny. Testyna także zaangażowana jest w tworzenie ognisk przylegania. Jest zbudowana z 421 aminokwasów o masie około 47kDa. Składa się z trzech domen LIM (lin-1, ils-1 i mec-3), z których każda zawiera po dwa palce cynkowe. Domeny LIM pełnią funkcję modulatorów interakcji białek, m.in. cytoszkieletu takich jak zyksyna, talina czy aktyna [9]. Kodowana jest przez gen *TES* znajdujący się na chromosomie 7q31.2, który stanowi kruche miejsce oznaczone jako FRA7G.

W 2007 roku testyna zyskała na znaczeniu poprzez opublikowanie przez Boëda i wsp. potencjalnego mechanizmu jej funkcji supresorowej. Wykazano, że trzecia domena LIM testyny posiada większe powinowactwo do białka MENA (ang. *Mammalian-Enabled*) niż jej ligand, tym samym wypierając je z typowych lokalizacji subkomórkowych (ogniska przylegania, krawędź czołowa komórki). Odkrycie to było znaczące, biorąc pod uwagę, że białko MENA jest często nadekspresjonowane w komórkach nowotworowych i uważa się, że jest częściowo odpowiedzialne za mobilność komórek nowotworowych, a tym samym wpływa

na potencjał tworzenia przerzutów nowotworowych [10]. Z drugiej strony ekspresja testyny często jest nieobecna w komórkach nowotworowych [11-13].

W dostępnych bazach literaturowych nie odnajduje się prac analizujących ekspresję testyny w zmianach przedinwazyjnych i w raku szyjki macicy. Wobec trudności diagnostycznych oraz niezadowalających wyników leczenia pacjentek z wyżej wymienionymi nowotworami, poszukiwanie nowych markerów stanowi obiecujący obszar badań naukowych. Potencjalny związek testyny z markerami nowotworowymi jak również danymi kliniczno-patologicznymi wymaga dalszych badań.

Jednym z kluczowych zjawisk odpowiedzialnych za tworzenie się przerzutów jest proces przejścia epitelialno-mezenchymalnego (ang. E*pithelial-Mesenchymal Transition*, EMT). Podstawą tego procesu jest pozbawienie komórek łączności z innymi komórkami, zwiększenie ich mobilności oraz uzyskanie zdolności do inwazji. Jest to możliwe dzięki pozyskaniu przez komórkę fenotypu mezenchymalnego głównie poprzez zmianę profilu ekspresji specyficznych białek [14-15]. Czynnikami stymulującymi proces przejścia epitelialno-mezenchymalnego są czynniki transkrypcyjne m.in. TWIST, SNAIL oraz SLUG. Badania nad markerami EMT zyskują na popularności w związku z możliwością wykorzystania ich zarówno do badań podstawowych w celu lepszego zrozumienia biologii nowotworu, jak również dla celów praktyki klinicznej m.in. stanowiąc wskaźnik prognostyczno-predykcyjny czy też punkty uchwytu nowoczesnych terapii [16-17].

Pomimo poprawy sytuacji w zakresie diagnostyki oraz możliwości terapeutycznych rak szyjki macicy stanowi nadal istotny problem kliniczny, społeczny, a także ekonomiczny. Niekorzystne dane epidemiologiczne oraz brak dostatecznej wiedzy na temat biologii tego nowotworu wskazują na potrzebę poszukiwania nowych, czułych oraz specyficznych markerów prognostycznych oraz predykcyjnych raka szyjki macicy.

## Piśmiennictwo

[1] E. A. Stier *et al.*, "Cervical cancer incidence stratified by age in women with HIV compared with the general population in the United States, 2002-2016," *Aids*, vol. 35, no. 11, pp. 1851–1856, 2021, doi: 10.1097/QAD.0000000000002962.

[2] S. D. Balasubramaniam, V. Balakrishnan, C. E. Oon, and G. Kaur, "Key molecular events in cervical cancer development," *Med.*, vol. 55, no. 7, 2019, doi: 10.3390/medicina55070384.

[3] L. Tao *et al.*, "Prevalence and risk factors for cervical neoplasia: A cervical cancer screening program in Beijing," *BMC Public Health*, 2014, doi: 10.1186/1471-2458-14-1185.

[4] S. S. Raab, N. S. Bishop, and M. S. Zaleski, "Long-term outcome and relative risk in women with atypical squamous cells of undetermined significance," *Am. J. Clin. Pathol.*, vol. 112, no. 1, pp. 57–62, 1999, doi: 10.1093/ajcp/112.1.57.

[5] P. Naucler *et al.*, "Efficacy of HPV DNA testing with cytology triage and/or repeat HPV DNA testing in primary cervical cancer screening," *J. Natl. Cancer Inst.*, vol. 101, no. 2, pp. 88–99, 2009, doi: 10.1093/jnci/djn444.

[6] F. Landoni *et al.*, "Randomised study of radical surgery versus radiotherapy for stage Ib-IIa cervical cancer.," *Lancet (London, England)*, vol. 350, no. 9077, pp. 535–540, Aug. 1997, doi: 10.1016/S0140-6736(97)02250-2.

[7] M. Rotman *et al.*, "A phase III randomized trial of postoperative pelvic irradiation in Stage IB cervical carcinoma with poor prognostic features: follow-up of a gynecologic oncology group study.," *Int. J. Radiat. Oncol. Biol. Phys.*, vol. 65, no. 1, pp. 169–176, May 2006, doi: 10.1016/j.ijrobp.2005.10.019.

[8] G. Delgado, B. Bundy, R. Zaino, B. U. Sevin, W. T. Creasman, and F. Major, "Prospective surgical-pathological study of disease-free interval in patients with stage IB squamous cell carcinoma of the cervix: a Gynecologic Oncology Group study.," *Gynecol. Oncol.*, vol. 38, no. 3, pp. 352–357, Sep. 1990, doi: 10.1016/0090-8258(90)90072-s.

[9] A. Popiel, C. Kobierzycki, and P. Dzięgiel, "The Role of Testin in Human Cancers," *Pathol. Oncol. Res.*, vol. 25, no. 4, pp. 1279–1284, 2019, doi: 10.1007/s12253-018-0488-3.

[10] B. Boëda *et al.*, "Tes, a Specific Mena Interacting Partner, Breaks the Rules for EVH1 Binding," *Mol. Cell*, vol. 28, no. 6, pp. 1071–1082, 2007, doi: 10.1016/j.molcel.2007.10.033.

[11] H. Li, K. Huang, L. Gao, L. Wang, Y. Niu, and H. Liu, "TES inhibits colorectal cancer progression through activation of p38," *Oncotagret*, vol. 7, no. 29, 2016.

[12] Z. Gu *et al.*, "TESTIN suppresses tumor growth and invasion via manipulating cell cycle progression in endometrial carcinoma," *Med. Sci. Monit.*, vol. 20, pp. 980–987, 2014, doi: 10.12659/MSM.890544.

13] E. Gunduz *et al.*, "Downregulation of TESTIN and Its Association With Cancer History and a Tendency Toward Poor Survival in Head and Neck Squamous Cell Carcinoma," *Arch. Otolaryngol. Neck Surg.*, vol. 135, no. 3, p. 254, 2009, doi: 10.1001/archoto.2008.560.

[14] R. Qureshi, H. Arora, and M. A. Rizvi, "EMT in cervical cancer: Its role in tumour progression and response to therapy," *Cancer Lett.*, vol. 356, no. 2, pp. 321–331, 2015, doi: 10.1016/j.canlet.2014.09.021.

[15] M. Y. Lee and M. R. Shen, "Epithelial-mesenchymal transition in cervical carcinoma," *Am. J. Transl. Res.*, vol. 4, no. 1, pp. 1–13, 2012.

[16] D. A. I. Bingling, Y. U. Runze, F. A. N. Mengying, T. Yang, B. Wang, and Y. Zhang, "HMQ-T-F2 suppresses migration of the human cervical cancer HeLa cells by reversing EMT via the PI3K/Akt signaling pathway," *Oncol. Rep.*, vol. 42, no. 4, pp. 1451–1458, 2019, doi: 10.3892/or.2019.7245.

[17] B. Weadick *et al.*, "EMT-Induced Gemcitabine Resistance in Pancreatic Cancer Involves the Functional Loss of Equilibrative Nucleoside Transporter 1.," *Mol. Cancer Ther.*, vol. 20, no. 2, pp. 410–422, Feb. 2021, doi: 10.1158/1535-7163.MCT-20-0316.

# 4. Założenia i cel pracy

Testyna jest białkiem posiadającym zdolność do interakcji z innymi białkami cytoszkieletu pełniąc rolę regulatora mobilności komórek. Wykazano, że obniżona ekspresja testyny zmniejsza kontakt pomiędzy komórkami zwiększając ich ruchliwość. Takie obserwacje poczyniono m.in. dla nowotworów jajnika, prostaty, szyjki macicy, jelita grubego, żołądka, płuc, piersi. Brakuje jednak badań nad testyną oraz markerami przejścia epitelialno-mezenchymalnego w zmianach przednowotworowych i nowotworowych szyjki macicy.

**Założeniem niniejszej rozprawy było zbadanie nasilenia ekspresji testyny oraz markerów przejścia epitelialno-mezenchymalnego w odniesieniu do dostępnych danych kliniczno-patologicznych w zmianach przedinwazyjnych oraz w rakach szyjki macicy.**

Zestawienie publikacji użytych do niniejszej rozprawy doktorskiej:

Aneta Popiel, Christopher Kobierzycki, Piotr Dzięgiel **The role of Testin in Human Cancers**. *Pathology Oncology Research. 2019*; 25(4):1279-1284.

Celem pracy było usystematyzowanie oraz zebranie najnowszych doniesień literaturowych opisujących rolę testyny w chorobach nowotworowych.

Aneta Popiel, Aleksandra Piotrowska, Patrycja Sputa-Grzegrzółka, Beata Smolarz, Hanna Romanowicz, Piotr Dzięgiel, Marzenna Podhorska-Okołów, Christopher Kobierzycki. **Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia**. *Biomedicines. 2021* Aug 13; 9(8):1010

Aneta Popiel-Kopaczyk**,** Jędrzej Grzegrzółka, Aleksandra Piotrowska, Mateusz Olbromski, Beata Smolarz, Hanna Romanowicz, Agnieszka Rusak, Monika Mrozowska, Piotr Dzięgiel, Marzenna Podhorska-Okołów, Christopher Kobierzycki. **The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics**. *Current Issues in Molecular Biology. 2023*; 45(1):490-500.

Celem prac było określenie ekspresji testyny, jej lokalizacji i korelacji z markerami proliferacji komórek w zmianach przednowotworowych i nowotworowych szyjki macicy.

**Aneta Popiel-Kopaczyk,** Aleksandra Piotrowska, Patrycja Sputa-Grzegrzółka, Beata Smolarz, Hanna Romanowicz, Piotr Dzięgiel, Marzenna Podhorska-Okołów, Christopher Kobierzycki. The Immunohistochemical Expression of Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer. *International Journal of Molecular Sciences. 2023,* 24, (9): 8063.

Celem pracy była ocena nasilenia ekspresji markerów przejścia epitelialno-mezenchymalnego w zmianach przedinwazyjnych oraz w raku szyjki macicy.

# 5. Streszczenie

Pomimo obserwowanego od połowy lat 70. malejącego trendu umieralności z powodu raka szyjki macicy, na skutek wprowadzonych badań przesiewowych oraz szczepienia przeciwko wirusowi brodawczaka ludzkiego (ang. *Human Papilloma Virus, HPV*), wciąż stanowi on istotny problem społeczny, ekonomiczny i medyczny.

Jednym z kluczowych czynników rokowniczych w raku szyjki macicy jest stadium zaawansowania klinicznego w momencie rozpoznania. W przypadku rozpoznania we wczesnym stadium choroby, 5-letnie przeżycie wynosi 92%, natomiast jeśli nowotwór rozprzestrzenił się regionalnie na okoliczne tkanki i węzły chłonne, wówczas wskaźnik ten wynosi 58%, ale drastycznie spada do 18% w przypadku wystąpienia przerzutów odległych. Wczesne stadium choroby (IA-IB2 wg. FIGO 2018) ograniczone do szyjki macicy może być leczone radykalnym zabiegiem chirurgicznym niejednokrotnie z równoczesną chemioterapią. Postępowanie chirurgiczne zależne jest od stopnia zaawansowania klinicznego. Poczynając od zmian przednowotworowych typu HSIL leczenie opiera się o procedurę elektrokonizacji (ang. *Loop Electrosurgical Excision Procedure, LEEP*), natomiast w przypadku rozpoznania nowotworu złośliwego proponowaną procedurą jest histerektomia radykalna z obustronną resekcją przydatków wraz z ewentualną towarzyszącą limfadenektomią. Po samej operacji prawdopodobieństwo nawrotu wynosi co najmniej 30%. Przerzuty choć nie są częste przy pierwotnym rozpoznaniu, rozwijają się u 15% do 61% kobiet z rakiem szyjki macicy, zwykle w ciągu pierwszych dwóch lat od zakończenia leczenia [1-3].

Przeprowadzono wiele badań w celu lepszego poznania mechanizmów inicjacji, promocji i progresji raka szyjki macicy. Ryzyko rozwoju inwazyjnego raka szyjki macicy z HSIL wynosi około 20% (10-40% według literatury) [4-6]. Zjawisko przejścia epitelialno-mezenchymalnego będąc procesem wieloetapowym odgrywa ważną rolę na etapie progresji nowotworu, znacząco pogarszając rokowanie.

Kluczowymi białkami, które kontrolują mobilność i inwazyjność komórek w procesie EMT są kadheryny. Zmniejszenie ekspresji E-kadheryny prowadzi do utraty polaryzacji komórek i zmniejszenia ich adhezji. W wielu ludzkich nowotworach złośliwych obniżenie poziomu E-kadheryny wiąże się ze złym rokowaniem. Kilka ważnych czynników transkrypcyjnych takich jak SNAIL, SLUG i TWIST indukując EMT działa jako represory E-kadheryny. Wcześniejsze badania opisywały ekspresję markerów EMT w raku szyjki macicy, jednak nie prowadzono badań dotyczących ich ekspresji w zmianach przedinwazyjnych. Z uwagi na brak badań postanowiłam sprawdzić, czy istnieją jakiekolwiek różnice w ekspresji

markerów EMT pomiędzy zmianami przedinwazyjnymi a inwazyjnymi oraz w prawidłowym nabłonku szyjki macicy.

Testyna jest białkiem obecnym w niemal wszystkich prawidłowych tkankach człowieka a jej ekspresja w komórce obserwowana jest zarówno w cytoplaźmie jak i błonie komórkowej, wzdłuż włókien aktyny. Testyna zaangażowana jest także w tworzenie ognisk przylegania. Kodowana jest przez gen *TES* znajdujący się na chromosomie 7q31.2, który stanowi kruche miejsce oznaczone jako FRA7G. Wiele autorów wskazuje, że wyższa ekspresja testyny indukuje apoptozę, hamuje proliferację komórek nowotworowych oraz zatrzymuje komórki w fazie G1 cyklu komórkowego. Ponadto, niska ekspresja testyny koreluje z wyższym stopniem złośliwości histologicznej oraz jest niekorzystnym markerem prognostycznym. W dostępnych bazach literaturowych nie odnajduje się prac analizujących ekspresję testyny w zmianach przedinwazyjnych i w raku szyjki macicy. Wobec trudności diagnostycznych oraz niezadowalających wyników leczenia pacjentek, poszukiwanie nowych markerów stanowi obiecujący obszar prac badawczych. Potencjalny związek testyny z markerami nowotworowymi jak również danymi kliniczno-patologicznymi wymaga dalszych badań.

W pracy pt. *The role of Testin in Human Cancers* zebrałam wszystkie najnowsze badania naukowe dotyczące roli ekspresji testyny w nowotworach człowieka. Zaobserwowałam, że testyna wykazuje obniżoną ekspresję m.in. w nowotworach jajnika, żołądka, płuc, głowy i szyi. W oparciu o przeprowadzone badania wykazano, że *TES* może pełnić rolę genu supresorowego dla wielu nowotworów. Odkrycie to może być przydatne w zindywidualizowanej terapii nowotworów. Zrozumienie molekularnych mechanizmów kancerogenezy jest ważnym krokiem naprzód w rozszerzaniu możliwości stosowania nowoczesnych metod terapeutycznych. Co więcej, istnieją dowody sugerujące możliwość wykorzystania testyny jako markera prognostycznego. Jednak konieczne są dalsze badania w tym obszarze.

W pracy pt. *Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia,* z wykorzystaniem reakcji immunohistochemicznych (IHC) oceniłam nasilenie ekspresji testyny oraz p16 i Ki-67 - markerów kontrolujących cykl komórkowy oraz wskazujących na proliferację komórek. Grupę badawczą stanowiło 229 przypadków neoplazji śródnabłonkowej (CIN1 - 31 przypadków; CIN2 - 75; CIN3 - 123). Grupę kontrolną stanowił materiał prawidłowej tkanki szyjki macicy pacjentek, które przeszły całkowitą histerektomię z powodu mięśniaków macicy.

Wyniki przeprowadzonych badań poddałam analizie statystycznej, która wykazała istotnie wyższą ekspresję testyny w zmianach neoplazji śródnabłonkowej w porównaniu do kontroli ($p<0,0001$). Ponadto wykazałam, że ekspresja testyny była wyższa w grupie zmian HSIL niż LSIL ($p<0,0024$). Cytoplazmatyczna ekspresja testyny pozytywnie korelowała z ekspresją markera proliferacji komórkowej Ki-67 ($r=0,4209$; $p<0,0001$) oraz ekspresją białka p16 ($r=0,5681$; $p<0,0001$), które jest kluczowym białkiem regulującym prawidłowy przebieg cyklu komórkowego. W przeprowadzonym badaniu ekspresja Ki-67, p16 i testyny wzrastała stopniowo wraz ze stopniem zaawansowania zmian od LSIL do HSIL. Użycie w diagnostyce zmian przedinwazyjnych tych trzech markerów mogłoby poprawić czułość i swoistość badania.

W pracy pt. *The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics* wykonałam badania IHC na 91 przypadkach raka szyjki macicy, w tym 73 przypadkach raka płaskonabłonkowego. Ponadto przeprowadziłam badania metodą Western Blot (WB) na trzech liniach komórkowych raka szyjki macicy: HeLa, SiHa (HPV pozytywna), C-33A (HPV negatywna) oraz linii komórkowej ludzkich keratynocytów – HaCaT jako kontroli.

Wykazałam, że ekspresja testyny jest niższa w komórkach nowotworowych w porównaniu do prawidłowej tkanki szyjki macicy ($p<0,0113$). Ekspresja markerów Ki-67 ($p<0,0001$) oraz p16 ($p<0,0001$) była natomiast wyższa w grupie raków szyjki macicy. Analiza statystyczna wykazała, że testyna negatywnie koreluje z Ki-67 ($r = - 0.2359$; $p=0,0278$) oraz p16 ($r = - 0.2104$; $p<0,0465$). Analiza ekspresji testyny metodą WB we wszystkich liniach komórkowych raka szyjki macicy odpowiadała wynikom IHC i wykazała słabszą ekspresję w porównaniu z ekspresją w linii komórkowej HaCaT. Uzyskane wyniki mogą być związane z supresorowymi właściwościami testyny.

W pracy pt. *The Immunohistochemical Expression of Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer* wykonałam badania IHC na 124 przypadkach raka szyjki macicy oraz 229 przypadkach śródnabłonkowej neoplazji. Wykazałam, wyższą ekspresję markerów przejścia epitelialno-mezenchymalnego TWIST, SNAIL oraz SLUG w zmianach HSIL niż w LSIL. Najsilniejszą ekspresję TWIST, SNAIL oraz SLUG stwierdziłam w zmianach CIN3 *vs*. CIN1 oraz CIN2 (odpowiednio TWIST $p<0,0001$; SNAIL $p<0,0013$; SLUG $p<0,0001$). W raku szyjki macicy zaobserwowałam wyższą ekspresję wszystkich badanych markerów EMT w porównaniu do prawidłowej tkanki szyjki macicy ($p<0,0001$). Ponadto analiza statystyczna wykazała, że ekspresja TWIST, SNAIL oraz SLUG znacząco różniła się pomiędzy stopniem zaawansowania histologicznego i była niższa w G1 *vs.* G2 (dla TWIST $p<0,0011$; SNAIL $p<0,0017$; SLUG $p<0,0001$), a także G1 *vs.* G3 (dla TWIST $p<0,0029$; SNAIL $p<0,0005$; SLUG $p<0,0001$). Wykazane różnice w ekspresji

badanych markerów w zmianach przednowotworowych oraz nowotworowych szyjki macicy mogą być wykorzystane w diagnostyce tego nowotworu.

W oparciu o powyższe badania dotyczące testyny wykonane na zmianach śródnabłonkowej neoplazji i rakach szyjki macicy wykazałam stopniowy wzrost ekspresji testyny w śródnabłonkowej neoplazji oraz jej obniżoną ekspresję w raku szyjki macicy. Ponadto wykazałam korelację pomiędzy ekspresją testyny a stosowanymi powszechnie w diagnostyce markerami proliferacji Ki-67 oraz p16. Badanie obejmujące markery EMT pozwoliło wykazać udział tego procesu w kancerogenezie raka szyjki macicy. Bardziej wnikliwe badania dotyczące testyny oraz mechanizmu EMT pozwolą na rozwój nowych narzędzi diagnostyczno-terapeutycznych, a w przyszłości mogą mieć wpływ na tworzenie terapii personalizowanych.

## Piśmiennictwo


1. Landoni, F.; Maneo, A.; Colombo, A.; Placa, F.; Milani, R.; Perego, P.; Favini, G.; Ferri, L.; Mangioni, C. Randomised study of radical surgery versus radiotherapy for stage Ib-IIa cervical cancer. *Lancet* 1997, 350, 535–540.
2. Rotman, M.; Sedlis, A.; Piedmonte, M.R.; Bundy, B.; Lentz, S.S.; Muderspach, L.I.; Zaino, R.J. A phase III randomized trial ofpostoperative pelvic irradiation in stage IB cervical carcinoma with poor prognostic features: Follow-up of a gynecologic oncology group study. *Int. J. Radiat. Oncol. Biol. Phys*. 2006, 65, 169–176. [CrossRef]
3. Delgado, G.; Bundy, B.; Zaino, R.; Sevin, B.-U.; Creasman, W.T.; Major, F. Prospective surgical-pathological study of disease-free interval in patients with stage IB squamous cell carcinoma of the cervix: A Gynecologic Oncology Group study. *Gynecol. Oncol*. 1990, 38, 352–357
4. C. Rosty, D.G. Hewett, I.S. Brown, B. a Leggett, V.L.J. Whitehall, Serrated polyps of the large intestine: current understanding of diagnosis, pathogenesis, and clinical management., *J. Gastroenterol*. 48 (2013) 287–302. https://doi.org/10.1007/s00535-012-0720-y.
5. Y. Ge, P.A. Christensen, E. Luna, D. Armylagos, J. Xu, J.W. Hsu, H. Zhou, M.R. Schwartz, D.R. Mody, Age-specific 3-year cumulative risk of cervical cancer and high-grade dysplasia on biopsy in 9434 women who underwent HPV cytology cotesting, *Cancer Cytopathol.* 127 (2019) 757–764. https://doi.org/10.1002/cncy.22192.
6. S.S. Raab, N.S. Bishop, M.S. Zaleski, Long-term outcome and relative risk in women with atypical squamous cells of undetermined significance, *Am. J. Clin. Pathol.* 112 (1999) 57–62. https://doi.org/10.1093/ajcp/112.1.5

# 6. Summary

Despite the trend of declining mortality from cervical cancer since the mid-1970s thanks to increased screening and the Human Papilloma Virus (HPV) vaccine, it is still a significant social, economic and medical problem.

One of the key prognostic factors in cervical cancer is the clinical stage at the time of diagnosis. If diagnosed at an early stage of the disease, the 5-year survival rate is 92%, however, if the cancer has spread regionally to surrounding tissues and lymph nodes, then the rate is 58%, but drops dramatically to 18% if distant metastasis occurs. Early stage disease (IA-IB2 according to FIGO 2018) confined to the cervix can be treated with radical surgery that may be accompanied by concurrent chemotherapy in specific cases. Surgical management depends on the clinical stage. Starting with premalignant lesions of the HSIL type, treatment is based on the Loop Electrosurgical Excision Procedure (LEEP), while in the case of a diagnosis of malignancy, the proposed procedure is radical hysterectomy with bilateral resection of the adnexa with possible accompanying lymphadenectomy. After surgery alone, the probability of recurrence is at least 30%. Metastasis, although not common at initial diagnosis, develops in 15% to 61% of women with cervical cancer, usually within the first two years of treatment [1-3].

Many studies have been conducted to better understand the mechanisms of cervical cancer initiation, promotion and progression. The risk of developing invasive cervical cancer with HSIL is about 20% (10-40% according to the literature) [4-6]. The phenomenon of epithelial-mesenchymal transition being a multistage process plays an important role at the stage of cancer progression, significantly worsening the prognosis of the patient.

The key proteins that control cell mobility and invasiveness in EMT are cadherins. Decreased E-cadherin expression leads to loss of cell polarity and reduced cell adhesion. In many human malignancies, decreased levels of E-cadherin are associated with a poor prognosis. Several important transcription factors such as SNAIL, SLUG and TWIST induce EMT by acting as repressors of E-cadherin. Previous studies have described the expression of EMT markers in cervical cancer, but there were no studies on their expression in pre-invasive lesions. Given the lack of studies, we decided to check if there were any differences in the expression of EMT markers between pre-invasive lesions, cervical cancer and normal cervical epithelium.

Testin is a protein present in almost all normal human tissues and its expression in the cell is observed both in the cytoplasm and the cell membrane, along actin filaments. Testin is also involved in the formation of adhesion foci. It is encoded by the TES gene located on

chromosome 7q31.2, which is a fragile site designated FRA7G. Many authors indicate that higher expression of testin induces apoptosis, inhibits tumor cell proliferation and arrests cells in the G1 phase of the cell cycle. In addition, low testin expression correlates with a higher degree of histological malignancy and is an unfavorable prognostic marker. The available literature databases do not find papers analyzing testin expression in pre-invasive lesions and cervical cancer. In view of the diagnostic difficulties and unsatisfactory results in patients, the search for new markers is a promising area of research work. The potential association of testin with tumor markers as well as clinicopathological data requires further research.

In the study *The role of Testin in Human Cancers*, I analysed the latest scientific studies on the role of testin expression in human cancers. I observed that testin shows weaker expression in ovarian, gastric, lung, head and neck cancers, among others. Based on the study, it was shown that *TES* may act as a tumor suppressor gene for many cancers. This discovery may be useful for individualized cancer therapy. Understanding the molecular mechanisms of carcinogenesis is an important step forward in expanding treatment options, such as the use of novel therapeutic methods. Moreover, there is evidence to suggest the possibility of using testin as a prognostic marker. However, further research is needed in this area.

In the paper titled *Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia* by using of immunohistochemical (IHC) reactions, I assessed the expression of testin and p16, Ki-67 - markers that control the cell cycle and indicate cell proliferation. The study group consisted of 229 cases of intraepithelial neoplasia (CIN1 - 31 cases; CIN2 - 75; CIN3 - 123). The control group consisted of normal cervical tissue of patients who underwent total hysterectomy for myomas.

The statistical analysis was performed, which showed significantly higher expression of testin in lesions of intramural neoplasia compared to controls ($p<0,0001$). In addition, I showed that testin expression was higher in the HSIL lesion group than in LSIL ($p<0,0024$). Cytoplasmic testin expression positively correlated with the expression of the cell proliferation marker Ki-67 ($r=0,4209$; $p<0,0001$) and the expression of p16 protein ($r=0,5681$; $p<0.0001$), which is a key protein regulating the normal course of the cell cycle. In the conducted study, the expression of Ki-67, p16 and testin increased progressively with the stage of the lesions from LSIL to HSIL. The use of these three markers in the diagnosis of pre-invasive lesions could improve the sensitivity and specificity of the test.

In my work entitled *The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics*, I performed IHC testing on 91 cases of cervical cancer, including 73 cases of squamous cell carcinoma. I performed Western Blot testing on three cervical cancer cell lines:

HeLa, SiHa (HPV positive), C-33A (HPV negative) and the human keratinocyte cell line - HaCaT as a control.

I showed that testin expression is lower in tumor cells compared to normal cervical tissue ($p<0,0113$). In contrast, the expression of the markers Ki-67 ($p<0,0001$) and p16 ($p<0.0001$) was higher in the cervical cancer group. Statistical analysis showed that testin negatively correlates with Ki-67 ($r = - 0,2359$; $p=0,0278$) and p16 ($r = - 0,2104$; $p<0,0465$). WB analysis of testin expression in all cervical cancer cell lines matched the IHC results and showed weaker expression compared to that in the HaCaT cell line. The results obtained may be related to the suppressive properties of testin.

In my study entitled *The Immunohistochemical Expression of Epithelial-Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer*, I performed IHC studies on 124 cases of cervical cancer and 229 cases of intraepithelial neoplasia. I showed that the epithelial-mesenchymal transition markers TWIST, SNAIL and SLUG had higher expression in HSIL lesions than in LSIL. The strongest expression of TWIST, SNAIL and SLUG was in CIN3 vs. CIN1 and CIN2 (correspondingly TWIST $p<0,0001$; SNAIL $p<0,0013$; SLUG $p<0,0001$). Higher expression of all tested EMT markers was observed in cervical cancer compared to normal cervical tissue ($p<0,0001$). In addition, statistical analysis revealed that the expression of TWIST, SNAIL and SLUG significantly differed between histological grades and was weaker in G1 vs. G2 (for TWIST $p<0,0011$; SNAIL $p<0,0017$; SLUG $p<0,0001$) and also G1 vs. G3 (for TWIST $p<0,0029$; SNAIL $p<0,0005$; SLUG $p<0,0001$). The results of the study clearly showed that the existing differences in the expression of the tested markers in precancerous lesions and cervical cancer can be used in the diagnosis of this cancer.

Based on the above studies on testin performed on lesions of intraepithelial neoplasia and cervical cancer, I showed a gradual increase in testin expression in intraepithelial neoplasia and its decreased expression in cervical cancer. In addition, a correlation between testin expression and the proliferation markers Ki-67 and p16, which are commonly used in diagnosis was revealed. A study involving EMT markers allowed me to demonstrate the involvement of this process in the carcinogenesis of cervical cancer. More in-depth studies of testin and the mechanism of EMT may lead to the development of new diagnostic and therapeutic tools which in the future. Such findings could be used in the creation of targeted and individualized therapies.

**References**

1. Landoni, F.; Maneo, A.; Colombo, A.; Placa, F.; Milani, R.; Perego, P.; Favini, G.; Ferri, L.; Mangioni, C. Randomised study of radical surgery versus radiotherapy for stage Ib-IIa cervical cancer. *Lancet* 1997, 350, 535–540.
2. Rotman, M.; Sedlis, A.; Piedmonte, M.R.; Bundy, B.; Lentz, S.S.; Muderspach, L.I.; Zaino, R.J. A phase III randomized trial ofpostoperative pelvic irradiation in stage IB cervical carcinoma with poor prognostic features: Follow-up of a gynecologic oncology group study. *Int. J. Radiat. Oncol. Biol. Phys*. 2006, 65, 169–176. [CrossRef]
3. Delgado, G.; Bundy, B.; Zaino, R.; Sevin, B.-U.; Creasman, W.T.; Major, F. Prospective surgical-pathological study of disease-free interval in patients with stage IB squamous cell carcinoma of the cervix: A Gynecologic Oncology Group study. *Gynecol. Oncol*. 1990, 38, 352–357
4. C. Rosty, D.G. Hewett, I.S. Brown, B. a Leggett, V.L.J. Whitehall, Serrated polyps of the large intestine: current understanding of diagnosis, pathogenesis, and clinical management., *J. Gastroenterol*. 48 (2013) 287–302. https://doi.org/10.1007/s00535-012-0720-y.
5. Y. Ge, P.A. Christensen, E. Luna, D. Armylagos, J. Xu, J.W. Hsu, H. Zhou, M.R. Schwartz, D.R. Mody, Age-specific 3-year cumulative risk of cervical cancer and high-grade dysplasia on biopsy in 9434 women who underwent HPV cytology cotesting, *Cancer Cytopathol*. 127 (2019) 757–764. https://doi.org/10.1002/cncy.22192.
6. S.S. Raab, N.S. Bishop, M.S. Zaleski, Long-term outcome and relative risk in women with atypical squamous cells of undetermined significance, *Am. J. Clin. Pathol*. 112 (1999) 57–62. https://doi.org/10.1093/ajcp/112.1.5

# 7. Publikacje

Publikacje będące podstawą mojej pracy doktorskiej:

I. **Aneta Popiel***, Christopher Kobierzycki, Piotr Dzięgiel The role of Testin in Human Cancers.
*Pathology Oncology Research. 2019*; 25(4):1279-1284.
**IF:2,826; Pkt. MNiSW: 70**

II. **Aneta Popiel***, Aleksandra Piotrowska, Patrycja Sputa-Grzegrzółka, Beata Smolarz, Hanna Romanowicz, Piotr Dzięgiel, Marzenna Podhorska-Okołów, Christopher Kobierzycki. Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia.
*Biomedicines. 2021*; 9(8):1010.
**IF: 4,757; Pkt. MNiSW: 20**

III. **Aneta Popiel-Kopaczyk,** Jędrzej Grzegrzółka, Aleksandra Piotrowska, Mateusz Olbromski, Beata Smolarz, Hanna Romanowicz, Agnieszka Rusak, Monika Mrozowska, Piotr Dzięgiel, Marzenna Podhorska-Okołów, Christopher Kobierzycki. The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics.
*Current Issues in Molecular Biology. 2023*; 45(1):490-500.
**IF: 2,976; Pkt. MNiSW: 70**

IV. **Aneta Popiel-Kopaczyk,** Aleksandra Piotrowska, Patrycja Sputa-Grzegrzółka, Beata Smolarz, Hanna Romanowicz, Piotr Dzięgiel, Marzenna Podhorska-Okołów, Christopher Kobierzycki. The Immunohistochemical Expression of Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer. *International Journal of Molecular Sciences. 2023;* 24, (9): 8063.
**IF: 6,208; Pkt. MNiSW: 140**

Pathology & Oncology Research
https://doi.org/10.1007/s12253-018-0488-3

REVIEW

# The Role of Testin in Human Cancers

Aneta Popiel[1,2] · Christopher Kobierzycki[1] · Piotr Dzięgiel[1]

Received: 29 November 2017 / Accepted: 10 October 2018


**Abstract**
Testin is a protein expressed in almost all normal human tissues. It locates in the cytoplasm along stress fibers being recruited to focal adhesions. Together with zyxin and vasodilator stimulated protein it forms complexes with various cytoskeleton proteins such as actin, talin and paxilin. They jointly play significant role in cell motility and adhesion. In addition, their involvement in the cell cycle has been demonstrated. Expression of testin protein level correlates positively with percentage of cells in G1 phase, while overexpression can induce apoptosis and decreased colony forming ability. Decreased testin expression associate with loss by cells epithelial morphology and gain migratory and invasive properties of mesenchymal cells. Latest reports indicate that *TES* is a tumor suppressor gene which can contribute to cancerogenesis but the mechanism of loss *TES* gene expression is still unknown. Some authors point out hypermethylation of the CpG island as a main factor, however loss of heterozygosity may also play an important role [4, 5]. The altered expression of testin was found in malignant neoplasm, *i.a.* ovarian, lung, head and neck squamous cell cancer, breast, endometrial, colorectal, prostate and gastric cancers [1–9]. Testin participate in the processes of tumor growth, angiogenesis, and metastasis [10]. Many researchers stated involvement of testin in tumor progression, what suggest its potential usage in immunotherapy [7, 11]. Understanding the molecular functions of testin may be crucial in development personalized treatment. In the present manuscript up-to-date review of literature can be found.

**Keywords** Testin · Cancerogenesis · Breast cancer · Ovarian cancer · Gastrointestinal cancers

## Introduction

**Testin in Physiology** Testin is a protein with a molecular mass 47 kDa encoded by *TES* gene located on the fragile site FRA7G at 7q31.2 [6]. It is localized in the cytoplasm, along stress fibers and is recruited to focal adhesions [12]. The protein is composed of N-terminal PET (Prickle, Espinas, *TES*) and C-terminal three LIM (lin–11, isl-1, mec-3) domains [13]. At its COOH terminal (Fig. 1), the testin protein has three zinc-binding domain linked by two amino-acids spacer which play role in focal adhesion. LIM family proteins have been found to be a part of cytoskeleton [14]. They are responsible for protein-protein interactions coordinating signaling intracellular pathways [15, 16]. The N-terminal and C-terminal halves of the protein can interact with each other, hence hindering interaction with other cytoskeleton associated protein such as: zyxin, vasodilator stimulated protein (VASP), talin, nuclear actin related protein (Arp7A) and actin [17]. They are together acting as integrating partners in focal adhesion. Coutts et al. noticed that fibroblasts stably overexpressing TES have an increased ability to spread, are larger and contain increased numbers of actin protrusions [16]. Testin protein overexpression had effect on cells spreading potential not on percentage of cells spread. In view of described built, testin plays significant role in the cell adhesion, cell spreading and in the reorganization at the actin cytoskeleton [5, 18, 19].

**Testin in Pathology** Molecular studies characterized *TES* as tumor suppressor gene and reported its downregulation in many human malignancies [1–3, 6–8, 20]. In 2007 Boëda et al. presented for the first time interaction between LIM3 domain of testin protein and Mena protein which is key modulator of cellular migration [18]. Moreover, it was shown, that decreased expression of testin protein increased cell motility, decreased cell-cell contact and therefore have potential to be a marker of cancer metastasis [4, 21, 22].

✉ Aneta Popiel
aneta.popiel@live.com

[1] Division of Histology and Embryology, Department of Human Morphology and Embryology, Wroclaw Medical University, Wroclaw, Poland

[2] Wroclaw Medical University, Wroclaw, Poland

Published online: 25 October 2018

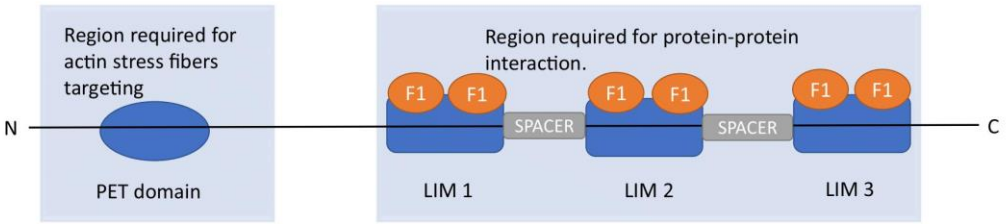


**Fig. 1** The structure of the testin protein

TES encodes testin protein containing a PET domain at the NH2- terminus which is involved in actin stress fibers targeting, and three LIM ((lin-1, ils-1 and mec-3) domains (LIM1, LIM2, LIM3) at the COOH-terminus. One LIM domain contain loosely conserved cysteine-rich consensus sequence including two separate zinc fingers-F1. They are separated from each other by SPACER.

Cancers are the only investigated diseases with *TES* gene disruptions. It is silencing promotes cell proliferation, invasiveness ability and angiogenesis [23, 24]. Important ways of *TES* inactivation are mechanisms of loss of heterozygosity (LOH) and hypermethylation (HMT). In LOH one or two alleles of the same gene are lost, whereas in HMT occurs abnormal DNA methylation which may inactivate suppressor genes. This phenomena were described in almost every type of cancer. Predominantly, in performed studies decreased *TES* gene expression associated with HMT of CpG islands nor LOH of chromosome 7q31 was found [3, 19, 25]. In addition, methylation of *TES* promoter region was described in various tumor types. Tobias et al. showed methylation of the CpG islands at the 5′ end in many types of tested tumor-derived cell lines [26]. Tatarelli et al. found fully methylated *TES* promoter in 1/10 breast, 1/8 pancreatic and 9/18 leukemia cell lines [19]. According to Ma et al. methylation of CpG in the *TES* promoter inactivate gene. Moreover, it was revealed that treatment with 5-aza-2'deoxycytidine (DAC), inhibitor of DNA methyltransferase activity, switched completely methylated *TES* promoter into partially or even fully unmethylated region in gastric cancer cell lines [3]. Upregulation of *TES* gene expression after treatment with DAC in glioblastoma cells confirmed that HMT play significant role in *TES* regulation, being responsible for gene silencing [27]. Only one study presented contrary results. Han et al. indicated overexpression of *TES* in GTL-16 gastric cancer cell line [28]. High frequency of LOH was found at 7q31 region in primary gastric cancer, they identified D7S486 to be the most frequent LOH locus [3]. As it was anticipated, there is an evidence presence of LOH in 7q31.2 in many types of neoplasms, e.g. ovary, breast, colorectal, gastric, head and neck, prostate, thyroid, pancreatic and kidney cancer as well in leukemias [29–37]. Ma et al. presented correlation between LOH presence and lack of testin protein expression in gastric cancer [1, 3]. Results are unequivocal as Chene et al. did not disclose such correlations in prostate cancer [4].

**Testin - Epigenetic Modifications, Copy Number Alterations and Mutations Based on Available Database (GDC Data Portal, NGS Data, GEO)** According to GDC Data Portal which analyzed 10,202 cases they identified 105 cases with different type of cancers (Fig. 2).

Analyzed patients was affected by 114 mutations of TES gene described in 21 projects. The largest number of cases was covered by endometrial carcinoma (42 cases), colorectal cancer (12) and breast cancer (7). During the process of transcription was identified missense, stop gained and frameshift mutations (Fig. 3).

The most frequent type of somatic mutation was substitution, described in 91 cases. Two authors cloned and described human TES gene. Tatarelli et al. determined that inactivation of TES is caused by methylation of CpG islands and revealed 3 missense mutation in 26 tumor cell lines [19]. In 2001 Tobias et al. showed also frequent occurrence of methylation of the CpG island at the 5-prime end in ovarian cancer and tumor-derived cell lines [26]. Research on TES gene revealed that TES gene may represent tumor supressor gene.

## Role of Testin in Cancers

**Ovarian Cancer** According to Knudson's hypothesis, HMT can be the "second hit" in tumor suppressor genes inactivation [38]. Previous studies showed also high frequency of *TES* gene HMT in the cancer cells [26]. Upregulation of *TES* gene by DAC in ovarian cancer cell line induces cell apoptosis and reduces colony formation preventing from rapid grow of cancer cells [1, 19, 27]. Qui et al. analyzed *TES* expression in regard HMT and LOH using microsatellite analysis and methylation-specific PCR (MS-PCR) in epithelial ovarian cancer cell lines (SKOV3 and A2780) and ovarian cancer samples. Additionally, they demonstrated by immunohistochemistry (IHC) weak testin expression in cancer samples and strong in normal ovarian tissues [1]. Mentioned study is the only one describing role of testin gene and protein in ovarian cancer. Authors did not correlate disclosed expressions with clinicopathological data.

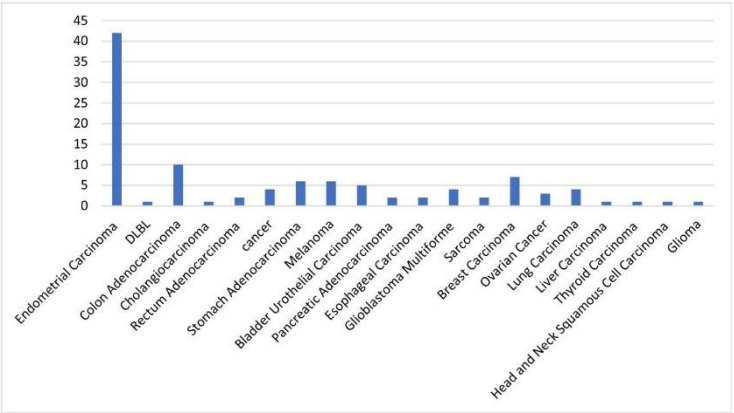


**Fig. 2** Number of cases and type of cancer affected by TES mutations

**Breast Cancer** Zhu et al. conducted studies analyzing association between *TES* gene expression and cell migratory potential tested by Transwell chamber assay as well their invasiveness (expression of matrix metalloproteinase-2; MMP-2) and angiogenesis (expression of CD34, marker of angiogenesis) by IHC. In the perspective of recognized markers they correlated with them *TES* expression. It seems that *TES* play important role not only in tumor formation but also in angiogenesis and metastasis. *TES* gene expression inversely correlates with expressions of MMP-2 and CD34 [24, 39, 40]. Expression of MMP-2 is regulated by miR-29b. Inhibition of MMP-2 through miR-29b can suppress tumor invasion, angiogenesis and metastasis [41]. Interaction between MMP-2 and miR-29b may be useful as therapeutic target for breast cancer (BC). Sarti et al. studied IHC expression of testin in BC patients in a view of their molecular subtype (luminal A, luminal B, basal-like called triple-negative and normal-like BC). Expression of testin was decreased in 74.7% of studied samples, whereas statistically significant downregulation of testin expression was observed in triple-negative and luminal B subtypes. Furthermore, frequency of HMT of CpG in testin varied between BC subtypes and was the highest in luminal B subtype [42]. Consequently, a different correlation levels between testin protein vs. triple-negative and luminal B subtypes may be the result of different grades of HMT of CpG in *TES* promoter region [6, 19, 26]. Low level of testin protein correlates with higher grades of histological malignancy and is unfavorable prognostic marker [25]. Zhu et al. demonstrated in breast cancer (BC) correlation between low *TES* gene expression and shortened survival rates e.g. breast relapse-free, cause-specific, distant metastasis free and overall survival [21].

**Endometrial Cancer** Dong et al. showed loss of *TES* gene expression in endometrial tissue using PCR. They hypothesized HMT as a main regulator of *TES* expression. Overexpressed *TES* gene significantly induce apoptosis, reduce cell proliferation and arrest cells in G1 phase of cell cycle [2]. Additionally, Gu et al. measured *TES* gene expression by PCR in five endometrial cancer cell lines (AN3CA, Ishikawa, KLE, ECC-1, HEC-1A). They presented influence of *TES* gene on MMP-2. which may control cellular invasion [20]. On the other hand in cancer progression important role plays

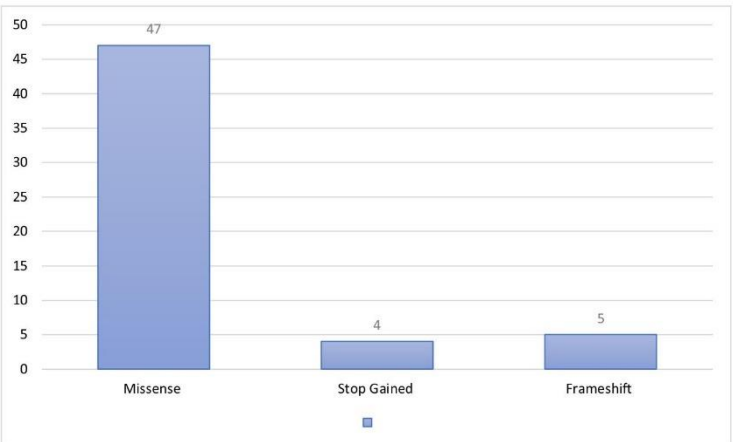


**Fig. 3** Shown the incidence of types of mutations with a predominance of missense mutations (47)

epithelial-mesenchymal transition (EMT). During this process biology and structure of epithelial cells is switched into features found in mesenchymal ones, i.e. cells lose their polarity, cell-cell adhesion and gain migratory and invasive properties [43]. Downregulation of *TES* gene was observed with decreased expression of epithelial marker- E-cadherin and increased expression of mesenchymal markers: N-cadherin, vimentin and snail. Presence of EMT markers expression significantly correlated with poor outcomes and invasiveness of cancer cell [2]. Immunoexpression of testin was various in different clinical stages and histological grades. IHC analysis revealed decreased testin expression in endometrial cancer cases compared to the adjacent normal endometrium. Decreased expression of testin protein correlated with advanced tumor stage, high grade and lymphatic vascular space invasion. No correlation between testin protein expression and patient age, pathologic types, myometrial invasion and lymphatic metastasis was shown [20].

**Colorectal Cancer** Li et al. described decreased expression of testin gene and protein in colorectal cancer (CRC). Additionally, they pointed correlation between low testin gene and protein expression and cell migratory as well invasive properties [5]. A disabled apoptotic response may be a major contributor of tumor growth, however mechanism in which testin reduce proliferation and induce apoptosis is still unclear. Study on CRC revealed that high expression of *TES* gene correlate with decreased levels of the anti-apoptotic proteins such as Bcl-2, survivin and increased levels of pro-apoptotic proteins i.e. p53, Puma, Bax. In pathogenesis of sporadic CRC take part uncertain communication between cells which is altered by deviation in p38 mitogen-activated protein kinase pathway (p38-MAPK) [5, 44]. Studies on the role of p38 in CRC cancerogenesis are divergent. Several reports described role of p38 in cell survival and invasion in advanced tumor types whereas involvement in induced cell cycle arrest, differentiation and apoptosis was shown [45, 46]. Western blot (WB) analysis performed by Li et al. on CRC cell lines overexpressing *TES* showed increased p38 phosphorylation. Moreover, inhibition of p38 by specific inhibitor of p38-MAPK (SB203580) markedly promoted proliferation and inhibited apoptosis of cancer cells [5]. This result indicate that activation of MAPK through phosphorylation of p38 in CRC with high expression of *TES* gene is associated with anti-proliferative and pro-apoptotic effect [3].

**Gastric Cancer** Ma et al. analyzed expression of *TES* gene in gastric normal and cancer cell lines. They comprehensive analysis did not show any specific *TES* gene mutation. However, HMT of CpG islands observed in the region of *TES* gene resulted in downregulation of *TES* gene and protein level in primary gastric cancer [3]. In another study, WB and IHC analysis showed that low protein level was associated with lower differentiation However, there was no relationship with age, sex, tumor size, metastasis, lymphatic invasion and clinical stage. Patients with positive *TES* expression have longer survival time [47].

**Lung Cancer** The role of testin in lung cancer is not widely described. The only work by Wang et al. describe weak *TES* gene and protein expression in non-small cell lung cancer (NSCLC) cell lines in regard to normal bronchial epithelial cells [46]. The authors explored suppressive effect of testin on proliferation, invasion and colony forming of NSCLC cells. Flow cytometric analysis revealed induced apoptosis in NSCLC cells overexpressing *TES*. Additionally, they presented inhibitory effect of TES on NSCLC cell xenograft formation and growth in vivo on athymic nude mice. These data suggest important role of testin in development and progression of NSCLC [48].

**Prostate Cancer** *TES* gene is localized at 7q31 region which encodes also others candidate tumor suppressor genes such as *CAV2, CAV1, MET, CAPZA2, ST7, WNT2* [19]. Quantified analysis by RT-PCR displayed that only *TES* gene showed decrease expression in all types of prostate tumors, supporting tumor suppressor gene hypothesis. This is in concordance with results of Tobias et al. who showed reduced growth potential in ovarian (OVCAR5) and cervical (HeLa) cancer cell lines with reduced *TES* gene expression. Chene et al. demonstrated decreased expression of *TES* in confined prostate tumors, tumors with extracapsullary extension and hormonal refractory prostate tumors. However, in hormonal refractory tumors *TES* gene expression was lower than in other types of prostate cancer [24]. Additionally, *TES* gene expression was higher in prostatic epithelial cell lines than in primary prostatic fibroblast [4]. However, results of study by Chene et al. did not find correlation between LOH and *TES* gene expression [26].

**Head and Neck Squamous Cell Cancer** Gunduz et al. analyzed *TES* expression in regard to clinical advancement (i.e. tumor, lymph nodes and metastasis status; TNM) and survival ratio in head and neck squamous cell cancers. They presented no association between TNM stage and *TES* gene expression, whereas worse survival rate (50% vs. 80%) was observed in cases with weak compared to normal and high *TES* expression [8]. In Li et al. work expressions of testin gene and protein were measured by PCR, WB and IHC. They disclosed lower expression of testin protein in the nasopharyngeal cancer in comparison to the normal tissue. Moreover, protein expression positively correlated with lymph node and distant metastasis and differentiation grade. This protein may be useful as a prognostic tool reference to metastasis in nasopharyngeal cancer [9].

## Conclusion

Various studies showed that *TES* gene silencing can contribute to cancerogenesis revealing its nature of tumor suppressor gene. This findings may be useful in individualized therapy. Understanding molecular mechanisms of cancerogenesis are an important step forward in expanding possibilities of treatment, e.g. usage of 5-aza-2'deoxycytidine. Moreover, there is an evidence suggesting possible usage of testin protein as a prognostic marker. Further studies are necessary to reveal, evaluate and confirm various interesting clinical implications.

**Availability of Data** The datasets analysed in the current study are available on the in the Genomic Data Commons (GDC) Data Portal at https://gdc.cancer.gov/.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.



## References

1. Qiu H, Zhu J, Yuan C, Yan S, Yang Q, Kong B (2010) Frequent hypermethylation and loss of heterozygosity of the testis derived transcript gene in ovarian cancer. Cancer Sci 101:1255–1260. https://doi.org/10.1111/j.1349-7006.2010.01497.x
2. Dong R, Pu H, Wang Y, Yu J, Lian K, Mao C (2015) TESTIN was commonly hypermethylated and involved in the epithelial-mesenchymal transition of endometrial cancer. APMIS 123:394–400. https://doi.org/10.1111/apm.12361
3. Ma H, Weng D, Chen Y, Huang W, Pan K, Wang H, Sun J, Wang Q, Zhou Z, Wang H, Xia J (2010) Extensive analysis of D7S486 in primary gastric cancer supports TESTIN as a candidate tumor suppressor gene. Mol Cancer 9:1–10
4. Chêne L, Giroud C, Desgrandchamps F, Boccon-Gibod L, Cussenot O, Berthon P, Latil A (2004) Extensive analysis of the 7q31 region in human prostate tumors supports TES as the best candidate tumor suppressor gene. Int J Cancer 111:798–804. https://doi.org/10.1002/ijc.20337
5. Li H, Huang K, Gao L, Wang L, Niu Y, Liu H (2016) TES inhibits colorectal cancer progression through activation of p38. Oncotagret 7
6. Sarti M, Sevignani C, Calin GA, Aqeilan R, Shimizu M, Pentimalli F, Picchio MC, Godwin A, Rosenberg A, Drusco A, Negrini M, Croce CM (2005) Adenoviral transduction of TESTIN gene into breast and uterine Cancer cell lines promotes apoptosis and tumor reduction in vivo. Clin Cancer Res 11:806–813
7. Ming W, Qian W, Peng W-J, Hu J-F, Wang Z-Y, Liu H, Huang L-N (2017) Testin is a tumor suppressor in non-small cell lung cancer. Oncol Rep 37:1027–1035. https://doi.org/10.3892/or.2016.5316
8. Gunduz E, Gunduz M, Beder L, Nagatsuka H, Fukushima K, Sutcu R, Delibas N, Yamanaka N, Shimizu K, Nagai N, CM C, AG K, M E, DM P, JM C, MI E, JC L, V S, S T, XL W, JC Z, JS Z, JC Z, JC Z, T O, M G, C T, M S, A D, L C, W M, F B, B T (2009) Downregulation of TESTIN and its association with Cancer history and a tendency toward poor survival in head and neck squamous cell carcinoma. Arch Otolaryngol Neck Surg 135:254. https://doi.org/10.1001/archoto.2008.560, 260
9. Li S, Xue M HL (2016) Relationship between Testin expression and clinicopathological characteristics in nasopharyngeal carcinoma patients. 310–3
10. Steponaitis G, Kazlauskas A, Skiriute D, Valiulyte I, Skauminas K, Tamasauskas A, Vaitkiene P (2016) Testin (TES) as a candidate tumour suppressor and prognostic marker in human astrocytoma. Oncol Lett 12:3305–3311. https://doi.org/10.3892/ol.2016.5077
11. Prieto-García E, Díaz-García CV, García-Ruiz I, Agulló-Ortuño MT (2017) Epithelial-to-mesenchymal transition in tumor progression. Med Oncol 34:122. https://doi.org/10.1007/s12032-017-0980-8
12. Sala S, Van Troys M, Medves S, Catillon M, Timmerman E, Staes A, Schaffner-Reckinger E, Gevaert K, Ampe C (2017) Expanding the interactome of TES by exploiting TES modules with different subcellular localizations. J Proteome Res acs.jproteome.7b00034. https://doi.org/10.1021/acs.jproteome.7b00034
13. Zhong Y, Zhu J, Wang Y, Zhou J, Ren K, Ding X, Zhang J (2009) LIM domain protein TES changes its conformational states in different cellular compartments. Mol Cell Biochem 320:85–92. https://doi.org/10.1007/s11010-008-9901-7
14. Khurana T, Khurana B, Noegel a a (2002) LIM proteins: association with the actin cytoskeleton. Protoplasma 219:1–12. https://doi.org/10.1007/s007090200000
15. Sala S, Catillon M, Hadzic E, Schaffner-reckinger E, Troys M Van, Ampe C (2017) The PET and LIM1–2 domains of testin contribute to intramolecular and homodimeric interactions. 1:1–21
16. Weeks RJ, Ludgate JL, LeMée G, Morison IM (2016) TESTIN induces rapid death and suppresses proliferation in childhood B acute lymphoblastic Leukaemia cells. PLoS One 11:e0151341. https://doi.org/10.1371/journal.pone.0151341
17. Oldenburg J, van der Krogt G, Twiss F, Bongaarts A, Habani Y, Slotman JA, Houtsmuller A, Huveneers S, de Rooij J (2015) VASP, zyxin and TES are tension-dependent members of focal Adherens junctions independent of the α-catenin-vinculin module. Sci Rep 5:17225. https://doi.org/10.1038/srep17225
18. Coutts AS (2003) TES is a novel focal adhesion protein with a role in cell spreading. J Cell Sci 116:897–906. https://doi.org/10.1242/jcs.00278
19. Tatarelli C, Linnenbach A, Mimori K, Croce CM (2000) Characterization of the human TESTIN gene localized in the FRA7G region at 7q31. 2 Genomics 12:1–12. https://doi.org/10.1006/geno.2000.6272
20. Gu Z, Ding G, Liang K, Zhang H, Guo G, Zhang L, Cui J (2014) TESTIN suppresses tumor growth and invasion via manipulating cell cycle progression in endometrial carcinoma. Med Sci Monit 20:980–987. https://doi.org/10.12659/MSM.890544
21. Boëda B, Briggs DC, Higgins T, Garvalov BK, Fadden AJ, McDonald NQ, Way M (2007) Tes, a specific Mena interacting partner, breaks the rules for EVH1 binding. Mol Cell 28:1071–1082. https://doi.org/10.1016/j.molcel.2007.10.033
22. Griffith E, Coutts AS, Black DM (2004) Characterisation of chicken TES and its role in cell spreading and motility. Cell Motil Cytoskeleton 57:133–142. https://doi.org/10.1002/cm.10162
23. Drusco A, Zanesi N, Roldo C, Trapasso F, Farber JL, Fong LY, Croce CM (2005) Knockout mice reveal a tumor suppressor function for Testin. PNAS 102:10947–10951

24. Zhu J, Li X, Kong X, Moran MS, Su P, Haffty BG, Yang Q (2012) Testin is a tumor suppressor and prognostic marker in breast cancer. Cancer Sci 103:2092–2101. https://doi.org/10.1111/cas.12020
25. Sarti M, Pinton S, Limoni C, Carbone GM, Pagani O, Cavalli F, Catapano CV (2013) Differential expression of testin and survivin in breast cancer subtypes. Oncol Rep 30:824–832. https://doi.org/10.3892/or.2013.2502
26. Tobias ES, Hurlstone AFL, Mackenzie E, Mcfarlane R, Black DM (2001) The TES gene at 7q31 . 1 is methylated in tumours and encodes a novel growth-suppressing LIM domain protein. Oncogene 20:2844–2853
27. Mueller W, Nutt CL, Ehrich M, Riemenschneider MJ, von Deimling A, van den Boom D, Louis DN (2007) Downregulation of RUNX3 and TES by hypermethylation in glioblastoma. Oncogene 26:583–593. https://doi.org/10.1038/sj.onc.1209805
28. Han SY, Druck T, Huebner K (2003) Candidate tumor suppressor genes at FRA7G are coamplified with MET and do not suppress malignancy in a gastric cancer. Genomics 81:105–107. https://doi.org/10.1016/S0888-7543(02)00029-0
29. Frey MK, Pothuri B (2017) Homologous recombination deficiency (HRD) testing in ovarian cancer clinical practice: a review of the literature. Gynecol Oncol Res Pract 4:4. https://doi.org/10.1186/s40661-017-0039-8
30. Bièche I, Khodja a DK, Lidereau R (1997) Genetic alteration mapping on chromosome 7 in primary breast cancer. Clin Cancer Res 3:1009–1016
31. Zenklusen JC, Thompson JC, Conti CJ, Klein-Szanto AJP (1995) Frequent loss of heterozygosity in human primary squamous cell and Colon carcinomas at 7q31.1: evidence for a broad range tumor suppressor gene. Cancer Res 55:1347–1350
32. Jia X, Shanmugam C, Paluri RK, Jhala NC, Behring P, Katkoori VR, Sugandha SP, Bae S (2017) Prognostic value of loss of heterozygosity and sub-cellular localization of SMAD4 varies with tumor stage in colorectal cancer. 8:20198–20212. https://doi.org/10.18632/oncotarget.15560
33. Matsuura K, Shiga K, Yokoyama J, Saijo S, Miyagi T, Takasaka T (1998) Loss of heterozygosity of chromosome 9p21 and 7q31 is correlated with high incidence of recurrent tumor in head and neck squamous cell carcinoma. Anticancer Res 18:453–458
34. Koike M, Tasaka T, Spira S, Tsuruoka N, Phillip Koeffler H (1999) Allelotyping of acute myelogenous leukemia: loss of heterozygosity at 7q31.1 (D7S486) and q33-34 (D7S498, D7S505). Leuk Res 23:307–310. https://doi.org/10.1016/S0145-2126(98)00159-3
35. Weeks RJ, Kees UR, Song S, Morison IM (2010) Silencing of TESTIN by dense biallelic promoter methylation is the most common molecular event in childhood acute lymphoblastic leukaemia. Mol Cancer 9:163. https://doi.org/10.1186/1476-4598-9-163
36. Shridhar V, Sun QC, Miller OJ, Kalemkerian GP, Petros J, Smith DI (1997) Loss of heterozygosity on the long arm of human chromosome 7 in sporadic renal cell carcinomas. Oncogene 15:2727–2733. https://doi.org/10.1038/sj.onc.1201448
37. Achille A, Biasi MO, Zamboni G, Bogina G, Magalini AR, Pederzoli P, Perucho M, Scarpa A (1996) Chromosome 7q allelic losses in pancreatic carcinoma. Cancer Res 56:3808–3813
38. Hino O, Kobayashi T (2017) Mourning Dr. Alfred G. Knudson: the two-hit hypothesis, tumor suppressor genes, and the tuberous sclerosis complex. Cancer Sci 108:5–11. https://doi.org/10.1111/cas.13116
39. Nasir A, Holzer TR, Man M, Benjamin LE, Melemed AS, Schade AE (2015) Differential expression of VEGFR2 protein in HER2 positive primary human breast cancer: potential relevance to newer anti-angiogenic therapies. Cancer Res 75:1–12. https://doi.org/10.1186/s12935-017-0427-5
40. Iyer RP, Patterson NL, Fields GB, Lindsey ML (2012) The history of matrix metalloproteinases : milestones , myths , and misperceptions. Trabslational Physiol. https://doi.org/10.1152/ajpheart.00577.2012
41. Fang J, Zhou H, Zeng C, Yang J, Liu Y, Huang X, Zhang J, Guan X, Zhuang S (2011) MicroRNA-29b suppresses tumor angiogenesis, invasion, and metastasis by regulating matrix metalloproteinase 2 expression. Hepatology 54:1729–1740. https://doi.org/10.1002/hep.24577
42. Holm K, Hegardt C, Staaf J, Vallon-Christersson J, Jönsson G, Olsson H, Borg A, Ringnér M (2010) Molecular subtypes of breast cancer are associated with characteristic DNA methylation patterns. Breast Cancer Res 12:R36. https://doi.org/10.1186/bcr2590
43. Kalluri R, R a W (2009) Review series the basics of epithelial-mesenchymal transition. J Clin Invest 119:1420–1428. https://doi.org/10.1172/JCI39104.1420
44. Grossi V, Peserico A, Tezil T, Simone C (2014) p38α MAPK pathway: a key factor in colorectal cancer therapy and chemoresistance. World J Gastroenterol 20:9744–9758. https://doi.org/10.3748/wjg.v20.i29.9744
45. Zhang Y, Guo Z, Du T, Chen J, Wang W, Xu K, Lin T, Huang H (2013) Prostate specific membrane antigen (PSMA): a novel modulator of p38 for proliferation, migration, and survival in prostate cancer cells. Prostate 73:835–841. https://doi.org/10.1002/pros.22627
46. Hui L, Bakiri L, Stepniak E, Wagner EF (2007) p38alpha: a suppressor of cell proliferation and tumorigenesis. Cell Cycle 6:2429–2433. https://doi.org/10.4161/cc.6.20.4774
47. Huang W, Weng DS, Pan ZZ, Pan K, Ding PR, Zhou JW, Wang H, Zhang HK, Li JJ, Xia JC (2008) Expression and clinical significance of TESTIN in primary gastric cancer. Chinese J Cancer 27:267–270
48. Wang M, Wang Q, Peng W, Hu J (2017) Testin is a tumor suppressor in non-small cell lung cancer. 1027–1035. https://doi.org/10.3892/or.2016.5316

biomedicines

MDPI

*Article*

# Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia

**Aneta Popiel [1,*], Aleksandra Piotrowska [1], Patrycja Sputa-Grzegrzolka [2], Beata Smolarz [3], Hanna Romanowicz [3], Piotr Dziegiel [1,4], Marzenna Podhorska-Okolow [5] and Christopher Kobierzycki [1]**

1 Division of Histology and Embryology, Department of Human Morphology and Embryology, Wroclaw Medical University, 50-368 Wroclaw, Poland; aleksandra.piotrowska@umed.wroc.pl (A.P.); piotr.dziegiel@umed.wroc.pl (P.D.); christopher.kobierzycki@umed.wroc.pl (C.K.)
2 Division of Anatomy, Department of Human Morphology and Embryology, Wroclaw Medical University, 50-368 Wroclaw, Poland; patrycja.sputa-grzegrzolka@umed.wroc.pl
3 Department of Pathology, Polish Mother's Memorial Hospital Research Institute, 93-338 Lodz, Poland; smolbea@wp.pl (B.S.); hanna-romanowicz@wp.pl (H.R.)
4 Department of Physiotherapy, University School of Physical Education, 51-612 Wroclaw, Poland
5 Division of Ultrastructural Research, Wroclaw Medical University, 50-368 Wroclaw, Poland; marzenna.podhorska-okolow@umed.wroc.pl
* Correspondence: popielaneta1@gmail.com

**Abstract:** Cervical cancer is one of the most common malignant cancers in women worldwide. The 5-year survival rate is 65%; nevertheless, it depends on race, age, and clinical stage. In the oncogenesis of cervical cancer, persistent HPV infection plays a pivotal role. It disrupts the expression of key proteins as Ki-67, p16, involved in regulating the cell cycle. This study aimed to identify the potential role of testin in the diagnosis of cervical precancerous lesions (CIN). The study was performed on selected archival paraffin-embedded specimens of CIN1 (31), CIN2 (75), and CIN3 (123). Moderate positive correlation was observed between testin and Ki-67 as well as testin and p16 expression in all dysplastic lesions ($r = 0.4209$, $r = 0.5681$; $p < 0.0001$ for both). Statistical analysis showed stronger expression of the testin in dysplastic lesions vs. control group ($p < 0.0001$); moreover, expression was significantly higher in HSIL than LSIL group ($p < 0.0024$). In addition, a significantly stronger expression of testin was observed in CIN3 vs. CIN1 and CIN3 vs. CIN2. In our study, expression of Ki-67, p16, and testin increased gradually as the lesion progressed from LSIL to HSIL. The three markers complemented each other effectively, which may improve test sensitivity and specificity when used jointly.

**Keywords:** testin; p16 protein; cervical cancer; cervical neoplasia; immunohistochemistry



**Citation:** Popiel, A.; Piotrowska, A.; Sputa-Grzegrzolka, P.; Smolarz, B.; Romanowicz, H.; Dziegiel, P.; Podhorska-Okolow, M.; Kobierzycki, C. Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia. *Biomedicines* **2021**, *9*, 1010. https://doi.org/10.3390/biomedicines9081010

Academic Editor: Naomi Nakayama

Received: 13 July 2021
Accepted: 10 August 2021
Published: 13 August 2021



## 1. Introduction

Cervical cancer is one of the most common malignant cancers in women worldwide. Each year, over 580,000 new cases of cervical cancer are diagnosed [1]. The American Cancer Society estimates that 4290 women will die due to cervical cancer in 2021 in the United States. In 2018, the number of deaths worldwide was 311,000, 90% of which occurred in low- and middle-income countries. The 5-year survival rate is 65%; nevertheless, it depends on race, age, and clinical stage. In developing countries in Africa, Asia, and South America, cervical cancer causes over 50% of early deaths in women of childbearing age. The WHO (World Health Organization) invests great effort into decreasing mortality and morbidity by means of promoting both primary and secondary preventive care for cervical cancer. In May 2018, WHO announced its project to eliminate cervical cancer as a public health issue between 2020 and 2030, reducing the age-correlated incidence rate to 4/100,000. WHO developed a triple intervention plan, which includes scaling up vaccination against HPV, twice-lifetime cervical screenings up to 70%, and treatment of preinvasive lesions and invasive cancer to 90% [2]. Informing patients that HPV is an important cause of cervical

cancer has led to significant advances in the primary and secondary prevention of cervical cancer. Ten years ago, the WHO introduced vaccines against two of the most cancerogenic types, HPV16 and HPV18. They are highly effective at preventing HPV infections when administered before sexual activity. Nowadays there are available vaccines against seven carcinogenic HPV types and two non-cancer types that cause warts [3]. Cervical cancer is most often diagnosed in women aged 35–44, whereas it rarely occurs before 20 years of age. Over 15% of all cases occur in women above 65 years of age; however, it is rare in women who undergo regular screening. The main risk factor of cervical cancer is persistent HPV infection. It promotes impaired growth and differentiation of cells, leading to dysplasia (cervical intraepithelial neoplasia, CIN) [4]. We distinguished three grades of cervical dysplasia: CIN1 (LSIL; low-grade squamous intraepithelial lesion), CIN2, and CIN3 (classified together as HSIL; high-grade squamous intraepithelial lesion). The risk of developing invasive cervical cancer from HSIL is approximately 20% (10–40% according to literature) [5–7]. In the oncogenesis of cervical cancer, persistent HPV infection plays a pivotal role as it is necessary for CIN changes to occur. In assessing the risk of progression of lesions and the proper selection of treatment, techniques of viral DNA identification are used, which may be helpful in the triage of patients with cytological diagnosis of atypical squamous cells of undetermined significance (ASCUS). HPV DNA tests are highly sensitive, but the specificity of HPV tests is low due to most HPV infections being naturally cleared [8]; this creates the need to search for more specific diagnostic methods. More specific markers of cervical cancer have been identified from HPV-induced oncogenesis studies. The oncogenic HPV viruses disrupt the expression of key proteins as Ki-67, p16 involved in regulating the cell cycle. Our study aimed to identify the potential role of testin protein in the diagnosis of cervical precancerous lesions. Research points to its possible role in cervical cancer, whereas in CIN it has not been analyzed yet [9,10]. In this article, we focus on the correlation between testin and known markers used in the diagnosis of cervical intraepithelial changes.

## 2. Materials and Methods

### 2.1. Material

The study was performed on selected archival paraffin-embedded specimens of CIN1 (31), CIN2 (75), and CIN3 (123). Patients aged from 25 to 86 years old were female (Table 1). The control group consisted of 125 cases of normal cervical tissue possessed from patients who underwent total hysterectomy due to uterine leiomyomas. Patients were operated on between 2014 and 2017 in the Polish Mother's Memorial Hospital in Lodz.

**Table 1.** Clinicopathological features of study group patients.

| Feature | N | % |
|---|---|---|
| Age | | |
| <35 | 119 | 51.97 |
| 35–45 | 75 | 32.75 |
| >45 | 35 | 15.28 |
| Cytology result | | |
| CIN 1 | 31 | 13.5 |
| CIN 2 | 75 | 32.8 |
| CIN 3 | 123 | 53.7 |
| Histology result | | |
| LSIL | 31 | 13.5 |
| HSIL | 198 | 86.5 |

### 2.2. TMA Construction

Hematoxylin and eosin-stained (HE) 6-µm thick paraffin sections were prepared to verify the histopathological diagnosis and assess the suitability of the sample for further analysis. In short, slides were scanned utilizing histologic scanner Pannoramic MIDI

(3DHistech Ltd., Sysmex Suisse AG, Horgen, Switzerland). Afterward, scans were examined by two independent pathologists who chose and electronically labeled areas of the CIN in the changed epithelium of the cervix. For TMA construction, from the corresponding paraffin donor blocks, triplicate tissue core punches (2 mm) for every case were obtained (TMA Grand Master; 3DHistech, Budapest, Hungary). The normal epithelial tissue of the cervix was marked as a control group.

### *2.3. Immunohistochemistry (IHC)*

Immunohistochemical reactions were performed on 4 µm paraffin sections obtained from TMA blocks in an automated staining platform, Autostainer Link48 (Dako, Glostrup, Denmark). Deparaffinization, rehydration, and antigen retrieval was performed using EnVision FLEX Target Retrieval Solution (97 °C, 20 min; pH 6 for Ki-67 and pH 9 for p16 and testin) in PT-Link. The activity of endogenous peroxidase was blocked by 5 min exposure to a peroxidase-blocking reagent (Dako). Monoclonal mouse anti-p16 antibody (1:100+linker, 550834, BP Pharmingen, San Diego, CA, USA), anti-Ki-67 (ready to use, IR626, Dako), and polyclonal rabbit anti-testin (1:400, NBP1-87987, Novus Biologicals, Centennial, CO, USA) were used as the primary antibody (20 min incubation) followed by incubation with a secondary antibody conjugated with horseradish peroxidase (EnVision™ FLEX/HRP—20 min incubation). 3,3′-diaminobenzidine (DAB) was utilized as the peroxidase substrate, and the sections were incubated for 10 min. Finally, all sections were counterstained with EnVision FLEX Hematoxylin (Dako) for 5 min. After dehydration in graded ethanol concentrations (70%, 96%, absolute) and xylene, all slides were closed with coverslips in SUB-X Mounting Medium in a coverslipper.

The slides were scanned using a histologic scanner, Pannoramic MIDI (3DHistech). Reactions were evaluated (Ki-67) with the use of Quant Center software (3DHistech) under researcher supervision. For every case, three TMA cores were quantified by the algorithm SCORE (range = 0–8), and the final result was an average count. Expression of testin was assessed using a Pannoramic Viewer Digital image analysis as well as a routinely used immunoreactive scale (IRS) by Remmele and Stegner, presented in Table 2. The expression of p16 antigen was evaluated by two parameters, a percentage of p16-positive cells, and reaction intensity. The percentage of positive cells was evaluated in the highest expression area ("hot spot") and graded as follows (grade 0) when no cells stained, positive cells >0–5% (grade 1), positive cells >5–25% (grade 2), positive cells >25% (grade 3). The intensity of the reaction was scored as negative (0), weak (1), moderate (2), and strong (3). The reaction was considered as positive when nuclear, or nuclear and cytoplasmic, strong, and diffuse p16 staining beginning from the basal cell layer of the epithelium was observed. Whereas non-specific patterns, focal, wispy, small clusters of cells and a complete lack of staining qualified as negative p16 expression. Testin cytoplasmatic expression was scored as follows:

**Table 2.** Immunoreactive scale scoring system.

| Score | Positively Stained Cells (PP) | Intensity of Staining (SI) | IRS Points (PP × SI) | IRS Classification |
|---|---|---|---|---|
| **0** | no staining | no color reaction | 0–1 | Negative |
| **1** | <10% | mild reaction | 2–3 | Positive, mild expression |
| **2** | 10–50% | moderate reaction | 4–8 | Positive, moderate expression |
| **3** | 51–80% | strong reaction | 9–12 | Positive, strong expression |
| **4** | >80% | | | |

### *2.4. Statistical Analysis*

The results were statistically analyzed using GraphPad Prism 5.0 software using Spearman correlation, Kruskal–Wallis, Dunn's multiple comparison, and Mann–Whitney tests. In all analyzed cases, the associations were considered statistically significant for $p < 0.05$.

## 3. Results

Expression of Ki-67 was observed in 100%, p16 in 84.6%, and testin in 98.25% of cervical dysplasia cases (Figure 1).

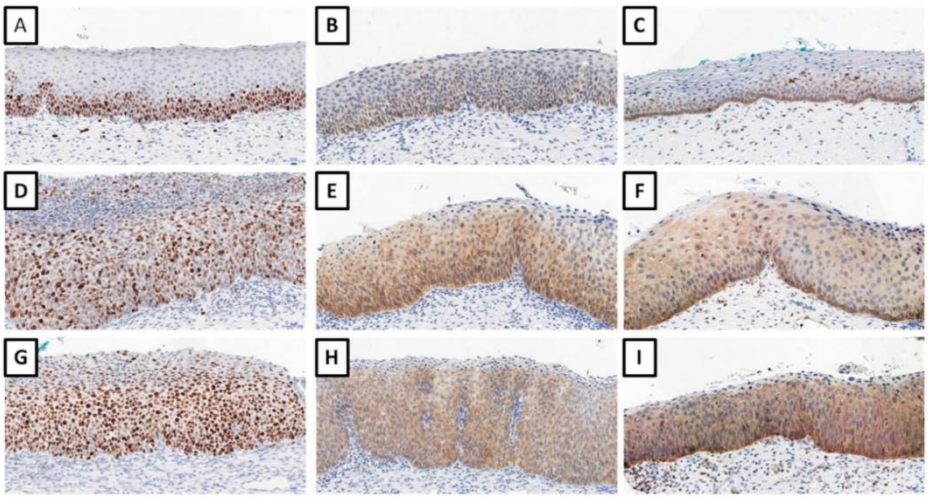


**Figure 1.** Immunohistochemical expression of Ki-67 ((**A**), CIN1; (**D**), CIN2; (**G**), CIN3); p16 protein ((**B**), CIN1; (**E**), CIN2; (**H**), CIN3), and testin ((**C**), CIN1; (**F**), CIN2; (**I**), CIN3). Magnification ×200.

A moderate positive correlation was observed between testin and Ki-67 as well testin and p16 expression in all dysplastic lesions (r = 0.4209, r = 0.5681; $p < 0.0001$ for both; Spearman correlation test). The relationships subdivided according to CINs are presented in Table 3.

**Table 3.** Spearman correlation test results.

| | CIN1 | | CIN2 | | CIN3 | |
|---|---|---|---|---|---|---|
| | **Ki-67** | **p16** | **Ki-67** | **p16** | **Ki-67** | **p16** |
| **testin** | r = 0.3678<br>$p < 0.0418$ | NS | r = 0.3441<br>$p < 0.0025$ | r = 0.6348<br>$p < 0.0001$ | r = 0.3406<br>$p < 0.0001$ | r = 0.4364<br>$p < 0.0001$ |
| **Ki-67** | | r = 0.5342<br>$p < 0.0028$ | | r = 0.6032<br>$p < 0.0001$ | | r = 0.3802<br>$p < 0.0001$ |

Statistical analysis showed stronger expression for testin in dysplastic lesions vs. control group ($p < 0.0001$; Mann–Whitney test; Figure 2A). Moreover, the expression was significantly higher in HSIL than the LSIL group ($p < 0.0024$; Mann–Whitney test; Figure 2B). Expression of p16 and Ki-67 was stronger in all dysplastic lesions vs. control group ($p < 0.0001$; Mann–Whitney test; Figure 2 C,D), and the expression of these two markers was higher in HSIL than in LSIL ($p < 0.0001$; Mann–Whitney test). The expression of p16 does not show statistically significant differences between normal cervical tissue and CIN1 ($p > 0.05$; Dunn's multiple comparison test). The expression of Ki-67 in normal cervical tissue was significantly lower than in CIN1 ($p < 0.05$; Dunn's multiple comparison

test). In addition, significantly stronger expression of testin was observed in CIN3 vs. CIN1 and CIN3 vs. CIN2 cases ($p < 0.05$ respectively; Dunn's multiple comparison test). There were no statistically significant differences in testin expression between CIN1 and CIN2 as well as in p16 expression between CIN2 and CIN3 ($p > 0.05$; Dunn's Multiple Comparison test). Additionally, moderate positive correlation was observed between the expression of testin and Ki67, testin and p16 also between Ki-67 and p16 in all dysplastic lesions ($p < 0.0001$, r = 0.3917; $p < 0.0001$, r = 0.5681; $p < 0.0001$, r = 0.5655 Spearmann correlation test; Figure 3A–C). No differences in the relationship between age groups and type of CIN were observed. The highest percentage of HSIL lesions occurred in the <35 age group (Table 4). In addition, there was no statistically significant relationship between age groups and Ki-67 (Figure 4) or p16 median (Figure 5), but the relationship between age group and testin was close to being statistically significant (Chi-square test; $p = 0.0667$; Table 5).

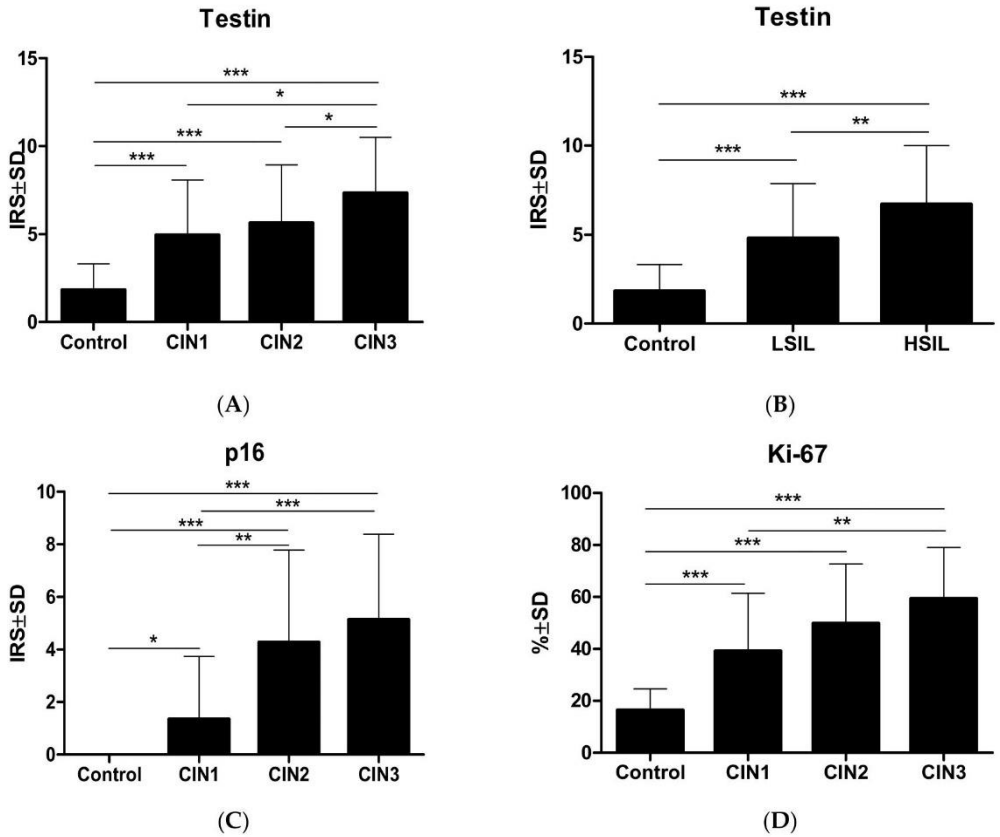


**Figure 2.** Immunohistochemical reaction in cervical intraepithelial neoplasia: (**A**) testin expression in CIN lesions; (**B**) testin expression in LSIL and HSIL lesions; (**C**) p16 expression in CIN lesions; (**D**) Ki-67 expression in CIN lesions. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

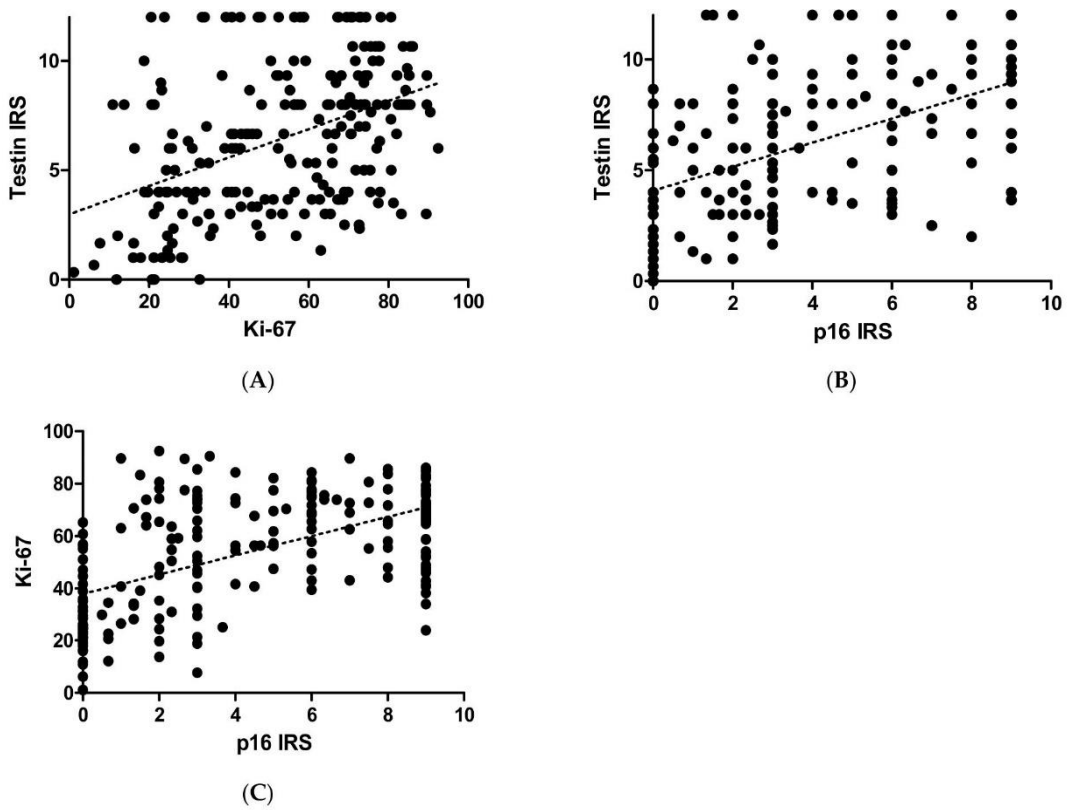


**Figure 3.** Immunohistochemical reaction in cervical intraepithelial neoplasia: (**A**) testin and Ki-67 correlation in CIN lesions; (**B**) testin and p16 correlation in CIN lesions; (**C**) Ki-67 and p16 correlation in CIN lesions.

**Table 4.** LSIL and HSIL in specific age groups.

| | LSIL | HSIL |
|---|---|---|
| **<35** | 13 | 106 |
| **35–45** | 13 | 62 |
| **>45** | 5 | 30 |

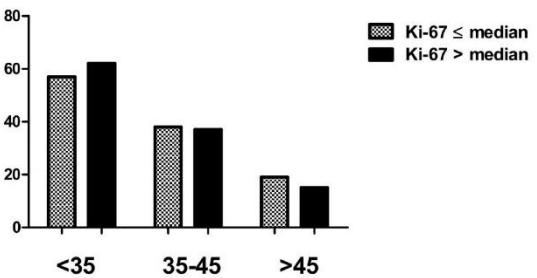


**Figure 4.** Median of the Ki-67 expression in age groups of CIN lesions.

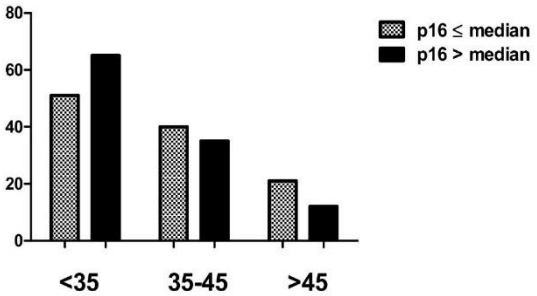


**Figure 5.** Median of the p16 expression in age groups of CIN lesions.

**Table 5.** Median of the testin expression in age groups of CIN lesions.

| | Testin < Median | Testin > Median |
|---|---|---|
| **<35** | 58 | 61 |
| **35–45** | 49 | 26 |
| **>45** | 18 | 17 |

## 4. Discussion

The term dysplasia refers to the replacement of normal squamous stratified epithelium by abnormal cells with pathological morphology spreading on successive layers of the epithelium. In the early 1980s, this term was changed into cervical intraepithelial neoplasia by the International Society of Gynecological Pathologists [11]. The terminology change was dictated by a detailed understanding of cervical cancerogenesis. All changes in the epithelium leading to the formation of CIN1, CIN2, CIN3, and cervical cancer are a series of related events, not isolated actions as previously believed. For clinicians, the most important aspect of diagnostics for CIN is to differentiate LSIL from HSIL. For this reason, many currently ongoing studies search for new markers that may streamline the diagnostic process. In this study, we focused on the correlation between testin and Ki-67 as a marker of proliferation and p16, indicating the impact of the HPV infection on the cervical epithelium. The latest report indicates that the TES gene is a tumor suppressor gene that can contribute to cancerogenesis, but the mechanism of the loss of TES gene expression is still unknown. HPV infection leads to the overexpression of E6 and E7 oncoproteins which disrupt the normal function of the tumor suppressor gene [12]. There is a lack of studies that analyze the expression of testin in LSIL and HSIL. The expression of this producer in CIN may prove an important factor in the development of cervical lesions.

P16, also known as p16INK4a, is encoded by the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene located on chromosome 9p21.3. It is a cell cycle protein that regulates cell proliferation in the G1-S phase due to the reciprocal relationship with another tumor suppressor protein-Rb. In persistent HPV infection, oncogenic proteins E6 and E7 bind to host regulatory proteins. In HPV, E6 oncoproteins lead to the dysfunction of the p53 suppressor gene through direct protein–protein interaction, inducing p53 protein degradation [13]. On the other hand, E7 oncoproteins form a complex with retinoblastoma (Rb) protein that blocks the phosphorylation of Rb protein, thereby increasing free E2F; this results in both abnormal cell cycle progression and overexpression of p16 protein. Overexpression of p16 is commonly found in cells infected by HPV. Currently, IHC expression of p16 together with the expression of Ki-67 is routinely used to improve diagnosis of cervical lesions [14–16]. In the present study, the correlation between the grade of cervical lesions and the expression of p16 was strong ($p < 0.0001$). A similar correlation was observed according to the expression of Ki-67. It directly confirms the observation of other authors' research and indicates the validity of using commercial kits (CINtec PLUS Kit) to diagnose LSIL and HSIL [17–20]. Our study confirms the results of other research and indicates that p16 expression positively

correlates with the degree of cervical lesions [21]. This study also shows no statistically significant difference between the expression of p16 in normal cervical tissue and CIN1, suggesting that p16 does not fully reflect the degree of cervical lesions. The absence of p16 expression can be used to eliminate associated high-grade squamous intraepithelial lesions in biopsy material. There is one limitation of p16 analysis as a CIN marker. P16 expression can sometimes be focal or diffuse in benign endocervical intercalated columnar cells, tubal metaplasia of the endometrium, and cervical endometriosis [22]. Despite that, the expression of p16 in these cells does not have the premalignant potential [23].

Ki-67 is a cellular marker of proliferation, detected in the non-G0 phase of the cell cycle [24]. In normal squamous cervical epithelium, Ki-67 is present only in basal and parabasal layers. In dysplasia and carcinoma, their expression extends above the basal one-third of squamous epithelium [25]. Many studies have shown that the elevated expression of Ki-67 is closely related to cell mitosis and cell proliferation [26–29]. Some scientists highlight this marker's role in distinguishing different degrees of cervical lesion [21,28,29]. The results of this study showed that the expression of Ki-67 was higher in the CIN1, CIN2, and CIN3 groups than in the control group ($p < 0.05$). The expression level of Ki-67 was significantly higher in HSIL than LSIL ($p < 0.05$), indicating that the expression level increased with the development of the HSIL lesions. Moreover, there is a significant difference in Ki-67 expression between normal cervical tissue and CIN1. Some studies show that Ki-67 is expressed in proliferative non-cancerous tissue, and in this study, patients with LSIL also have a positive expression of Ki-67 [30]. High expression of Ki-67 is associated with the severity of cervical lesions but not with HPV infection [31]. There is evidence that Ki-67 has a prognostic value superior to the standard histopathological grading to prognosticate CIN progression. Several studies have shown that diffuse expression of Ki-67 is present in almost all cases of HSIL or cervical cancer [32–34].

Testin is a protein with molecular mass 47kDa. The human TES gene is localized on the fragile site FRA7G at 7q31.2 [35]. Testin protein comprises three LIM (Linl-1, Isl-1, Mec-3) domains, and each consists of two zinc fingers linked by two amino acid spacers [36]. It was observed that the N- and C-terminal parts could interact with each other and create open and close conformations in cells. When expressed separately in cells, these two halves show partially different subcellular localizations with a dominant role of LIM domains targeting focal adhesion. Testin is localized along the actin stress fibers at the cell–cell junction and focal adhesion. Testin can interact with cytoskeletal proteins such as zyxin, talin, and VASP [9,37]. Together, they play a significant role in cell motility and adhesion. In chicken and human fibroblast, testin notably activates cell spreading, but there is a loss of testin increased cell motility. These suggest that testin appears crucial in regulating cellular migration, invasion, and process of epithelial-mesenchymal transition. Additionally, testin is involved in the cell cycle. The expression of testin protein positively correlates with a percentage of cells in the G1 phase; however, overexpression can induce apoptosis and decreased colony-forming ability. The expression of testin has been described in many types of human malignancies, but there is fewer data about the expression of testin in cervical intraepithelial neoplasia. The mechanism and pathways of testin's influence on cancerogenesis are still unknown. Some of the authors point out hypermethylation of the gene as the main factor. [35,36,38–43]. In this study, the expression of testin was significantly stronger in all dysplastic lesions compared to the control group. Testin expressed stronger in HSIL than LSIL; this indicates testin as a good diagnostic marker for distinguishing cervical lesions (CIN1 vs. CIN2 and CIN3). Zhong et al. support that testin has open and close conformations in cells by detecting the anti-TES serum in the nucleolus and anti-TESC as well anti-TESN sera in the cytoplasm. Cellular location is important for protein function because many phosphates, kinases, and transcription factors regulate their activity by controlling subcellular distribution [44]. Testin shuttling among cellular compartments may have divergent functions with different interacting partner proteins. The multiple conformational states and different locations in cellular compartments impact various expressions of testin protein; this may be the factor that

suggests low testin expression in HSIL lesions. Future studies should be performed to find functions correlating with different cellular locations [45]. We plan on expanding our research with further studies to include cancer tissue. We plan on researching correlations between testin and lymphovascular space invasion, nodes metastasis, angiogenesis, and epithelial-mesenchymal transition markers in the upcoming months.

## 5. Conclusions

In our study, the expressions of Ki-67, p16, and testin gradually increased as the lesion progressed from LSIL to HSIL. The three markers complemented each other effectively, which may improve test sensitivity and specificity when used jointly.

**Author Contributions:** Conceptualization, A.P. (Aneta Popiel) and C.K.; methodology, A.P. (Aleksandra Piotrowska); software, P.S.-G.; validation, A.P. (Aneta Popiel), P.D., and M.P.-O.; formal analysis, A.P. (Aneta Popiel); investigation, A.P. (Aleksandra Piotrowska); resources, H.R. and B.S.; data curation, A.P. (Aneta Popiel); writing—original draft preparation, A.P. (Aneta Popiel); writing—review and editing, C.K.; visualization, P.S.-G.; supervision, P.D.; project administration, M.P.-O.; funding acquisition, A.P. (Aneta Popiel). All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Science Centre, Poland grant number, 2018/29/N/NZ5/01911. The APC was funded by the National Science Centre.

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Medical University in Wroclaw (6 May 2019; protocol code 411/2019).

**Informed Consent Statement:** Patient consent was waived due to use of anonymized archival material, which in no way influenced the diagnostic and therapeutic process.

**Data Availability Statement:** The data presented in this study are available upon request from the corresponding author. The data are not publicly available due to privacy issue.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Cubie, H.A.; Campbell, C. Cervical cancer screening—The challenges of complete pathways of care in low-income countries: Focus on Malawi. *Women's Health* **2020**, *16*, 1745506520914804. [CrossRef]
2. Canfell, K.; Kim, J.J.; Brisson, M.; Keane, A.; Simms, K.T.; Caruana, M.; Burger, E.A.; Martin, D.; Nguyen, D.T.N.; Bénard, É.; et al. Mortality impact of achieving WHO cervical cancer elimination targets: A comparative modelling analysis in 78 low-income and lower-middle-income countries. *Lancet* **2020**, *395*, 591–603. [CrossRef]
3. Wentzensen, N.; Schiffman, M.; Palmer, T.; Arbyn, M. Triage of HPV positive women in cervical cancer screening. *J. Clin. Virol. Off. Publ. Pan Am. Soc. Clin. Virol.* **2016**, *76*, 49–55. [CrossRef] [PubMed]
4. Moreno-Acosta, P.; Romero-Rojas, A.; Vial, N.; Huertas, A.; Acosta, J.; Mayorga, D.; Schyrly, C.; Molano, M.; Gamboa, O.; Cotes, M.; et al. Persistent High-Risk HPV Infection and Molecular Changes Related to the Development of Cervical Cancer. *Case Rep. Obstet. Gynecol.* **2020**, *2020*, 6806857. [CrossRef]
5. Tao, L.; Han, L.; Li, X.; Gao, Q.; Pan, L.; Wu, L.; Luo, Y.; Wang, W.; Zheng, Z.; Guo, X. Prevalence and risk factors for cervical neoplasia: A cervical cancer screening program in Beijing. *BMC Public Health* **2014**, *14*, 1185. [CrossRef]
6. Kim, H.Y.; Kim, S.M.; Seo, J.-H.; Park, E.-H.; Kim, N.; Lee, D.-H. Age-specific prevalence of serrated lesions and their subtypes by screening colonoscopy: A retrospective study. *BMC Gastroenterol.* **2014**, *14*, 82. [CrossRef] [PubMed]
7. Raab, S.S.; Bishop, N.S.; Zaleski, M.S. Long-term outcome and relative risk in women with atypical squamous cells of undetermined significance. *Am. J. Clin. Pathol.* **1999**, *112*, 57–62. [CrossRef] [PubMed]
8. Naucler, P.; Ryd, W.; Törnberg, S.; Strand, A.; Wadell, G.; Elfgren, K.; Rådberg, T.; Strander, B.; Forslund, O.; Hansson, B.G.; et al. Efficacy of HPV DNA testing with cytology triage and/or repeat HPV DNA testing in primary cervical cancer screening. *J. Natl. Cancer Inst.* **2009**, *101*, 88–99. [CrossRef] [PubMed]
9. Gu, Z.; Ding, G.; Liang, K.; Zhang, H.; Guo, G.; Zhang, L.; Cui, J. TESTIN suppresses tumor growth and invasion via manipulating cell cycle progression in endometrial carcinoma. *Med. Sci. Monit.* **2014**, *20*, 980–987. [CrossRef] [PubMed]
10. Dong, R.; Pu, H.; Wang, Y.; Yu, J.; Lian, K.; Mao, C. TESTIN was commonly hypermethylated and involved in the epithelial-mesenchymal transition of endometrial cancer. *Apmis* **2015**, *123*, 394–400. [CrossRef]
11. Buckley, C.; Butler, E.; Fox, H. Cervical intraepithelial neoplasia. *J. Clin. Pathol.* **1982**, *35*, 1–13. [CrossRef] [PubMed]

12. Balasubramaniam, S.D.; Balakrishnan, V.; Oon, C.E.; Kaur, G. Key molecular events in cervical cancer development. *Medicina* **2019**, *55*, 384. [CrossRef]
13. Lu, D.W.; El-Mofty, S.K.; Wang, H.L. Expression of p16, Rb, and p53 proteins in squamous cell carcinomas of the anorectal region harboring human papillomavirus DNA. *Mod. Pathol.* **2003**, *16*, 692–699. [CrossRef] [PubMed]
14. Hebbar, A.; Murthy, V.S. Role of p16/INK4a and Ki-67 as specific biomarkers for cervical intraepithelial neoplasia: An institutional study. *J. Lab. Phys.* **2017**, *9*, 104–110. [CrossRef] [PubMed]
15. Kanthiya, K.; Khunnarong, J.; Tangjitgamol, S.; Puripat, N.; Tanvanich, S. Expression of the p16 and Ki67 in cervical squamous intraepithelial lesions and cancer. *Asian Pac. J. Cancer Prev.* **2016**, *17*, 3201–3206.
16. de Melo, F.L.P.; Lancellotti, C.L.P.; da Silva, M.A.L.G. Expression of the Immunohistochemical Markers p16 and Ki-67 and Their Usefulness in the Diagnosis of Cervical Intraepithelial Neoplasms. *Rev. Bras. Ginecol. Obstet.* **2016**, *38*, 82–87. [CrossRef]
17. Takacs, F.Z.; Radosa, J.C.; Bochen, F.; Juhasz-Böss, I.; Solomayer, E.F.; Bohle, R.M.; Breitbach, G.P.; Schick, B.; Linxweiler, M. Sec62/Ki67 and p16/Ki67 dual-staining immunocytochemistry in vulvar cytology for the identification of vulvar intraepithelial neoplasia and vulvar cancer: A pilot study. *Arch. Gynecol. Obstet.* **2019**, *299*, 825–833. [CrossRef]
18. Desai, F.; Singh, L.S.; Majachunglu, G.; Kamei, H. Diagnostic accuracy of conventional Cell Blocks along with p16INK4 and Ki67 biomarkers as triage tests in resource-poor organized cervical cancer screening programs. *Asian Pac. J. Cancer Prev.* **2019**, *20*, 917. [CrossRef]
19. Miyamoto, S.; Hasegawa, J.; Morioka, M.; Hirota, Y.; Kushima, M.; Sekizawa, A. The association between p16 and Ki-67 immunohistostaining and the progression of cervical intraepithelial neoplasia grade 2. *Int. J. Gynecol. Obstet.* **2016**, *134*, 45–48. [CrossRef]
20. Sangwaiya, A.; Gill, M.; Bairwa, S.; Chaudhry, M.; Sen, R.; Prakash Kataria, S. Utility of P16/INK4a and KI-67 in preneoplasticand neoplastic lesions of cervix. *Iran. J. Pathol.* **2018**, *13*, 308–316. [PubMed]
21. Nam, E.J.; Kim, J.W.; Hong, J.W.; Jang, H.S.; Lee, S.Y.; Jang, S.Y.; Lee, D.W.; Kim, S.W.; Kim, J.H.; Kim, Y.T.; et al. Expression of the p16INK4a and Ki-67 in relation to the grade of cervical intraepithelial neoplasia and high-risk human papillomavirus infection. *J. Gynecol. Oncol.* **2008**, *19*, 162–168. [CrossRef]
22. Tringler, B.; Gup, C.J.; Singh, M.; Groshong, S.; Shroyer, A.L.; Heinz, D.E.; Shroyer, K.R. Evaluation of p16INK4a and pRb expression in cervical squamous and glandular neoplasia. *Hum. Pathol.* **2004**, *35*, 689–696. [CrossRef]
23. Silva, D.C.; Gonçalves, A.K.; Cobucci, R.N.; Mendonça, R.C.; Lima, P.H.; Júnior, G.C. Immunohistochemical expression of p16, Ki-67 and p53 in cervical lesions—A systematic review. *Pathol. Res. Pract.* **2017**, *213*, 723–729. [CrossRef]
24. Min, Z.; Pu, X.; Gu, Z. Correlative analysis of the expression of IL-10 and Ki-67 in human cervical cancer and cervical intraepithelial neoplasias and human papillomavirus infection. *Oncol. Lett.* **2018**, *16*, 7189–7194. [CrossRef]
25. Ancuţa, E.; Ancuţa, C.; Cozma, L.G.; Iordache, C.; Anghelache-Lupaşcu, I.; Anton, E.; Carasevici, E.; Chirieac, R. Tumor biomarkers in cervical cancer: Focus on Ki-67 proliferation factor and E-cadherin expression. *Rom. J. Morphol. Embryol.* **2008**, *50*, 413–418.
26. Li, L.T.; Jiang, G.; Chen, Q.; Zheng, J.N. Predic Ki67 is a promising molecular target in the diagnosis of cancer (Review). *Mol. Med. Rep.* **2015**, *11*, 1566–1572. [CrossRef]
27. Juríková, M.; Danihel, Ľ.; Polák, Š.; Varga, I. Ki67, PCNA, and MCM proteins: Markers of proliferation in the diagnosis of breast cancer. *Acta Histochem.* **2016**, *118*, 544–552. [CrossRef]
28. Denkert, C.; Budczies, J.; von Minckwitz, G.; Wienert, S.; Loibl, S.; Klauschen, F. Strategies for developing Ki67 as a useful biomarker in breast cancer. *Breast* **2015**, *24*, 67–72. [CrossRef] [PubMed]
29. Ma, X.; Wu, Y.; Zhang, T.; Song, H.; Jv, H.; Guo, W.; Ren, G. Ki67 proliferation index as a histopathological predictive and prognostic parameter of oral mucosal melanoma in patients without distant metastases. *J. Cancer* **2017**, *18*, 3828. [CrossRef]
30. Shi, Q.; Xu, L.; Yang, R.; Meng, Y.; Qiu, L. Ki-67 and P16 proteins in cervical cancer and precancerous lesions of young women and the diagnostic value for cervical cancer and precancerous lesions. *Oncol. Lett.* **2019**, *18*, 1351–1355. [CrossRef] [PubMed]
31. Davidson, B.; Goldberg, I.; Lerner-Geva, L.; Gotlieb, W.H.; Ben-Baruch, G.; Novikov, I.; Kopolovic, J. Expression of topoisomerase II and Ki-67 in cervical carcinoma-clinicopathological study using immunohistochemistry. *Apmis* **2000**, *108*, 209–215. [CrossRef] [PubMed]
32. Qian, Q.P.; Zhang, X.; Ding, B.; Jiang, S.W.; Li, Z.M.; Ren, M.L.; Shen, Y. Performance of P16/Ki67 dual staining in triaging hr-HPV-positive population during cervical Cancer screening in the younger women. *Clin. Chim. Acta* **2018**, *483*, 281–285. [CrossRef] [PubMed]
33. Li, Y.; Liu, J.; Gong, L.; Sun, X.; Long, W. Combining HPV DNA load with p16/Ki-67 staining to detect cervical precancerous lesions and predict the progression of CIN1-2 lesions. *Virol. J.* **2019**, *16*, 1–9. [CrossRef] [PubMed]
34. Piri, R.; Ghaffari, A.; Azami-Aghdash, S.; Ali-Akbar, Y.P.; Saleh, P.; Naghavi-Behzad, M. Ki-67/MIB-1 as a prognostic marker in cervical cancer—a systematic review with meta-analysis. *Asian Pac. J. Cancer Prev.* **2015**, *16*, 6997–7002.
35. Sarti, M.; Sevignani, C.; Calin, G.A.; Aqeilan, R.; Shimizu, M.; Pentimalli, F.; Picchio, M.C.; Godwin, A.; Rosenberg, A.; Drusco, A.; et al. Adenoviral Transduction of testin Gene into Breast and Uterine Cancer Cell Lines Promotes Apoptosis and Tumor Reduction In vivo. *Clin. Cancer Res.* **2005**, *11*, 806–813. [PubMed]
36. Tobias, E.S.; Hurlstone, A.F.L.; Mackenzie, E.; MacFarlane, R.; Black, D.M. The TES gene at 7q31. 1 is methylated in tumours and encodes a novel growth-suppressing LIM domain protein. *Oncogene* **2001**, *20*, 2844–2853. [CrossRef]
37. Popiel, A.; Kobierzycki, C.; Dzięgiel, P. The Role of Testin in Human Cancers. *Pathol. Oncol. Res.* **2019**, *25*, 1279–1284. [CrossRef]

38. Li, H.; Huang, K.; Gao, L.; Wang, L.; Niu, Y.; Liu, H.; Wang, Z.; Wang, L.; Wang, G.; Wang, J. TES inhibits colorectal cancer progression through activation of p38. *Oncotagret* **2016**, *7*, 45819. [CrossRef]
39. Ma, H.; Weng, D.; Chen, Y.; Huang, W.; Pan, K.; Wang, H.; Sun, J.; Wang, Q.; Zhou, Z.; Wang, H.; et al. Extensive analysis of D7S486 in primary gastric cancer supports TESTIN as a candidate tumor suppressor gene. *Mol. Cancer* **2010**, *9*, 190. [CrossRef]
40. Chêne, L.; Giroud, C.; Desgrandchamps, F.; Boccon-Gibod, L.; Cussenot, O.; Berthon, P.; Latil, A. Extensive analysis of the 7q31 region in human prostate tumors supports TES as the best candidate tumor suppressor gene. *Int. J. Cancer* **2004**, *111*, 798–804. [CrossRef]
41. Sarti, M.; Pinton, S.; Limoni, C.; Carbone, G.M.; Pagani, O.; Cavalli, F.; Catapano, C.V. Differential expression of testin and survivin in breast cancer subtypes. *Oncol. Rep.* **2013**, *30*, 824–832. [CrossRef]
42. Wang, M.; Wang, Q.; Peng, W.; Hu, J. Testin is a tumor suppressor in non-small cell lung cancer. *Oncol. Rep.* **2017**, *37*, 1027–1035. [CrossRef] [PubMed]
43. Qiu, H.; Zhu, J.; Yuan, C.; Yan, S.; Yang, Q.; Kong, B. Frequent hypermethylation and loss of heterozygosity of the testis derived transcript gene in ovarian cancer. *Cancer Sci.* **2010**, *101*, 1255–1260. [CrossRef]
44. Szymańska-Chabowska, A.; Juzwiszyn, J.; Jankowska-Polańska, B.; Tański, W.; Chabowski, M. Chitinase 3-Like 1, Nestin, and Testin Proteins as Novel Biomarkers of Potential Clinical Use in Colorectal Cancer: A Review. *Health Med.* **2020**, *1279*, 1–8.
45. Zhong, Y.; Zhu, J.; Wang, Y.; Zhou, J.; Ren, K.; Ding, X.; Zhang, J. LIM domain protein TES changes its conformational states in different cellular compartments. *Mol. Cell. Biochem.* **2009**, *320*, 85–92. [CrossRef] [PubMed]

current issues in molecular biology

MDPI

*Article*

# The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics

**Aneta Popiel-Kopaczyk [1,*], Jedrzej Grzegrzolka [1], Aleksandra Piotrowska [1], Mateusz Olbromski [1], Beata Smolarz [2], Hanna Romanowicz [2], Agnieszka Rusak [1], Monika Mrozowska [1], Piotr Dziegiel [1,3], Marzenna Podhorska-Okolow [4] and Christopher Kobierzycki [1]**

[1] Division of Histology and Embryology, Department of Human Morphology and Embryology, Wroclaw Medical University, 50-367 Wroclaw, Poland
[2] Department of Pathology, Polish Mother's Memorial Hospital Research Institute, 93-338 Lodz, Poland
[3] Department of Physiotherapy, University School of Physical Education, 51-612 Wroclaw, Poland
[4] Division of Ultrastructural Research, Wroclaw Medical University, 50-368 Wroclaw, Poland
* Correspondence: popielaneta1@gmail.com or aneta.popiel@student.umed.wroc.pl

**Abstract:** Testin is a protein expressed in normal human tissues, being responsible, with other cytoskeleton proteins, for the proper functioning of cell–cell junction areas and focal adhesion plaques. It takes part in the regulation of actin filament changes during cell spreading and motility. Loss of heterozygosity in the testin-encoding gene results in altered protein expression in many malignancies, as partly described for cervical cancer. The aim of our study was the assessment of the immunohistochemical (IHC) expression of testin in cervical cancer and its analysis in regard to clinical data as well the expression of the Ki-67 antigen and p16 protein. Moreover, testin expression was assessed by Western blot (WB) in commercially available cell lines. The IHC analysis disclosed that the expression of testin inversely correlated with p16 ($r = -0.2104$, $p < 0.0465$) and Ki-67 expression ($r = -0.2359$, $p < 0.0278$). Moreover, weaker testin expression was observed in cancer cases vs. control ones ($p < 0.0113$). The WB analysis of testin expression in the cervical cancer cell lines corresponded to the IHC results and showed a weaker expression compared to that in the control cell line. When we compared the expression of testin in cervical cancer cell lines, we found a weaker expression in HPV-negative cell lines. In summary, we found that the intensity of testin expression and the number of positive cells inversely correlated with the expression of Ki-67 (a marker of proliferation) and p16 (a marker of cell cycle dysregulation). This study shows that the combined assessment of testin, Ki-67 and p16 expression may improve cervical cancer diagnostics.

**Keywords:** TES protein; p16 protein; antigen Ki-67; cervical cancer; immunohistochemistry



**Citation:** Popiel-Kopaczyk, A.; Grzegrzolka, J.; Piotrowska, A.; Olbromski, M.; Smolarz, B.; Romanowicz, H.; Rusak, A.; Mrozowska, M.; Dziegiel, P.; Podhorska-Okolow, M.; et al. The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics. *Curr. Issues Mol. Biol.* **2023**, *45*, 490–500. https://doi.org/10.3390/cimb45010032

Academic Editor: Peter C. Hart

Received: 8 December 2022
Revised: 29 December 2022
Accepted: 2 January 2023
Published: 5 January 2023



## 1. Introduction

Cervical cancer is one of the most common cancers worldwide in women, with almost 350,000 deaths noted in 2020 [1]. Over 85% of cervical cancer deaths occurred in low and middle-income countries. In South Africa and South America countries, cervical cancer is the leading cause of death in women. Approximately, only 66.3% of diagnosed patient will survive 5 years or more. Due to the unsatisfactory prophylaxis and ineffective screening, the incidence of cervical cancer is still increasing in developing countries [2].

The main identified risk factor for cervical cancer is persistent HPV (human papilloma virus) infection, which induced the development of low- and subsequently high-grade cervical squamous intraepithelial lesions (LSIL and HSIL). Imbalances and instabilities caused by the introduction of various HPV-derived oncogenic factors into the host genome result in long-term tumor progression, but the outcomes depend on the specific HPV subtypes, mostly the high-risk subtypes (HR-HPV) [3]. Currently, 216 HPV subtypes were identified; HR-HPV 16 is responsible for more than 50% of cancer cases, while HR-HPV 18 is responsible for up to 16.5% of cancer cases [4]. Presently, the performed studies

focus on seeking additional factors such as genetic and epigenetic triggers needed for the promotion and progression of cancerogenesis in patients with persistent HR-HPV infection [5–8]. Epigenetic changes are modifications of the DNA in a genome that regulate whether genes are turned off without changing the sequence of the DNA [9]. They include DNA methylation, histone modification and miRNA silencing [10].

In 2014, the estimated HPV vaccination rates in young women were around 30% in developed countries but less than 3% in least-developed countries. This clearly supports the World Health Organization goals for cervical cancer elimination, which primarily should be focused on prevention, screening and treatment of preinvasive and invasive forms of cervical cancer in low- and middle-income countries [11].

Testin is a protein encoded by the TES gene located in the fragile chromosomal region FRA7G at 7q31.1/2. In this locus, loss of heterozygosity induces the development of many malignancies as this region contains putative tumor suppressor genes such as caveolin-1 and TES [12,13]. In loss of heterozygosity, one or two alleles of the same gene are lost, so suppressor genes are inactivated. This is one of the mechanisms that decrease testin expression in cancer cells. The second mechanism leading to the loss of testin expression is associated with hypermethylation of CpG islands [14,15].

Testin is also expressed in normal human tissues. It is localized along actin stress fibers in cell–cell junction areas and in focal adhesion plaques where it can interact with other cytoskeleton-associated proteins such as talin (TLN), actin (ACT), vasodilator-stimulated phosphoprotein (VASP) and mammalian Ena homolog (Mena) [16–18]. Testin can take part in the regulation of actin filament changes during cell spreading and motility. The structure of testin includes three C-terminal LIM domains which have three zinc-binding domains that are responsible for protein–protein interactions and coordinate intra- and extracellular connections [19,20]. The NH-2 terminal of the TES protein can bind to its COOH- terminus. Therefore Boyan K. Garvalov et al. proposed that TES might have two conformational states: open and closed [17]. Zhong et al. confirmed that the NH-2 terminus binds to the third LIM domain but additionally found different cellular localizations of the protein. Besides the well-known localizations such as focal adhesions and cell junctions, they found the testin protein in nucleoli and the endoplasmic reticulum. This means that testin, just like other LIM protein members, is able to shuttle between the cytoplasmic and the nuclear compartments of the cell to influence gene expression [21–23]. The altered expression of the testin protein was described in many malignancies such as breast, colorectal, endometrial, gastric, head and neck, ovarian and prostate cancers as well in leukemia [24–31]. To date, the expression of testin was disclosed only in two cervical cancer studies [32,33].

The aim of our study was the assessment of the immunohistochemical (IHC) expression of testin in cervical cancer and its analysis in regard to clinical data as well the expression of the Ki-67 antigen and the p16 protein. Moreover, testin expression was assessed by Western blot (WB) on commercially available cell lines.

## 2. Materials and Methods

The study was approved by the Ethics Commission at the Wroclaw Medical University (approval no. 412/2019). Patient consent was waived due to the use of anonymized archival material only, which in no way influenced the diagnostic and therapeutic process.

A total of 91 cervical cancer samples were collected from women treated in the Polish Mother's Memorial Hospital in Lodz between 2011 and 2017. The control group consisted of 92 normal tissue cases from patients who underwent total hysterectomy due to uterine leiomyomas in the same hospital. The characterization of the study group is described in Table 1.

**Table 1.** Characterization of the study group and histological types of the cervical cancer samples.

| Parameters | No. | (%) |
|---|---|---|
| **Age (years)** | | |
| <35 | 7 | 7.69 |
| 35–45 | 22 | 24.18 |
| >45 | 62 | 68.13 |
| **Grade** | | |
| G1 | 18 | 19.78 |
| G2 | 57 | 62.64 |
| G3 | 16 | 17.58 |
| **Histological types** | | |
| **Carcinoma planoepithelial** | 73 | 80.22 |
| Adenocarcinoma | 12 | 13.19 |
| Other than carcinoma planoepithelial and adenocarcinoma | 6 | 6.59 |

### *2.1. Preparation of Tissue Microarrays (TMAs)*

Hematoxylin-and-eosin-stained (HE) 6 µm thick paraffin sections were prepared to verify the histopathological diagnosis and assess the suitability of the samples for further analysis. In short, slides were scanned utilizing the histologic scanner Pannoramic MIDI (3DHistech, Sysmex Suisse AG, Horgen, Switzerland). The scans were then examined by two independent pathologists to select and electronically label the areas of cervical cancer cells. Then, for TMA construction, from the corresponding paraffin donor blocks, triplicate tissue core punches (2 mm) for every case were obtained (TMA Grand Master; 3DHistech). Normal cervical tissues were used as the control group.

### *2.2. Immunohistochemistry (IHC)*

The immunohistochemical reactions were performed on 4 µm paraffin sections obtained from the TMA blocks using an automated staining platform, Autostainer Link48 (Dako, Glostrup, Denmark). At the beginning, deparaffinization, rehydration and antigen retrieval were performed using the EnVision FLEX Target Retrieval Solution (97 °C, 20 min; pH 6 for Ki-67 and pH 9 for p16 and testin) in PT-Link. The activity of endogenous peroxidase was blocked by 5 min exposure to Peroxidase-Blocking Reagent (Dako). The monoclonal mouse antibodies anti-p16 (1:100 + linker, 550834, BP Pharmingen, San Diego, CA, USA) and anti-Ki-67 (ready to use, IR626, Dako) and the polyclonal rabbit anti-testin (1:400, NBP1-87987, Novus Biologicals, Centennial, CO, USA) antibody were used as the primary antibodies (20 min. incubation) followed by incubation with secondary antibodies conjugated with horseradish peroxidase (EnVision™ FLEX/HRP—20 min. incubation). After the incubation, 3,3′-diaminobenzidine (DAB) was used as the peroxidase substrate, and the sections were incubated for 10 min. At the end, all sections were counterstained with EnVision FLEX Hematoxylin (Dako) for 5 min. Dehydration in graded ethanol concentrations (70%, 96%, absolute) and in xylene were the last steps, and then all slides were covered with coverslips in SUB-X Mounting Medium using a coverslipper.

### *2.3. Evaluation of the IHC Reactions*

The slides were scanned with the histologic scanner Pannoramic MIDI (3DHistech). The expression was nuclear for Ki-67 and cytoplasmic for p16 and testin. The reactions were evaluated (Ki-67) with the use of Quant Center Software (3DHistech) under the researchers' supervision. For every case, three TMA cores were quantified by the algorithm SCORE (range = 0–8), and the final result was an average count. Two parameters were used to evaluate the p16 antigen: percentage of p16-positive cells and reaction intensity. The percentage of positive cells was evaluated in the highest expression area ("hot spot") and graded as follows: grade 0 when no cells were stained, positive cells >0–5% (grade 1), positive cells >5–25% (grade 2), positive cells >25% (grade 3). The intensity of the reaction was scored as negative (0), weak (1), moderate (2) and strong (3). The reaction

was considered positive when nuclear or nuclear and cytoplasmic, strong and diffuse p16 expression was observed starting from the basal cell layer of the epithelium. The negative p16 expression was assessed when a non-specific pattern, or focal, wispy, small clusters of cells and complete lack of staining were observed. The expression of testin was assessed with the use of Pannoramic Viewer Digital image. The analysis was carried out using Immunoreactive Scale (IRS) by Remmele and Stegner, as shown in Table 2. Testin cytoplasmatic expression was assessed by using the Remmele–Stegner immunoreactive score [34]. This scale uses the percentage of positively stained cells (A) and the staining intensity of the reaction (B). The final result is the product of these two parameters (A × B).

**Table 2.** Scoring system by Remmele and Stegner (IRS, Immunoreactive Score) taking into account the percentage of cells and the intensity of the reaction.

| Score | Percentage of Positively Stained Cells (PP) | Intensity of Staining (SI) | IRS Points (PP × SI) | IRS Classification |
|---|---|---|---|---|
| **0** | no staining | no color reaction | 0–1 | Negative |
| **1** | <10% | weak reaction | 2–3 | Positive, weak expression |
| **2** | 10–50% | moderate reaction | 4–8 | Positive, moderate expression |
| **3** | 51–80% | strong reaction | 9–12 | Positive, strong expression |
| **4** | >80% | | | |

### 2.4. Cell Lines

The human cervical cancer cells SiHa and C-33 A were cultured in Eagle's minimum essential medium (EMEM, Lonza, Basel, Switzerland) supplemented with 1% sodium pirogronate and non-essential amino acids (Sigma-Aldrich, St. Louis, MO, USA). HeLa cells were cultured in EMEM (Lonza). The human epidermal keratinocytes HaCaT (DKFZ, Heidelberg, Germany) cell line was cultured in DMEM medium (Lonza) [35]. All media contained 1% L-glutamine, a penicillin/streptomycin solution and 10% FBS (fetal bovine serum) (Sigma-Aldrich). The cell cultures were maintained at 37 °C/5% CO2 and 95% humidity. The medium was changed twice a week, and the cells were passaged with a trypsin/EDTA solution (Sigma-Aldrich) when confluency reached about 70%.

### 2.5. Western Blot

The Western blot technique was used to determine testin expression in cervical cancer cell lines. Whole protein lysates were obtained by using the CelLytic TM MT Cell Lysis Reagent (Sigma Aldrich) with the addition of a cocktail of inhibitors (Sigma-Aldrich), 250 U of benzonase (Merck Millipore, USA) and 2 mM PMSF. The protein lysates were mixed with 4xSDS-PAGE gel loading buffer (200 mM Tris-HCl—pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol), loaded on a 10% acrylamide gel and separated by SDS-PAGE under reducing conditions, then finally transferred onto a PVDF membrane in the XCell SureLockTM Mini Gel Electrophoresis System (Life Technologies, Carlsbad, USA, USA). After protein transfer, the membranes were incubated in a blocker solution (4% BSA in TBST buffer) for 1 h at RT, followed by overnight incubation at 4 °C with the anti-testin monoclonal mouse antibody, diluted 1:500. The next step included washing the membranes with TBST buffer and incubating them for 1 h at RT with secondary donkey anti-rabbit antibodies conjugated with HRP, diluted 1:3000 (Jacksons ImmunoResearch, Mill Valley, CA, USA). Afterwards, the membranes were rinsed and treated with the Immun-Star HRP Chemiluminescent Kit (Bio-Rad, Hercules, CA, USA). Rabbit anti-human β-tubulin antibodies (2128, CellSignaling, Danvers, MA, USA) diluted 1:1000 were used as an internal control. The WB results were analyzed in the ChemiDoc MP System (Bio-Rad).

### *2.6. Statistical Analysis*

All data were analyzed with GraphPad Prism 5.0 software using Spearman correlation, Kruskal–Wallis and Mann–Whitney tests. The analysis of the normal distribution of the obtained data was made by the Kolmogorov–Smirnov test. In all analyzed cases, $p$-values < 0.05 were considered statistically significant.

## 3. Results

### *3.1. IHC on Tissue Samples*

The immunohistochemical reactions for testin, p16 and Ki-67 expression were performed on all tissue specimens, i.e., 91 cervical cancer samples and 92 normal cervical tissue samples (Table 1). The histopathological analysis disclosed 73 cases of carcinoma planoepithelial, 12 cases of adenocarcinoma, 1 case of adenosquamous carcinoma and 5 cases of other histological types. The IHC analysis by Spearman correlation test showed that the expression of testin inversely correlated with the expression of p16 (r = −0.2104, $p < 0.0465$; Figure 1A) and Ki-67 (r = −0.2359, $p = 0.0278$; Figure 1B). Additionally, a positive correlation was observed between the expression of Ki-67 and p16 ($p < 0.0082$, r = 0.2819 Spearman correlation test; Figure 1C). The expressions of p16 and Ki-67 were significantly stronger in cancer cases than in the control group ($p < 0.0001$ for both; Mann–Whitney test; Figure 1E,F). In contrast, the expression of testin was significantly weaker in cancer cases than in the control group ($p < 0.0113$; Mann–Whitney test; Figure 1D). We observed a positive cytoplasmic reaction for testin (Figure 2A,C) and p16 protein (Figure 2G–I). The nuclear positive expression of Ki-67 correlated with the histological grade (Figure 2D–F).

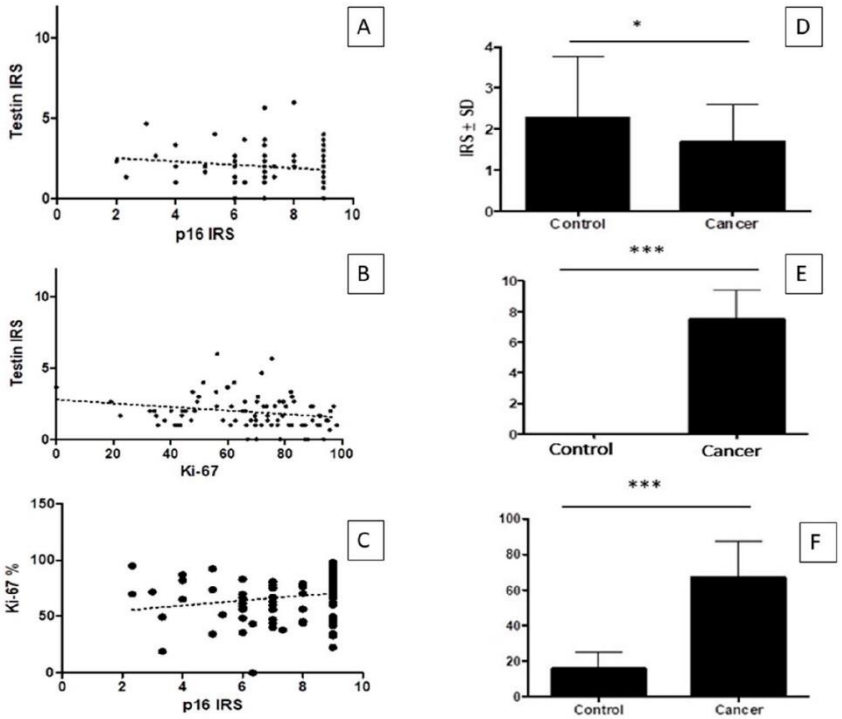


**Figure 1.** Immunohistochemical reactions in cervical cancer samples: (**A**) testin and p16 correlation in cervical cancer; (**B**) testin and Ki-67 correlation in cervical cancer; (**C**) Ki-67 and p16 correlation in cervical cancer; (**D**) testin expression in cervical cancer and control group; (**E**) p16 expression in cervical cancer and control group; (**F**) Ki-67 expression in cervical cancer and control group * $p < 0.05$ and *** $p < 0.001$.

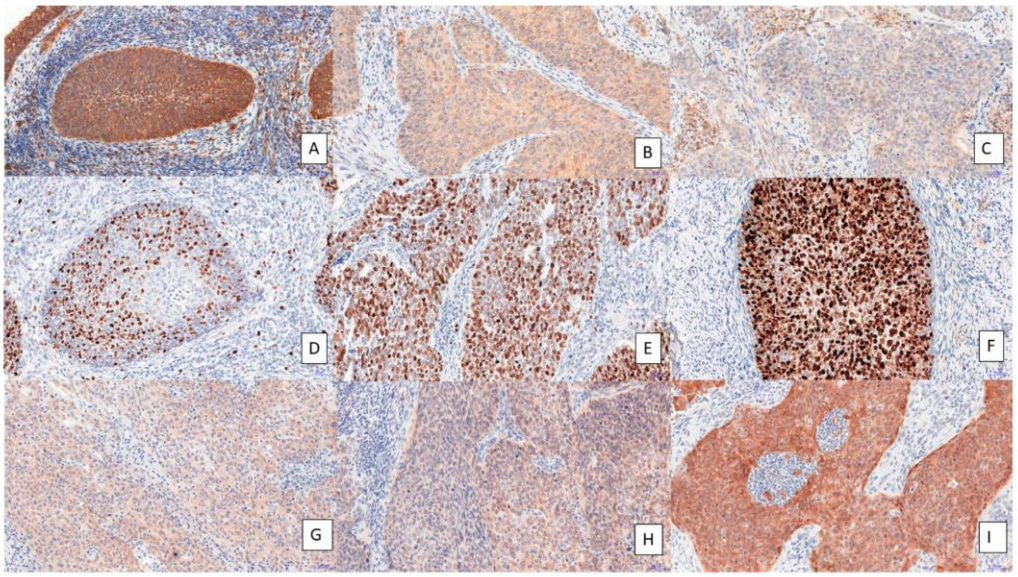


**Figure 2.** Immunohistochemical expression of testin in cervical cancer samples of different histological grades ((**A**), G1; (**B**), G2; (**C**), G3), Ki-67 ((**D**), G1; (**E**), G2; (**F**), G3) and p16 protein ((**G**), G1; (**H**), G2; (**I**), G3) in cervical cancer cases. Magnification ×200.

### *3.2. WB on Cell Lines*

The analysis of testin expression was performed on the cervical cancer cell lines HeLa, SiHa (HPV positive) and C-33 A (HPV negative), as well on a human keratinocyte cell line (HaCaT) as a control. The WB analysis of testin expression in all cervical cancer cell lines corresponded to the IHC results and showed a weaker expression compared to that in the HaCaT cell line. When we compared the expression of testin in the cervical cancer cell lines, we found a weaker expression of testin in HPV-negative cell lines (Figures 3 and 4). The expression of two isoforms of the p16 protein was lower in HaCaT, HeLa and SiHa cell lines compared to testin expression (Figure 5). The above results evaluated the presence of the protein within the cells. Table 3 present the quantification of the results of WB.

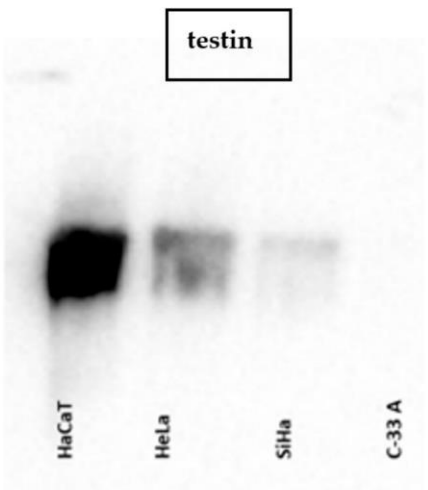


**Figure 3.** WB: testin expression in HaCat, HeLa, SiHa and C-33 A cell lines.

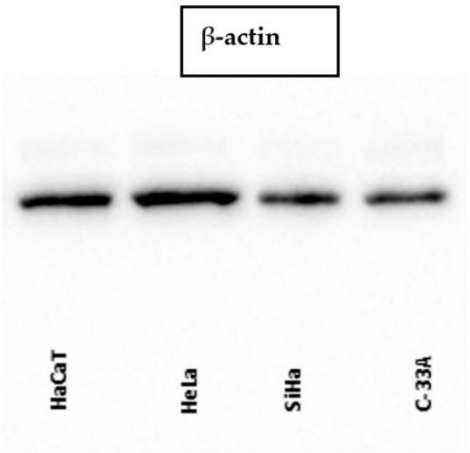


**Figure 4.** WB: β-actin expression in HaCat, HeLa, SiHa and C-33 A cell lines. The molecular weight of the proteins is: testin 54 kDa, β-actin 42 kDa.

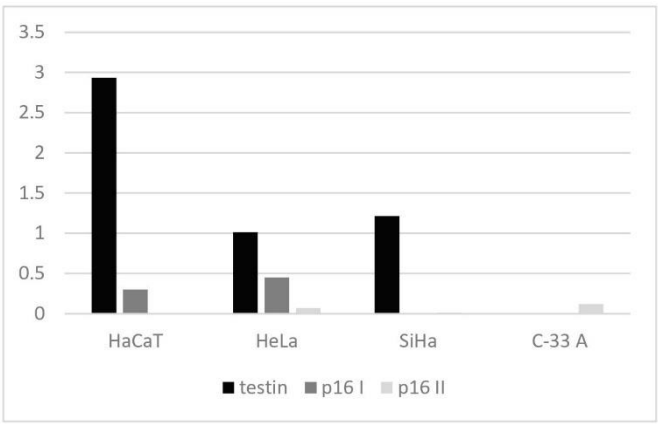


**Figure 5.** WB: testin and p16 expression in HaCat, HeLa, SiHa and C-33 A cell lines.

**Table 3.** Quantification of the WB bands for testin and β-actin.

| | Control | | | Testin | | | |
|---|---|---|---|---|---|---|---|
| | **β-Actin I** | **β-Actin II** | **β-Actin Average** | **Testin I** | **Testin II** | **Testin Average** | **Testin Normallized** |
| **HaCaT** | 2,179,147 | 1,980,546 | 2,079,847 | 6,246,846 | 5,946,842 | 6,096,844 | 2.931391331 |
| **HeLa** | 2,362,978 | 1,897,653 | 2,130,316 | 2,209,494 | 2,106,849 | 2,158,171.5 | 1.013075997 |
| **SiHa** | 2,008,224 | 2,012,950 | 2,010,587 | 471,170 | 4,405,306 | 2,438,238 | 1.212699575 |
| **C-33A** | 1,989,734 | 2,106,843 | 2,048,289 | 12,936 | 10,310 | 11,623 | 0.005674494 |

## 4. Discussion

Cervical cancer is one of the most malignant cancers among women. With a better understanding of the etiopathogenesis of this disease supported by appropriate screening, we may reduce the risk of death. Nowadays, cervical cancer screening is based on HPV testing and cytology. Depending on the local gynecological society recommendations, routine screening may vary in certain countries. Barely, these tests are based on age, belonging to a risk group or/and the HR-HPV status. In some cases, we expand the screening to clarify the diagnosis. For this reason, we need to perform histological additional testing. With

the development of cervical cancer screening, immunohistochemical reactions in cytology has shown great promise for cervical screening [36,37]. Biomarkers which could provide detailed information about cancer prognosis and progression are in urgent need. In this study, we analyzed for the first time the relationship between widely used markers such as p16 and Ki-67 and testin. The low testin expression in cervical cancer cells is in line with that shown by Gu et al., who indicated in both in vitro and in vivo models the function of testin in cell proliferation and invasion in endometrial cancer [38]. Testin is expressed in almost all normal human tissues. We found various expression levels of the testin protein in cervical cancer-derived cell lines and compared it with the expression in human epidermal keratinocytes, i.e., the HaCaT cell line. Previously, Gu et al. showed that testin mRNA expression depends on the cervical cancer cell line. It was shown that testin suppressed cell proliferation through inhibiting cell cycle progression by arresting the cells in the G1 phase of the cell cycle [33]. Zhu et al. indicated the role of testin in cell migration and invasion and pointed out the protective capacity of testin in tumor metastasis and angiogenesis [38]. IHC expression analysis of p16 with Ki-67 is routinely performed in cervical cancer screening using commercial kits, e.g., CINtec, Dalton [37,39,40]. The expression of p16 slows the progression of the cell cycle from the G1 phase to the S-phase and inhibits the phosphorylation of the retinoblastoma protein (pRb) mediated through cyclin-dependent kinases [41]. Persistent HPV infection leads to p16 overexpression and consequently to Rb functionally inactivation by the HPV E7 oncoprotein [42,43]. In the present study, the correlation between testin and p16 expression was moderate and negative. However, there was a strong positive correlation between Ki-67 and p16 expression. The expression of p16 did not show any difference depending on the histological grade of cervical cancer. The combined overexpression of p16 and Ki-67 in normal cervical tissue is less likely to occur [37,44]. Thus, the analysis assessment of the expression of these markers is widely used in cervical cancer screening as a valuable tool for evaluating cell cycle deregulation and cell transformation due to HPV infection [45,46]. Ki-67 is a marker strictly associated with cell proliferation, expressed in all active phases of the cell cycle (G1, S, G2, M) except the G0 phase. In normal cervical tissue, the expression of Ki-67 is restricted to one-third of the basal layer of the epithelium but in dysplasia and cancer, it is expanded above the basal and parabasal layers, and the number of positive cells increases [47]. Our study disclosed that Ki-67 overexpression was present in 100% of invasive cervical cancer samples. This is concordant with Shi et al. study [42]. The positivity rate of Ki-67 expression was significantly higher in cervical cancer cells than in control cells.

*Limitations*

Our study has some limitations. The major limitation is the small study sample. Moreover, the number of analytical methods used (IHC and WB) was limited; however, additional techniques such as at least reverse transcription–polymerase chain reaction (RT-PCR) are planned in the future.

## 5. Conclusions

In summary, we found that the intensity of testin expression and the number of positive cells reversely correlated with the expression of Ki-67 (a marker of proliferation) and p16 (a marker of cell cycle dysregulation). These three markers may complement each other, and their combined assessment may improve cervical cancer diagnostics. As in this study protein expression was presented, gene expression remains to be explored. Not-withstanding and unquestionably, further studies are necessary to discuss the above-presented results in the broadest context as possible.

**Author Contributions:** Conceptualization, A.P.-K., P.D. and C.K.; methodology, A.P., A.R., M.O., A.P.-K. and M.M.; software, J.G.; validation, J.G., M.P.-O., A.P.-K. and A.P.; formal analysis, J.G.; investigation, A.P.-K.; resources, B.S. and H.R.; data curation, J.G.; writing—original draft preparation, A.P.-K., B.S., H.R., A.R., J.G. and P.D.; writing—review and editing, C.K. and P.D.; visualization, A.P.-K., A.P. and M.O.; supervision, P.D., C.K. and M.P.-O.; project administration, A.P.-K.; funding acquisition, A.P.-K. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Science Centre, Poland grant number, 2018/29/N/NZ5/01911. The APC was funded by the National Science Centre.

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Medical University in Wroclaw (6 May 2019; protocol code 411/2019).

**Informed Consent Statement:** Patient consent was waived due to the use of anonymized archival material only, which in no way influenced the diagnostic and therapeutic process.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy issues.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J. Clin.* **2021**, *71*, 209–249. [CrossRef] [PubMed]
2. Cervical Cancer—Cancer Stat Facts, (n.d.). Available online: https://seer.cancer.gov/statfacts/html/cervix.html (accessed on 22 May 2022).
3. Olusola, P.; Banerjee, H.N.; Philley, J.V.; Dasgupta, S. Human Papilloma Virus-Associated Cervical Cancer and Health Disparities. *Cells* **2019**, *8*, 622. [CrossRef] [PubMed]
4. Crow, J.M. HPV: The global burden. *Nature* **2012**, *488*, S2–S3. [CrossRef] [PubMed]
5. Fullár, A.; Karászi, K.; Hollósi, P.; Lendvai, G.; Oláh, L.; Reszegi, A.; Papp, Z.; Sobel, G.; Dudás, J.; Kovalszky, I. Two ways of epigenetic silencing of TFPI2 in cervical cancer. *PLoS ONE* **2020**, *15*, e0234873. [CrossRef]
6. Cáceres-Durán, M.Á.; Ribeiro-Dos-Santos, Â.; Vidal, A.F. Roles and Mechanisms of the Long Noncoding RNAs in Cervical Cancer. *Int. J. Mol. Sci.* **2020**, *21*, 9742. [CrossRef]
7. Revathidevi, S.; Murugan, A.K.; Nakaoka, H.; Inoue, I.; Munirajan, A.K. APOBEC: A molecular driver in cervical cancer pathogenesis. *Cancer Lett.* **2020**, *496*, 104–116. [CrossRef]
8. Zhang, L.; Tian, S.; Pei, M.; Zhao, M.; Wang, L.; Jiang, Y.; Yang, T.; Zhao, J.; Song, L.; Yang, X. Crosstalk between histone modification and DNA methylation orchestrates the epigenetic regulation of the costimulatory factors, Tim-3 and galectin-9, in cervical cancer. *Oncol. Rep.* **2019**, *42*, 2655–2669. [CrossRef]
9. Laengsri, V.; Kerdpin, U.; Plabplueng, C.; Treeratanapiboon, L.; Nuchnoi, P. Cervical Cancer Markers: Epigenetics and microRNAs. *Lab. Med.* **2018**, *49*, 97–111. [CrossRef]
10. Sharma, S.; Kelly, T.K.; Jones, P.A. Epigenetics in cancer. *Carcinogenesis* **2010**, *31*, 27–36. [CrossRef]
11. Canfell, K. Towards the global elimination of cervical cancer. *Papillomavirus Res.* **2019**, *8*, 100170. [CrossRef]
12. Tatarelli, C.; Linnenbach, A.; Mimori, K.; Croce, C.M. Characterization of the Human TESTIN Gene Localized in the FRA7G Region at 7q31.2. *Genomics* **2000**, *12*, 1–12. [CrossRef] [PubMed]
13. Tobias, E.S.; Hurlstone, A.F.L.; MacKenzie, E.; McFarlane, R.; Black, D.M. The TES gene at 7q31.1 is methylated in tumours and encodes a novel growth-suppressing LIM domain protein. *Oncogene* **2001**, *20*, 2844–2853. [CrossRef]
14. Gong, Y.; Yang, Y.; Tian, S.; Chen, H. Different Role of Caveolin-1 Gene in the Progression of Gynecological Tumors. *Asian Pac. J. Cancer Prev.* **2019**, *20*, 3259–3268. [CrossRef] [PubMed]
15. Weeks, R.J.; Kees, U.R.; Song, S.; Morison, I.M. Silencing of TESTIN by dense biallelic promoter methylation is the most common molecular event in childhood acute lymphoblastic leukaemia. *Mol. Cancer* **2010**, *9*, 163. [CrossRef] [PubMed]
16. Coutts, A.; MacKenzie, E.; Griffith, E.; Black, D.M. TES is a novel focal adhesion protein with a role in cell spreading. *J. Cell Sci.* **2003**, *116*, 897–906. [CrossRef] [PubMed]
17. Garvalov, B.K.; Higgins, T.E.; Sutherland, J.D.; Zettl, M.; Scaplehorn, N.; Köcher, T.; Piddini, E.; Griffiths, G.; Way, M. The conformational state of Tes regulates its zyxin-dependent recruitment to focal adhesions. *J. Cell Biol.* **2003**, *161*, 33–39. [CrossRef]
18. Griffith, E.; Coutts, A.S.; Black, D.M. Characterisation of chicken TES and its role in cell spreading and motility. *Cell Motil. Cytoskelet.* **2004**, *57*, 133–142. [CrossRef]
19. Steponaitis, G.; Kazlauskas, A.; Skiriute, D.; Valiulyte, I.; Skauminas, K.; Tamasauskas, A.; Vaitkiene, P. Testin (TES) as a candidate tumour suppressor and prognostic marker in human astrocytoma. *Oncol. Lett.* **2016**, *12*, 3305–3311. [CrossRef]
20. Popiel, A.; Kobierzycki, C.; Dzięgiel, P. The Role of Testin in Human Cancers. *Pathol. Oncol. Res.* **2019**, *25*, 1279–1284. [CrossRef]

21. Khurana, T.; Noegel, A.A. LIM proteins: Association with the actin cytoskeleton. *Protoplasma* **2002**, *219*, 1–12. [CrossRef]
22. Cattaruzza, M.; Lattrich, C.; Hecker, M. Focal Adhesion Protein Zyxin Is a Mechanosensitive Modulator of Gene Expression in Vascular Smooth Muscle Cells. *Hypertension* **2004**, *43*, 726–730. [CrossRef]
23. Kadrmas, J.L.; Beckerle, M.C. The LIM domain: From the cytoskeleton to the nucleus. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 920–931. [CrossRef]
24. Edelson, M.I.; Scherer, S.W.; Tsui, L.C.; Welch, W.R.; Bell, D.A.; Berkowitz, R.S.; Mok, S.C. Identification of a 1300 kilobase deletion unit on chromosome 7q31.3 in invasive epithelial ovarian carcinomas. *Oncogene* **1997**, *14*, 2979–2984. [CrossRef] [PubMed]
25. Bieche, I.; Khodja, A.; Driouch, K.; Lidereau, R. Genetic alteration mapping on chromosome 7 in primary breast cancer. *Clin. Cancer Res.* **1997**, *3*, 1009–1016. [PubMed]
26. Matsuura, K.; Shiga, K.; Yokoyama, J.; Saijo, S.; Miyagi, T.; Takasaka, T. Loss of heterozygosity of chromosome 9p21 and 7q31 is correlated with high incidence of recurrent tumor in head and neck squamous cell carcinoma. *Anticancer Res.* **1998**, *18*, 453–458. [PubMed]
27. Nishizuka, S.; Tamura, G.; Terashima, M.; Satodate, R. Commonly deleted region on the long arm of chromosome 7 in differentiated adenocarcinoma of the stomach. *Br. J. Cancer* **1997**, *76*, 1567–1571. [CrossRef]
28. Koike, M.; Tasaka, T.; Spira, S.; Tsuruoka, N.; Koeffler, H.P. Allelotyping of acute myelogenous leukemia: Loss of heterozygosity at 7q31.1 (D7S486) and q33-34 (D7S498, D7S505). *Leuk. Res.* **1999**, *23*, 307–310. [CrossRef]
29. Latil, A.; Cussenot, O.; Fournier, G.; Baron, J.C.; Lidereau, R. Loss of heterozygosity at 7q31 is a frequent and early event in prostate cancer. *Clin. Cancer Res.* **1995**, *1*, 1385–1389. [CrossRef]
30. Wang, M.; Wang, Q.; Peng, W.-J.; Hu, J.-F.; Wang, Z.-Y.; Liu, H.; Huang, L.-N. Testin is a tumor suppressor in non-small cell lung cancer. *Oncol. Rep.* **2017**, *37*, 1027–1035. [CrossRef]
31. Szymańska-Chabowska, A.; Juzwiszyn, J.; Jankowska-Polańska, B.; Tański, W.; Chabowski, M. Chitinase 3-Like 1, Nestin, and Testin Proteins as Novel Biomarkers of Potential Clinical Use in Colorectal Cancer: A Review. In *Advances in Experimental Medicine and Biology*; Springer: Cham, Switzerland, 2020; pp. 1–8. [CrossRef]
32. Dong, R.; Pu, H.; Wang, Y.; Yu, J.; Lian, K.; Mao, C. TESTIN was commonly hypermethylated and involved in the epithelial-mesenchymal transition of endometrial cancer. *APMIS* **2015**, *123*, 394–400. [CrossRef]
33. Cui, J.; Gu, Z.; Ding, G.; Liang, K.; Zhang, H.; Guo, G.; Zhang, L. TESTIN suppresses tumor growth and invasion via manipulating cell cycle progression in endometrial carcinoma. *J. Pharmacol. Exp. Ther.* **2014**, *20*, 980–987. [CrossRef] [PubMed]
34. Remmele, W.; Stegner, H.E. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. *Pathologe* **1987**, *8*, 138–140. [PubMed]
35. Boukamp, P. Normal Keratinization in a Spontaneously Immortalized. *J. Cell Biol.* **1988**, *106*, 761–771. [CrossRef] [PubMed]
36. van Dam, P.A.; Rolfo, C.; Ruiz, R.; Pauwels, P.; Van Berckelaer, C.; Trinh, X.B.; Gandia, J.F.; Bogers, J.P.; Van Laere, S. Potential new biomarkers for squamous carcinoma of the uterine cervix. *ESMO Open* **2018**, *3*, e000352. [CrossRef] [PubMed]
37. Sun, H.; Shen, K.; Cao, D. Progress in immunocytochemical staining for cervical cancer screening. *Cancer Manag. Res.* **2019**, *11*, 1817–1827. [CrossRef]
38. Zhu, J.; Li, X.; Kong, X.; Moran, M.S.; Su, P.; Haffty, B.G.; Yang, Q. Testin is a tumor suppressor and prognostic marker in breast cancer. *Cancer Sci.* **2012**, *103*, 2092–2101. [CrossRef]
39. Li, Y.; Fu, Y.; Cheng, B.; Xie, X.; Wang, X. A Comparative Study on the Accuracy and Efficacy between Dalton and CINtec® PLUS p16/Ki-67 Dual Stain in Triaging HPV-Positive Women. *Front. Oncol.* **2022**, *11*, 815213. [CrossRef]
40. Zhang, S.-K.; Jia, M.-M.; Zhao, D.-M.; Wu, Z.-N.; Guo, Z.; Liu, Y.-L.; Guo, P.-P.; Chen, Q.; Cao, X.-Q.; Liu, S.-Z.; et al. Evaluation of p16/Ki-67 dual staining in the detection of cervical precancer and cancer in China. *Cancer Epidemiol.* **2019**, *59*, 123–128. [CrossRef]
41. Sarwath, H.; Bansal, D.; Husain, N.E.; Mohamed, M.; Sultan, A.A.; Bedri, S. Introduction of p16INK4a as a surrogate biomarker for HPV in women with invasive cervical cancer in Sudan. *Infect. Agents Cancer* **2017**, *12*, 50. [CrossRef]
42. Shi, Q.; Xu, L.; Yang, R.; Meng, Y.; Qiu, L. Ki-67 and P16 proteins in cervical cancer and precancerous lesions of young women and the diagnostic value for cervical cancer and precancerous lesions. *Oncol. Lett.* **2019**, *18*, 1351–1355. [CrossRef]
43. Song, F.; Du, H.; Xiao, A.; Wang, C.; Huang, X.; Yan, P.; Liu, Z.; Qu, X.; Belinson, J.L.; Wu, R. Evaluating the Performance of p16INK4a Immunocytochemistry in Cervical Cancer Screening. *Cancer Manag. Res.* **2020**, *12*, 9067–9075. [CrossRef] [PubMed]
44. Iaconis, L.; Hyjek, E.; Ellenson, L.H.; Pirog, E.C. p16 and Ki-67 Immunostaining in Atypical Immature Squamous Metaplasia of the Uterine Cervix: Correlation With Human Papillomavirus Detection. *Arch. Pathol. Lab. Med.* **2007**, *131*, 1343–1349. [CrossRef] [PubMed]
45. Peeters, E.; Wentzensen, N.; Bergeron, C.; Arbyn, M. Meta-analysis of the accuracy of p16 or p16/Ki-67 immunocytochemistry versus HPV testing for the detection of CIN2+/CIN3+ in triage of women with minor abnormal cytology. *Cancer Cytopathol.* **2019**, *127*, 169–180. [CrossRef] [PubMed]

46. Zhu, Y.; Ren, C.; Yang, L.; Zhang, X.; Liu, L.; Wang, Z. Performance of p16/Ki67 immunostaining, HPV E6/E7 mRNA testing, and HPV DNA assay to detect high-grade cervical dysplasia in women with ASCUS. *BMC Cancer* **2019**, *19*, 271. [CrossRef]
47. Keating, J.T.; Cviko, A.; Riethdorf, S.; Riethdorf, L.; Quade, B.J.; Sun, D.; Duensing, S.; Sheets, E.E.; Munger, K.; Crum, C.P. Ki-67, Cyclin E, and p16 INK4 Are Complimentary Surrogate Biomarkers for Human Papilloma Virus-Related Cervical Neoplasia. *Am. J. Surg. Pathol.* **2001**, *25*, 884–891. [CrossRef] [PubMed]

International Journal of
*Molecular Sciences*

MDPI

*Communication*

# The Immunohistochemical Expression of Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer

**Aneta Popiel-Kopaczyk [1,*], Aleksandra Piotrowska [1], Patrycja Sputa-Grzegrzolka [2], Beata Smolarz [3], Hanna Romanowicz [3], Piotr Dziegiel [1,4], Marzenna Podhorska-Okolow [5] and Christopher Kobierzycki [1]**

1 Division of Histology and Embryology, Department of Human Morphology and Embryology, Wroclaw Medical University, 50-368 Wroclaw, Poland; aleksandra.piotrowska@umw.edu.pl (A.P.); piotr.dziegiel@umw.edu.pl (P.D.); christopher.kobierzycki@umw.edu.pl (C.K.)
2 Division of Anatomy, Department of Human Morphology and Embryology, Wroclaw Medical University, 50-368 Wroclaw, Poland; patrycja.sputa-grzegrzolka@umw.edu.pl
3 Department of Pathology, Polish Mother's Memorial Hospital Research Institute, 93-338 Lodz, Poland; smolbea@wp.pl (B.S.); hanna-romanowicz@wp.pl (H.R.)
4 Department of Physiotherapy, University School of Physical Education, 51-612 Wroclaw, Poland
5 Division of Ultrastructural Research, Wroclaw Medical University, 50-368 Wroclaw, Poland; marzenna.podhorska-okolow@umw.edu.pl
* Correspondence: popielaneta1@gmail.com

**Abstract:** In the epithelial–mesenchymal transition (EMT) process, cells lose their epithelial phenotype and gain mesenchymal features. This phenomenon was observed in the metastatic phase of neoplastic diseases, e.g., cervical cancer. There are specific markers that are expressed in the EMT. The aim of this study was to determine the localization of and associations between the immunohistochemical (IHC) expression of TWIST, SNAIL, and SLUG proteins in precancerous lesions and cervical cancer. The IHC analysis disclosed higher expressions of EMT markers in precancerous lesions and cervical cancer than in the control group. Moreover, stronger expression of TWIST, SNAIL, and SLUG was observed in cervical intraepithelial neoplasia grade 3 (CIN3) vs. CIN1, CIN3 vs. CIN2, and CIN2 vs. CIN1 cases ($p < 0.05$). In cervical cancer, IHC reactions demonstrated differences in TWIST, SNAIL, and SLUG expression in grade 1 (G1) vs. grade 2 (G2) ($p < 0.0011$; $p < 0.0017$; $p < 0.0001$, respectively) and in G1 vs. grade 3 (G3) ($p < 0.0029$; $p < 0.0005$; $p < 0.0001$, respectively). The results of our study clearly showed that existing differences in the expression of the tested markers in precancerous vs. cancerous lesions may be utilized in the diagnosis of cervical cancer. Further studies on bigger populations, as well as in comparison with well-known markers, may improve our outcomes.

**Keywords:** SNAIL; TWIST; SLUG; EMT; cervical cancer; cervical intraepithelial neoplasia; immunohistochemistry; HSIL; LSIL



**Citation:** Popiel-Kopaczyk, A.; Piotrowska, A.; Sputa-Grzegrzolka, P.; Smolarz, B.; Romanowicz, H.; Dziegiel, P.; Podhorska-Okolow, M.; Kobierzycki, C. The Immunohistochemical Expression of Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer. *Int. J. Mol. Sci.* **2023**, *24*, 8063. https://doi.org/10.3390/ijms24098063

Academic Editor: Andrea Tinelli

Received: 24 March 2023
Revised: 26 April 2023
Accepted: 28 April 2023
Published: 29 April 2023



## 1. Introduction

Cervical cancer is one of the leading malignancies of the female genital system. In the United States alone, in 2023, over 13,960 women will be diagnosed with cervical cancer, and 4280 of them will die. There are still 341,831 deaths among women per year worldwide, despite reductions in the mortality and incidence of cervical cancer brought on by the human papilloma virus (HPV) vaccine and increased screening [1,2]. A squamous intraepithelial lesion is formed by the abnormal growth of squamous cells on the surface of the cervix. The degree of dysplasia is determined by the percentage of cervical epithelium that contains dysplastic cells. When compared to the more serious CIN2 and CIN3 (high-grade), which proceed to involve the full thickness of the epithelium, CIN1 (low-grade) only affects the lower one-third or less of the epithelium. When dysplasia penetrates the basement membrane, it develops into cancer. Cervical cancer is usually diagnosed

between the ages of 35 and 44, with a reported average 5-year survival rate of 66% [3]. There are several factors that influence this value. One of them is the clinical stage at diagnosis. When diagnosed at an early stage, the 5-year survival rate is 92%; if the cancer has spread regionally to surrounding tissue and lymph nodes, then the rate is 58%, but it dramatically declines to 18% when distant metastases occur [4]. Patients with locally advanced cervical cancer (stage IB3 to IVA) have a higher rate of recurrence. The early stage of the disease limited to the cervix and uterus (stage IA to IB2) can be treated by radical surgery or concomitant chemotherapy, which is based on patient characteristics and the volume of the disease [5]. After surgery alone, the probability of relapse is at least 30% [6,7]. Although it is not common at initial diagnosis, metastases develop in 15% to 61% of women with cervical cancer, usually within the first two years after finishing treatment [8]. The presence of invasion and metastasis is the major cause of most cancer-related deaths. The epithelial–mesenchymal transition (EMT) process is closely related to tumor metastasis. During the process of EMT, polarized epithelial tumor cells gain invasive and migratory characteristics, leave the primary site, invade the basement membrane, intravasate into blood or lymph vessels, transport through the circulation, extravasate from the circulation, disseminate into a secondary site, and finally, grow at the metastatic site. EMT can be triggered by the dysregulation of oncogenes, tumor suppressors, miRNAs, and growth factor signals. Several transcription factors influence the process of EMT: i.a., twist family bHLH transcription factor 1 (TWIST), snail family zinc finger 1 (SNAIL), and snail family zinc finger 2 (SLUG) [9].

TWIST belongs to the helix–loop–helix transcription factors engaged in the EMT process. Its expression leads to a loss of E-cadherin-mediated cell–cell adhesion, activates mesenchymal markers, and initiates cell motility. Li et al. showed that the expression of TWIST is crucial for the activation of the β-catenin and Akt pathway in HeLa cells to maintain the EMT process [10]. High expression of TWIST was linked with chemo- and radiotherapy resistance [11–13]. Moreover, TWIST overexpression is associated with lower patient survival rates and cervical cancer progression [9,11,14–16].

The SNAIL family is composed of zinc-finger-containing transcription factors and includes SNAIL, SLUG, and SMUC. SNAIL is one of the most widely studied regulators of the EMT process. Its expression is controlled at many levels: transcriptional, translational, and post-translational [11]. As a transcriptional factor, SNAIL rules genes related to EMT-independent functions such as cell survival, motility, anti-apoptosis, immune suppression, stem cell properties, and chemo-resistance [17–21].

SLUG is a member of the SNAIL superfamily and has a pivotal role in the EMT process. Increased expression of SLUG can lead to reduced E-cadherin expression and the onset of EMT. There are reports in the literature that SLUG initiates EMT and promotes metastasis through its trans-repression effect on E-cadherin regulation in cervical cancer [22,23]. Xian Liu et al. showed that exogenously expressed SLUG in HeLa and SiHa cells significantly enhanced cell motility in vitro and promoted distant metastasis in vivo [24]. On the other hand, Nan Cui et al. demonstrated that SLUG acts as a suppressor gene, inhibiting the proliferation of cervical cancer in vitro and tumor formation in vivo [25]. The scientific findings presented above indicate a strong need to expand research on EMT marker expression in cervical cancer.

The aim of this study was, thus, to determine the localization of and associations between immunohistochemical (IHC) expression of the TWIST, SNAIL, and SLUG proteins in precancerous lesions and cervical cancer.

## 2. Results

We determined the IHC expression of TWIST, SNAIL, and SLUG in 124 cervical cancer cases, 229 CIN cases, and 145 patients in the control group. We observed interesting cellular expression patterns. For TWIST, cytoplasmic localization in cancer (Figure 1E), CIN lesions (Figure 1B–D), and normal tissue (Figure 1A) were found. The expression of the SNAIL protein in normal tissue (Figure 2A) was nuclear, but in cervical cancer (Figure 2E) and CIN

lesions (Figure 2B–D), the reaction was nuclear–cytoplasmic. The expression of the SLUG protein was nuclear–cytoplasmic in cancer cells (Figure 3E) and nuclear in CIN lesions (Figure 3B–D) and normal tissue (Figure 3A).

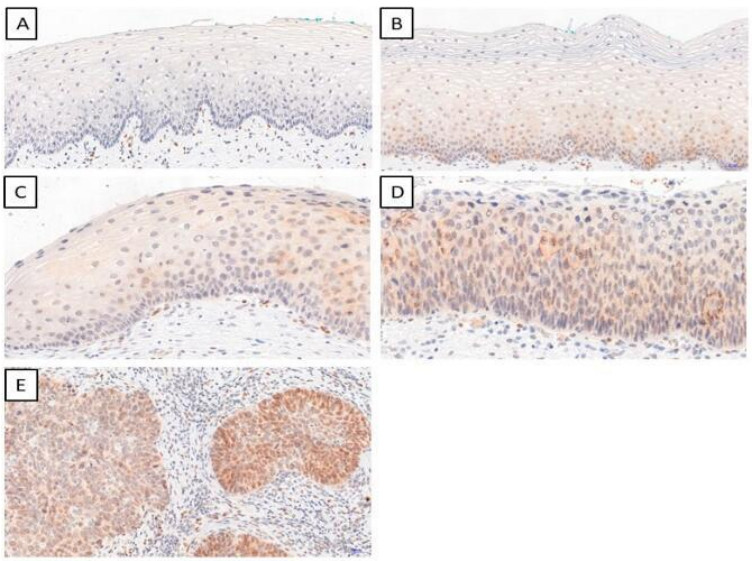


**Figure 1.** The IHC expression of TWIST (normal cervical tissue (**A**); CIN1 (**B**); CIN2 (**C**); CIN3 (**D**); cervical cancer (**E**)); magnification ×200.

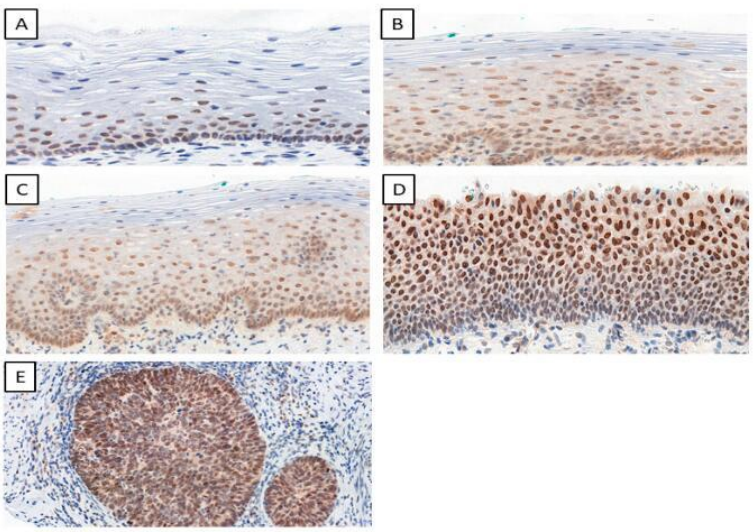


**Figure 2.** The IHC expression of SNAIL (normal cervical tissue (**A**); CIN1 (**B**); CIN2 (**C**); CIN3 (**D**); cervical cancer (**E**)); magnification ×200.

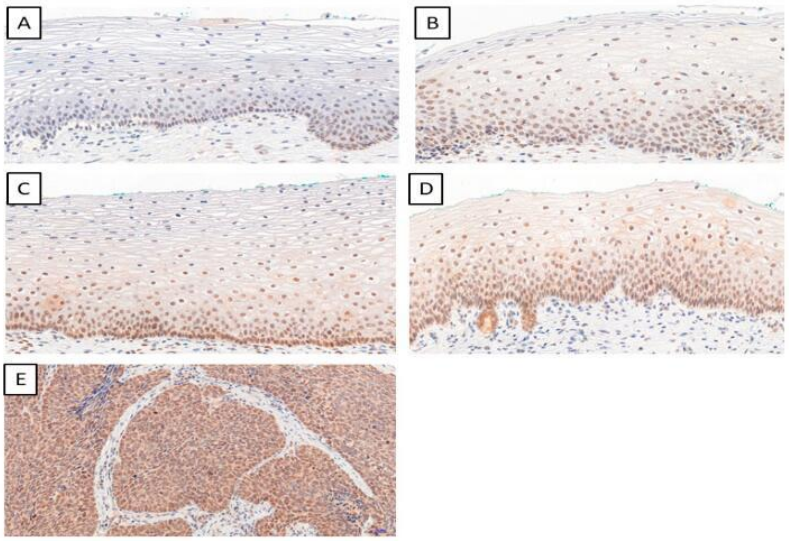


**Figure 3.** The IHC expression of SLUG (normal cervical tissue (**A**); CIN1 (**B**); CIN2 (**C**); CIN3 (**D**); cervical cancer (**E**)); magnification ×200.

### *2.1. Precancerous Lesions*

The highest TWIST, SNAIL, and SLUG expression was noted in CIN3 and was significantly higher as compared to that in CIN1 and CIN2 (respectively, TWIST $p < 0.0001$, SNAIL $p < 0.0013$; SLUG $p < 0.0001$, Figure 4A; Mann–Whitney test). Moreover, expression was significantly higher in the high-grade squamous intraepithelial lesion (HSIL) group than in the low-grade squamous intraepithelial lesion (LSIL) group ($p < 0.0001$; Mann–Whitney test; Figure 4B). In addition, significantly stronger expression of TWIST, SNAIL, and SLUG was observed in CIN3 vs. CIN1, CIN3 vs. CIN2, and CIN2 vs. CIN1 cases ($p < 0.05$ for all; Dunn's multiple comparison tests). Moreover, the Spearman correlation test revealed a significant correlation between EMT markers in CIN lesions (Table 1; Figure 5A–C).

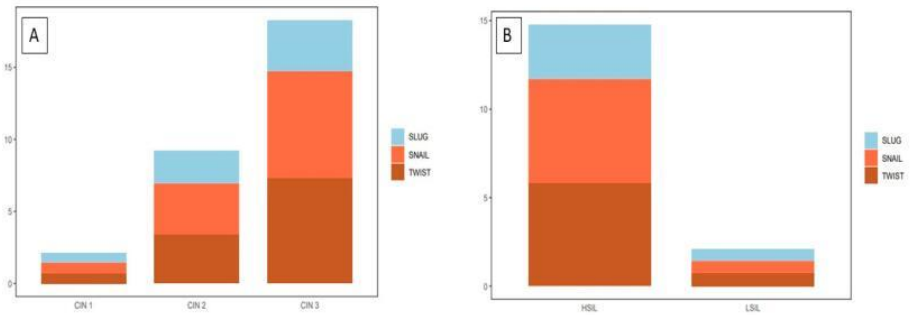


**Figure 4.** A comparison of the IHC expression of the tested markers in precancerous lesions: (**A**) TWIST expression in CIN1, CIN2, and CIN3; SNAIL expression in CIN1, CIN2, and CIN3; SLUG expression in CIN1, CIN2, and CIN3); (**B**) TWIST expression in HSIL and LSIL; SNAIL expression in HSIL and LSIL; SLUG expression in HSIL and LSIL Mann–Whitney test).

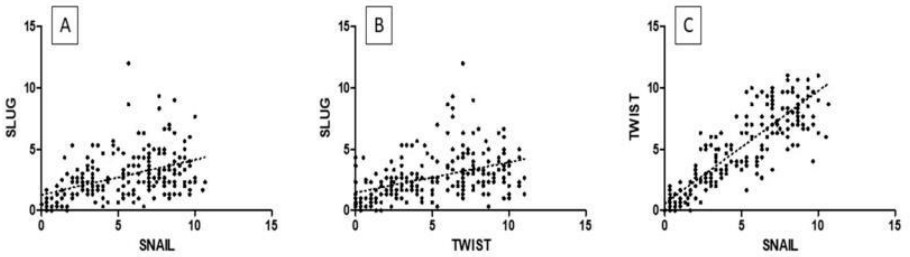


**Figure 5.** The correlations between IHC expression levels in precancerous lesions: (**A**) SLUG vs. SNAIL; (**B**) SLUG vs. TWIST; (**C**) TWIST vs. SNAIL (Spearman correlation test).

**Table 1.** Spearman correlation test results. All correlations except SLUG vs. TWIST in cervical cancer were statistically significant.

| | CIN | | Cervical Cancer | |
|---|---|---|---|---|
| | **SLUG** | **TWIST** | **SLUG** | **TWIST** |
| SNAIL | r = 0.4787<br>$p < 0.0001$ | r = 0.8470<br>$p < 0.0001$ | r = 0.3764<br>$p < 0.0001$ | r = 0.4338<br>$p < 0.0001$ |
| TWIST | r = 0.2157<br>$p < 0.0021$ | NA | r = 0.1066<br>$p < 0.2483$ | NA |

### *2.2. Cervical Cancer*

In cervical cancer cases, the expression of EMT markers was higher than in the control group ($p < 0.0001$ for TWIST, SNAIL, and SLUG; Mann–Whitney test; Figure 6A). Moreover, we demonstrated a considerable difference in TWIST, SNAIL, and SLUG expression between G1 and G2 (respectively, $p < 0.0011$, Figure 6B; $p < 0.0017$, $p < 0.0001$, Mann–Whitney test), as well as between G1 and G3 (accordingly, $p < 0.0029$, Figure 6B; $p < 0.0005$, $p < 0.0001$; Mann–Whitney test). Between G2 and G3, we found no significant differences in the expression of EMT markers (TWIST: $p < 0.8304$, Figure 6B; SNAIL: $p < 0.1208$, SLUG: $p < 0.7947$; Mann–Whitney test). The Spearman correlation test showed that the expression of TWIST positively correlated with the expression of the SNAIL protein in cervical cancer (r = 0.4338, $p < 0.0001$; Figure 7C; Table 1). A positive correlation was also shown between SLUG protein expression and SNAIL (r = 0.3764, $p < 0.0001$; Figure 7A; Table 1). There were no significant correlations between SLUG protein expression and TWIST protein expression in cervical cancer (r = 0.1066, $p < 0.2483$; Figure 7B; Table 1). The expression of all EMT markers in the LSIL, HSIL, G1, G2 and G3 was significantly different in Kruskal–Wallis test (Figure 8; $p < 0.0001$). Dunn's multiple comparison test showed statistically significant difference of TWIST expression between groups: LSIL vs. HSIL, LSIL vs. G1, LSIL vs. G2, LSIL vs. G3; SNAIL expression were different in groups: LSIL vs. HSIL, LSIL vs. G1, LSIL vs. G2, LSIL vs. G3, HSIL vs. G2, HSIL vs. G3 and the SLUG expression were statistically different in the groups: LSIL vs. HSIL, LSIL vs. G1, LSIL vs. G2, LSIL vs. G3, HSIL vs. G1, HSIL vs. G2, HSIL vs. G3, G1 vs. G2, G1 vs. G3.

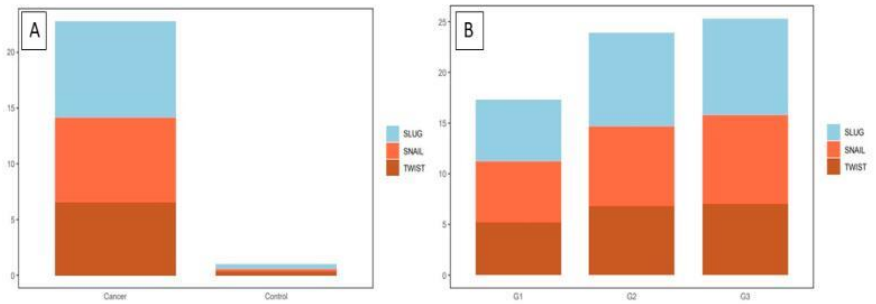


**Figure 6.** The IHC expression of the tested markers in cervical cancer: (**A**) TWIST expression in cervical cancer and control group; SNAIL expression in cervical cancer and control group; SLUG expression in cervical cancer and control group (Mann–Whitney test). (**B**) A comparison of the IHC expression of TWIST, SNAIL, and SLUG in regard to the histological malignancy G (Mann–Whitney test).

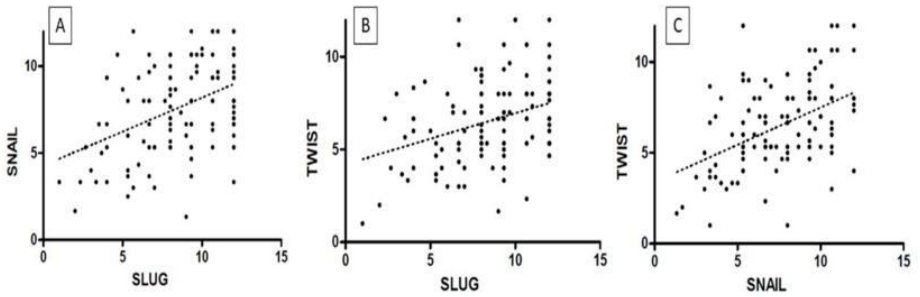


**Figure 7.** The correlations between IHC expression levels in cervical cancer: (**A**) SLUG vs. SNAIL; (**B**) SLUG vs. TWIST; (**C**) SNAIL vs. TWIST (Spearman correlation test).

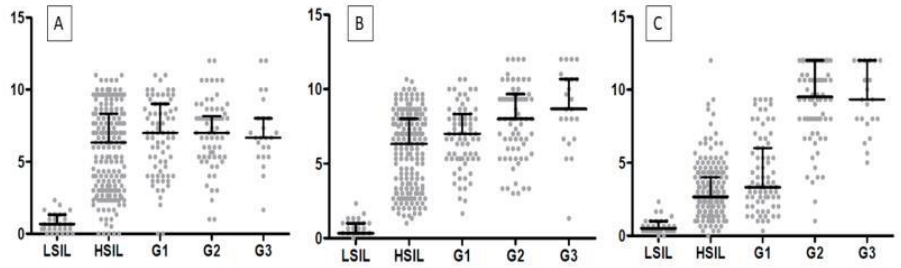


**Figure 8.** A comparison of the IHC expression of (**A**) TWIST, (**B**) SNAIL, and (**C**) SLUG in regard to the LSIL, HSIL, and histological malignancy G ($p < 0.05$, Mann–Whitney test).

## 3. Discussion

The epithelial–mesenchymal transition phenomenon plays an important role in cervical cancer progression. Many studies were conducted to investigate the mechanism of the progression of cervical cancer from CIN. The formation of metastases from cervical cancer is a process that involves multiple steps and a cascade of reactions. The prognosis significantly decreases when distant metastases occur because the treatment of local lesions is more effective than systemic therapy [26]. The cadherins are key components that contribute to cell motility and invasiveness via EMT. A reduction in E-cadherin expression leads to a loss of cell polarity and decreased cell adhesion [27]. In many human malignancies, downregulation of E-cadherin is associated with a poor prognosis and is a key feature of

cancerogenesis, such as tumor spreading [28,29]. Several important genes induce EMT and act as E-cadherin repressors, such as SNAIL, SLUG, and TWIST. The risk of developing invasive cervical cancer from HSIL is approximately 20% (10–40% according to the literature) [30–32]. Previous studies described the expression of EMT markers in cervical cancer; however, there were no prior data on their expression in preinvasive lesions. Due to the lack of such studies, we decided to examine whether there were any differences in EMT marker expression between precancerous lesions, cervical cancer, and normal cervical epithelium. We showed that the expression of SLUG, SNAIL, and TWIST was significantly higher in CIN lesions than in the control group. In addition, we demonstrated that EMT marker expression changed with the histological stage and rose with the stage (TWIST: $p < 0.0001$, Figure 4; SNAIL: $p < 0.0013$, Figure 4; SLUG: $p < 0.0001$, Figure 4; Mann–Whitney test). This may suggest that EMT has a role in the progression of CIN lesions into cervical cancer. Several studies suggested the involvement of SNAIL, SLUG, and TWIST in the development of cervical cancer [15,33–35]. The analysis of IHC expression showed higher expression of SLUG, SNAIL, and TWIST in cervical cancer than in the control group. Tian et al. showed that high SNAIL expression predicts a lower survival rate and is correlated with highly aggressive FIGO stage and LNM (lymph node metastasis) status in cervical cancer patients [36]. The overexpression of SLUG observed in cervical cancer is consistent with the work of Liu et al., who revealed a significant effect of SLUG on the EMT process [24]. In clinical practice, markers of EMT play an increasingly important role and are crucial for many treatments of cervical cancer. Dai et al. described novel therapeutic strategies based on negative regulation of the Wnt signaling pathway and reversing the EMT process. HMQ-T-F2 (F2) was shown to suppress the expression of SNAIL [37]. Due to EMT-induced anti-apoptotic abilities, enhanced DNA damage repair, and a changed drug metabolism route, tumor cells become resistant to therapy and cytotoxicity [38]. Tumor radiation sensitivity rapidly declines as EMT advances because EMT is inextricably linked to tumor radiation resistance. Increased radiation resistance in cervical cancer may be caused by TRIP4 overexpression in tumor tissues and cancerous cells, which may encourage EMT and activate the PI3K/Akt and MAPK/ERK signaling pathways [39]. XAV939 is an inhibitor of the Wnt signaling pathway that was shown to increase cervical cancer cells' radiation sensitivity [40,41]. The expression of EMT markers seems to be an important aspect in planning the treatment of patients and predicting the response to treatment. Work on finding efficient therapeutic targets for cervical cancer metastasis seems to be justified.

## 4. Materials and Methods

This study was performed on selected archival paraffin-embedded specimens. Patients were operated on between 2014 and 2017 at the Polish Mother's Memorial Hospital in Lodz. The patients, aged from 25 to 86 years old, were of the female sex. The control group consisted of normal cervical tissue obtained from patients who underwent total hysterectomy due to uterine leiomyomas. The study group consisted of CIN1 (31), CIN2 (75), CIN3 (123), and cervical cancer (124) cases, whereas the control group was composed of 145 cases. The study was approved by the Ethics Committee of Medical University in Wroclaw (21 December 2022; protocol code 1003/2022).

### *4.1. TMA Construction*

To confirm the histological diagnosis and determine whether the material was suitable for further examination, 6 m thick paraffin sections were produced and stained with hematoxylin and eosin (HE). Briefly, slides were scanned using the histologic Pannoramic MIDI scanner (3DHistech Ltd., Sysmex Suisse AG, Horgen, Switzerland). The sites of CIN in the altered cervix epithelium were then selected and digitally tagged after two independent pathologists reviewed the scans. Next, duplicate tissue core punches (2 mm) for each case were taken from the appropriate paraffin donor blocks for use in the preparation of TMAs (TMA Grand Master; 3DHistech). Normal epithelial tissue from the cervix was designated as the control group.

### 4.2. Immunohistochemistry (IHC)

All IHC reactions were performed on 4 µm thick paraffin slides from TMA using a Dako Autostainer Link48 (Dako, Glostrup, Denmark). The following primary antibodies were used: SLUG (1:50, sc-166476, Santa Cruz Biotechnology, Santa Cruz, CA, USA), TWIST (1:50, ab-50887, Abcam, Cambridge, UK), and SNAIL (1:400, 13099-1-AP, Proteintech, Rosemont, IL, USA). All procedures were conducted as previously described [42]. EnVision FLEX (Dako) was used for the visualization of antibodies, in accordance with the manufacturer's instructions. Cytoplasmic reactions of SLUG, SNAIL, and TWIST were evaluated via Pannoramic Viewer Digital (3DHistech) image analysis and the routinely used immunoreactive scale (IRS) by Remmele and Stegner. This scale evaluates the percentage of positive cancer cells (A) and the staining intensity of the reaction (B). The final result is the product of these two values (AxB). The nuclear expression of SNAIL and SLUG was evaluated semi-quantitatively based on the percentage of positively stained cells of the whole section (3 slides per case) and encoded as follows: 0: absence of staining; 1: 1–10% cells stained; 2: 11–25% cells stained; 3: 26–50% cells stained; and 4: over 50% cells stained.

### 4.3. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad, La Jolla, CA, USA) with Spearman correlation, Kruskal–Wallis, Dunn's multiple comparison, and Mann–Whitney tests. $p$-values $< 0.05$ were considered to be statistically significant. The image GP program was used to create the diagrams [43].

## 5. Conclusions

In our study, the expression of TWIST, SNAIL, and SLUG increased gradually as lesions progressed from LSIL to HSIL. We are the first to show a gradual increase in EMT markers in CIN lesions. Moreover, we confirmed a higher expression of TWIST, SNAIL, and SLUG in cervical cancer than in the control group. The aforementioned data support the idea that EMT elements play a role in the development of cancer.

**Author Contributions:** Conceptualization, A.P.-K. and C.K.; methodology, A.P.; software, P.S.-G.; validation, A.P.-K., P.D. and M.P.-O.; formal analysis, A.P.-K.; investigation, A.P.-K.; resources, H.R. and B.S.; data curation, P.S.-G.; writing—original draft preparation, A.P.-K.; writing—review and editing, A.P.-K. and C.K.; visualization, A.P.; supervision, P.D., C.K. and M.P.-O.; project administration, A.P.-K.; funding acquisition, A.P.-K., P.D. and C.K. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Science Centre, Poland, grant number, 2018/29/N/NZ5/01911. The APC was funded by the National Science Centre.

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of Medical University in Wroclaw (21 December 2022; protocol code 1003/2022).

**Informed Consent Statement:** Patient consent was waived due to use of anonymized archival material only, which in no way influenced the diagnostic and therapeutic process.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy issues.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Li, Y.; Fu, Y.; Cheng, B.; Xie, X.; Wang, X. A Comparative Study on the Accuracy and Efficacy Between Dalton and CINtec® PLUS p16/Ki-67 Dual Stain in Triaging HPV-Positive Women. *Front. Oncol.* **2021**, *11*, 815213. [CrossRef]
2. Cervical Cancer—Cancer Stat Facts. Available online: https://seer.cancer.gov/statfacts/html/cervix.html (accessed on 22 May 2022).
3. Popiel, A.; Kobierzycki, C.; Dzięgiel, P. The Role of Testin in Human Cancers. *Pathol. Oncol. Res.* **2019**, *25*, e12770. [CrossRef]

4. Cervical Cancer: Statistics | Cancer.Net. Available online: https://www.cancer.net/cancer-types/cervical-cancer/statistics (accessed on 13 June 2022).
5. Landoni, F.; Maneo, A.; Colombo, A.; Placa, F.; Milani, R.; Perego, P.; Favini, G.; Ferri, L.; Mangioni, C. Randomised study of radical surgery versus radiotherapy for stage Ib-IIa cervical cancer. *Lancet* **1997**, *350*, 535–540. [CrossRef]
6. Rotman, M.; Sedlis, A.; Piedmonte, M.R.; Bundy, B.; Lentz, S.S.; Muderspach, L.I.; Zaino, R.J. A phase III randomized trial of postoperative pelvic irradiation in stage IB cervical carcinoma with poor prognostic features: Follow-up of a gynecologic oncology group study. *Int. J. Radiat. Oncol. Biol. Phys.* **2006**, *65*, 169–176. [CrossRef]
7. Delgado, G.; Bundy, B.; Zaino, R.; Sevin, B.-U.; Creasman, W.T.; Major, F. Prospective surgical-pathological study of disease-free interval in patients with stage IB squamous cell carcinoma of the cervix: A Gynecologic Oncology Group study. *Gynecol. Oncol.* **1990**, *38*, 352–357. [CrossRef]
8. SEER Cancer Statistics Review 1975–2003—Previous Version—SEER Cancer Statistics. Available online: https://seer.cancer.gov/archive/csr/1975_2003/ (accessed on 13 June 2022).
9. Lee, M.-Y.; Chou, C.-Y.; Tang, M.-J.; Shen, M.-R. Epithelial-Mesenchymal Transition in Cervical Cancer: Correlation with Tumor Progression, Epidermal Growth Factor Receptor Overexpression, and Snail Up-Regulation. *Clin. Cancer Res.* **2008**, *14*, 4743–4750. [CrossRef]
10. Li, J.; Zhou, B.P. Activation of β-catenin and Akt pathways by Twist are critical for the maintenance of EMT associated cancer stem cell-like characters. *BMC Cancer* **2011**, *11*, 49. [CrossRef] [PubMed]
11. Lee, M.-Y.; Shen, M.-R. Epithelial-mesenchymal transition in cervical carcinoma. *Am. J. Transl. Res.* **2012**, *4*, 1–13. [PubMed]
12. Shibata, K.; Kajiyama, H.; Ino, K.; Terauchi, M.; Yamamoto, E.; Nawa, A.; Nomura, S.; Kikkawa, F. Twist expression in patients with cervical cancer is associated with poor disease outcome. *Ann. Oncol.* **2008**, *19*, 81–85. [CrossRef] [PubMed]
13. Zhu, K.; Chen, L.; Han, X.; Wang, J.; Wang, J. Short hairpin RNA targeting Twist1 suppresses cell proliferation and improves chemosensitivity to cisplatin in HeLa human cervical cancer cells. *Oncol. Rep.* **2012**, *27*, 1027–1034. [CrossRef] [PubMed]
14. Wang, T.; Li, Y.; Wang, W.; Tuerhanjiang, A.; Wu, Z.; Yang, R.; Yuan, M.; Ma, D.; Wang, W.; Wang, S. Twist2, the key Twist isoform related to prognosis, promotes invasion of cervical cancer by inducing epithelial-mesenchymal transition and blocking senescence. *Hum. Pathol.* **2014**, *45*, 1839–1846. [CrossRef]
15. Fan, Q.; Qiu, M.-T.; Zhu, Z.; Zhou, J.-H.; Chen, L.; Zhou, Y.; Gu, W.; Wang, L.-H.; Li, Z.-N.; Xu, Y.; et al. Twist induces epithelial-mesenchymal transition in cervical carcinogenesis by regulating the TGF-β/Smad3 signaling pathway. *Oncol. Rep.* **2015**, *34*, 1787–1794. [CrossRef]
16. Kyo, S.; Sakaguchi, J.; Ohno, S.; Mizumoto, Y.; Maida, Y.; Hashimoto, M.; Nakamura, M.; Takakura, M.; Nakajima, M.; Masutomi, K. High Twist expression is involved in infiltrative endometrial cancer and affects patient survival. *Hum. Pathol.* **2006**, *37*, 431–438. [CrossRef] [PubMed]
17. Baygi, M.E.; Soheili, Z.S.; Schmitz, I.; Sameie, S.; Schulz, W.A. Snail regulates cell survival and inhibits cellular senescence in human metastatic prostate cancer cell lines. *Cell Biol. Toxicol.* **2010**, *26*, 553–567. [CrossRef]
18. Zha, Y.-H.; He, J.-F.; Mei, Y.-W.; Yin, T.; Mao, L. Zinc-finger transcription factor Snail accelerates survival, migration and expression of matrix metalloproteinase-2 in human bone mesenchymal stem cells. *Cell Biol. Int.* **2007**, *31*, 1089–1096. [CrossRef]
19. Vega, S.; Morales, A.V.; Ocaña, O.H.; Valdés, F.; Fabregat, I.; Nieto, M.A. Snail blocks the cell cycle and confers resistance to cell death. *Genes Dev.* **2004**, *18*, 1131–1143. [CrossRef] [PubMed]
20. Kudo-Saito, C.; Shirako, H.; Takeuchi, T.; Kawakami, Y. Cancer Metastasis Is Accelerated through Immunosuppression during Snail-Induced EMT of Cancer Cells. *Cancer Cell* **2009**, *15*, 195–206. [CrossRef] [PubMed]
21. Wu, Y.; Zhou, B.P. Snail: More than EMT. *Cell Adhes. Migr.* **2010**, *4*, 199–203. [CrossRef] [PubMed]
22. Jiang, B.; Sun, R.; Fang, S.; Qin, C.; Pan, X.; Peng, L.; Li, Y.; Li, G. Lnc-CC3 increases metastasis in cervical cancer by increasing Slug expression. *Oncotarget* **2016**, *7*, 41650–41661. [CrossRef] [PubMed]
23. Lai, S.-Y.; Guan, H.-M.; Liu, J.; Huang, L.-J.; Hu, X.-L.; Chen, Y.-H.; Wu, Y.-H.; Wang, Y.; Yang, Q.; Zhou, J.-Y. Long noncoding RNA SNHG12 modulated by human papillomavirus 16 E6/E7 promotes cervical cancer progression via ERK/Slug pathway. *J. Cell. Physiol.* **2020**, *235*, 7911–7922. [CrossRef]
24. Liu, X.; Feng, Q.; Zhang, Y.; Zheng, P.; Cui, N. Absence of EpCAM in cervical cancer cells is involved in slug induced epithelial-mesenchymal transition. *Cancer Cell Int.* **2021**, *21*, 163. [CrossRef] [PubMed]
25. Cui, N.; Yang, W.-T.; Zheng, P.-S. Slug inhibits the proliferation and tumor formation of human cervical cancer cells by up-regulating the p21/p27 proteins and down-regulating the activity of the Wnt/β-catenin signaling pathway via the trans-suppression Akt1/p-Akt1 expression. *Oncotarget* **2016**, *7*, 26152–26167. [CrossRef] [PubMed]
26. Liao, Y.; Huang, J.; Liu, P.; Zhang, C.; Liu, J.; Xia, M.; Shang, C.; Ooi, S.; Chen, Y.; Qin, S.; et al. Downregulation of LNMAS orchestrates partial EMT and immune escape from macrophage phagocytosis to promote lymph node metastasis of cervical cancer. *Oncogene* **2022**, *41*, 1931–1943. [CrossRef] [PubMed]
27. Hugo, H.J.; Kokkinos, M.I.; Blick, T.; Ackland, M.L.; Thompson, E.W.; Newgreen, D.F. Defining the E-Cadherin Repressor Interactome in Epithelial-Mesenchymal Transition: The PMC42 Model as a Case Study. *Cells Tissues Organs* **2011**, *193*, 23–40. [CrossRef] [PubMed]
28. Onder, T.T.; Gupta, P.B.; Mani, S.A.; Yang, J.; Lander, E.S.; Weinberg, R.A. Loss of E-Cadherin Promotes Metastasis via Multiple Downstream Transcriptional Pathways. *Cancer Res.* **2008**, *68*, 3645–3654. [CrossRef]

29. Bourboulia, D.; Han, H.; Jensen-Taubman, S.; Gavil, N.; Isaac, B.; Wei, B.; Neckers, L.; Stetler-Stevenson, W.G. TIMP-2 modulates cancer cell transcriptional profile and enhances E-cadherin/beta-catenin complex expression in A549 lung cancer cells. *Oncotarget* **2013**, *4*, 166–176. [CrossRef]
30. Rosty, C.; Hewett, D.G.; Brown, I.S.; Leggett, B.A.; Whitehall, V.L.J. Serrated polyps of the large intestine: Current understanding of diagnosis, pathogenesis, and clinical management. *J. Gastroenterol.* **2012**, *48*, 287–302. [CrossRef] [PubMed]
31. Ge, Y.; Christensen, P.A.; Luna, E.; Armylagos, D.; Xu, J.; Hsu, W.-C.; Zhou, H.; Schwartz, M.R.; Mody, D.R. Age-specific 3-year cumulative risk of cervical cancer and high-grade dysplasia on biopsy in 9434 women who underwent HPV cytology cotesting. *Cancer Cytopathol.* **2019**, *127*, 757–764. [CrossRef]
32. Raab, S.S.; Bishop, N.S.; Zaleski, M.S. Long-term outcome and relative risk in women with atypical squamous cells of undetermined significance. *Am. J. Clin. Pathol.* **1999**, *112*, 57–62. [CrossRef] [PubMed]
33. Zhao, W.; Zhou, Y.; Xu, H.; Cheng, Y.; Kong, B. Snail family proteins in cervical squamous carcinoma: Expression and significance. *Clin. Investig. Med.* **2013**, *36*, E223–E233. [CrossRef]
34. Qureshi, R.; Arora, H.; Rizvi, M. EMT in cervical cancer: Its role in tumour progression and response to therapy. *Cancer Lett.* **2015**, *356*, 321–331. [CrossRef] [PubMed]
35. Campo, L.; Zhang, C.; Breuer, E.K. EMT-Inducing Molecular Factors in Gynecological Cancers. *Biomed Res Int.* **2015**, *2015*, 420891. [CrossRef]
36. Tian, Y.; Qi, P.; Niu, Q.; Hu, X. Combined Snail and E-cadherin Predicts Overall Survival of Cervical Carcinoma Patients: Comparison Among Various Epithelial-Mesenchymal Transition Proteins. *Front. Mol. Biosci.* **2020**, *7*, 22. [CrossRef] [PubMed]
37. Dai, B.; Yu, R.; Fan, M.; Yang, T.; Wang, B.; Zhang, Y. HMQ-T-F2 suppresses migration of the human cervical cancer HeLa cells by reversing EMT via the PI3K/Akt signaling pathway. *Oncol. Rep.* **2019**, *42*, 1451–1458. [CrossRef]
38. Weadick, B.; Nayak, D.; Persaud, A.K.; Hung, S.W.; Raj, R.; Campbell, M.J.; Chen, W.; Li, J.; Williams, T.M.; Govindarajan, R. EMT-Induced Gemcitabine Resistance in Pancreatic Cancer Involves the Functional Loss of Equilibrative Nucleoside Transporter 1. *Mol. Cancer Ther.* **2021**, *20*, 410–422. [CrossRef]
39. Alafate, W.; Li, X.; Zuo, J.; Zhang, H.; Xiang, J.; Wu, W.; Xie, W.; Bai, X.; Wang, M.; Wang, J. Elevation of CXCL1 indicates poor prognosis and radioresistance by inducing mesenchymal transition in glioblastoma. *CNS Neurosci. Ther.* **2020**, *26*, 475–485. [CrossRef]
40. Zhang, J.; Si, J.; Gan, L.; Guo, M.; Yan, J.; Chen, Y.; Wang, F.; Xie, Y.; Wang, Y.; Zhang, H. Inhibition of Wnt signalling pathway by XAV939 enhances radiosensitivity in human cervical cancer HeLa cells. *Artif. Cells Nanomed. Biotechnol.* **2020**, *48*, 479–487. [CrossRef]
41. Chen, S.; Yuan, X.; Xu, H.; Yi, M.; Liu, S.; Wen, F. WNT974 Inhibits Proliferation, Induces Apoptosis, and Enhances Chemosensitivity to Doxorubicin in Lymphoma Cells by Inhibiting Wnt/β-Catenin Signaling. *Experiment* **2020**, *26*, e923799. [CrossRef]
42. Popiel-Kopaczyk, A.; Grzegrzolka, J.; Piotrowska, A.; Olbromski, M.; Smolarz, B.; Romanowicz, H.; Rusak, A.; Mrozowska, M.; Dziegiel, P.; Podhorska-Okolow, M.; et al. The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics. *Curr. Issues Mol. Biol.* **2023**, *45*, 490–500. [CrossRef] [PubMed]
43. Chen, T.; Liu, Y.; Huang, L. ImageGP: An easy-to-use data visualization web server for scientific researchers. *Imeta* **2022**, *1*, e5. [CrossRef]

# 8.Podsumowanie i wnioski

Niniejsza rozprawa doktorska przedstawia analizę ekspresji testyny oraz markerów przejścia epitelialno-mezenchmalnego w śródnabłonkowej neoplazji i w raku szyjki macicy. W załączonej pracy poglądowej przenalizowałam aktualne, opublikowane badania dotyczące roli testyny w chorobach nowotworowych. Prace eksperymentalne miały na celu określić lokalizację oraz ocenić nasilenie ekspresji testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych.

Celem pracy poglądowej było zebranie najnowszych danych literaturowych na temat roli testyny w chorobach nowotworowych takich jak: rak jajnika, płuc, piersi, szyjki macicy, żołądka, jelita grubego, prostaty oraz nowotworów głowy i szyi. Dane literaturowe wskazują, że wyższa ekspresja testyny indukuje apoptozę, redukuje proliferację komórek nowotworowych oraz zatrzymuje komórki w fazie G1 cyklu komórkowego. Ponadto, niska ekspresja testyny koreluje z wyższym stopniem złośliwości histologicznej oraz jest niekorzystnym markerem prognostycznym.

Celem pierwszej pracy oryginalnej było określenie lokalizacji oraz ekspresji testyny w zmianach śródnabłonkowej neoplazji szyjki macicy z wykorzystaniem metody immunohistochemicznej (mikromacierze tkankowe) i porównanie z ekspresją testyny w prawidłowej tkance szyjki macicy. Wykazałam korelację testyny z powszechnie używanymi markerami: Ki-67 oraz p16. Po raz pierwszy stwierdziłam cytoplazmatyczną ekspresję testyny w zmianach przedinwazyjnych. Ponadto wykazałam wyższą ekspresję testyny w zmianach HSIL *vs*. LSIL.

Druga praca eksperymentalna przedstawia ekspresję testyny w raku szyjki macicy. Badania wykonałam metodą IHC na mikromacierzach tkankowych oraz metodą Western Blot na trzech liniach komórkowych raka szyjki macicy: HeLa, SiHa (HPV pozytywna), C-33A (HPV negatywna) oraz linii komórkowej ludzkich keratynocytów – HaCaT jako kontroli. Wykazałam, że ekspresja testyny korelowała negatywnie z ekspresją Ki-67 oraz p16. W raku szyjki macicy zaobserwowałam słabszą ekspresję testyny w porównaniu do prawidłowej tkanki szyjki macicy. Wyniki badania metodą Western Blot korelowały z wynikami IHC, testyna wykazała wyższą ekspresję w linii prawidłowych keratynocytów (HaCaT) w porównaniu z liniami komórkowymi raka szyjki macicy (SiHa, C-33 A, HeLa).

Celem ostatniej pracy z cyklu publikacji było określenie lokalizacji, ekspresji oraz korelacji markerów przejścia epitelialno-mezenchymalnego (TWIST, SNAIL i SLUG) w śródnabłonkowej neoplazji oraz raku szyjki macicy. Analiza ekspresji TWIST, SNAIL oraz SLUG wykazała ich silniejszą ekspresją w zmianach przedinwazyjnych oraz w rakach szyjki macicy niż w prawidłowej tkance szyjki macicy. Zaobserwowałam silniejszą ekspresję TWIST, SNAIL i SLUG w śródnabłonkowej neoplazji szyjki macicy w porównaniu do prawidłowej tkanki. Ekspresja markerów przejścia epitelialno-mezenchymalnego była niższa w CIN1 *vs*. CIN3, CIN2 *vs*. CIN3 i CIN1 *vs*. CIN2 ($p < 0,05$). Stwierdziłam także istotne statystycznie różnice w ekspresji TWIST, SNAIL i SLUG w rakach szyjki macicy w odniesieniu do stopnia zaawansowania histologicznego gdzie ekspresja markerów była znacząco niższa w G1 *vs.* G2 (odpowiednio, $p < 0,0011$; $p < 0,0017$; $p < 0,0001$) oraz w G1 *vs.* G3 (odpowiednio, $p < 0,0029$; $p < 0,0005$; $p < 0,0001$). Wykazałam tym samym po raz pierwszy, że wyższa ekspresja markerów EMT koreluje dodatnio ze stopniem złośliwości histologicznej zarówno w śródnabłonkowej neoplazji jak i raku szyjki macicy.

**Wnioski:**

1) Wzrost nasilenia ekspresji testyny w zmianach przednowotworowych może wskazywać na udział tego białka w procesie transformacji nowotworowej.
2) Negatywna korelacja ekspresji testyny z białkami zaangażowanymi w proliferację komórkową w rakach szyjki macicy może świadczyć o supresorowej roli tego białka w kancerogenezie.
3) Wyższa ekspresja markerów przejścia epitelialno-mezenchymalnego (TWIST, SNAIL, SLUG) w zmianach przedinwazyjnych oraz rakach szyjki macicy w porównaniu do prawidłowej tkanki szyjki macicy może świadczyć o udziale badanych białek w procesie nowotworzenia szyjki macicy.

# 9. Załączniki

## 9.1 Oświadczenia współautorów publikacji stanowiących podstawę pracy doktorskiej.

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Wrocław, 05.05.2023

Lek. Aneta Popiel-Kopaczyk

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

**Oświadczenie**

Oświadczam, że w pracach:

1. Popiel A, Kobierzycki C, Dzięgiel P. **The Role of Testin in Human Cancers. Pathol Oncol Res. 2019 Oct;25(4):1279-1284. doi: 10.1007/s12253-018-0488-3**
2. Popiel A, Piotrowska A, Sputa-Grzegrzółka P, Smolarz B, Romanowicz H, Dzięgiel P, Podhorska-Okołów M, Kobierzycki C. **Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia.** Biomedicines 2021, 9, 1010. https://doi.org/10.3390/biomedicines9081010
3. Popiel-Kopaczyk A, Grzegrzółka J, Piotrowska A, Olbromski M, Smolarz B, Romanowicz H, Rusak A, Mrozowska M, Dzięgiel P, Podhorska-Okołów M, Kobierzycki C. **The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics.** Curr.Issues Mol. Biol. 2023, 45, 490–500.https://doi.org/10.3390/ cimb45010032
4. Popiel-Kopaczyk, A.; Piotrowska, A.; Sputa-Grzegrzolka, P.; Smolarz, B.; Romanowicz, H.; Dziegiel, P.; Podhorska-Okolow, M.; Kobierzycki, C. **The Immunohistochemical Expression of**

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

**Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer.**

*Int. J. Mol. Sci.* 2023, *24*, 8063. https://doi.org/10.3390/ijms24098063

mój udział polegał na: opracowaniu koncepcji prac, postawieniu hipotez, zaplanowaniu badań i wyborze metodyki badań, ocenie reakcji immunohistochemicznych, interpretacji wyników i opracowaniu wniosków, pisaniu prac oraz graficznym przedstawieniu wyników, zebraniu piśmiennictwa a także korekcie prac przed złożeniem do druku.

Lek. Aneta Popiel-Kopaczyk

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Prof. dr hab. Piotr Dzięgiel

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 10.03.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel A, Kobierzycki C, Dzięgiel P. **The Role of Testin in Human Cancers. Pathol Oncol Res. 2019 Oct;25(4):1279-1284. doi: 10.1007/s12253-018-0488-3**. mój udział polegał na: konsultacji i opiece merytorycznej oraz korekcie pracy przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Prof. dr hab. Piotr Dzięgiel

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Dr Christopher Kobierzycki

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 10.03.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel A, Kobierzycki C, Dzięgiel P. **The Role of Testin in Human Cancers. Pathol Oncol Res. 2019 Oct;25(4):1279-1284. doi: 10.1007/s12253-018-0488-3** mój udział polegał na: konsultacji, opiece merytorycznej oraz korekcie językowej pracy przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Dr Christopher Kobierzycki

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Wrocław, 9.03.2023

Prof. dr hab. Piotr Dzięgiel

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel A, Piotrowska A, Sputa-Grzegrzółka P, Smolarz B, Romanowicz H, Dziegiel P, Podhorska-Okołów M, Kobierzycki C. **Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia. Biomedicines 2021, 9, 1010. https://doi.org/10.3390/biomedicines9081010** mój udział polegał na: konsultacji i opiece merytorycznej, ocenie reakcji immunohistochemicznych, interpretacji wyników, zdobywaniu środków finansowych na badania oraz korekcie pracy przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Prof. dr hab. Piotr Dzięgiel

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Wrocław, 10.03.2023

Dr Aleksandra Piotrowska

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel A, Piotrowska A, Sputa-Grzegrzółka P, Smolarz B, Romanowicz H, Dzięgiel P, Podhorska-Okołów M, Kobierzycki C. **Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia. Biomedicines 2021, 9, 1010.** **https://doi.org/10.3390/biomedicines9081010** mój udział polegał na: konsultacji w wyborze metodyki badań oraz wykonaniu reakcji immunohistochemicznych. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Dr Aleksandra Piotrowska

Aleksandra Piotrowska

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Prof. dr hab. Marzenna Podhorska-Okołów

Zakład Badań Ultrastrukturalnych

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 9.03.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel A, Piotrowska A, Sputa-Grzegrzółka P, Smolarz B, Romanowicz H, Dziegiel P, Podhorska-Okołów M, Kobierzycki C. **Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia. Biomedicines 2021, 9, 1010.** **https://doi.org/10.3390/biomedicines9081010** mój udział polegał na: konsultacji, opiece merytorycznej oraz korekcie pracy przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Prof. dr hab. Marzenna Podhorska-Okołów

M. Podhorska-Okołów

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Dr Christopher Kobierzycki

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 9.03.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel A, Piotrowska A, Sputa-Grzegrzółka P, Smolarz B, Romanowicz H, Dziegiel P, Podhorska-Okołów M, Kobierzycki C. **Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia. Biomedicines 2021, 9, 1010.** **https://doi.org/10.3390/biomedicines9081010** mój udział polegał na: konsultacji, opiece merytorycznej, interpretacji wyników, zdobywaniu środków finansowych na badania oraz korekcie pracy przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Dr Christopher Kobierzycki

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Lek. Patrycja Sputa-Grzegrzółka

Zakład Anatomii Prawidłowej

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 9.03.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel A, Piotrowska A, Sputa-Grzegrzółka P, Smolarz B, Romanowicz H, Dzięgiel P, Podhorska-Okołów M, Kobierzycki C. **Preliminary Study on the Expression of Testin, p16 and Ki-67 in the Cervical Intraepithelial Neoplasia. Biomedicines 2021, 9, 1010.** **https://doi.org/10.3390/biomedicines9081010** mój udział polegał na: graficznym przedstawieniu danych zastosowanych w badaniu. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Lek. Patrycja Sputa-Grzegrzółka

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Wrocław, 9.03.2023

Prof. dr hab. Piotr Dzięgiel

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk A, Grzegrzółka J, Piotrowska A, Olbromski M, Smolarz B, Romanowicz H, Rusak A, Mrozowska M, Dzięgiel P, Podhorska-Okołów M, Kobierzycki C.
**The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics. Curr.Issues Mol. Biol. 2023, 45, 490–500.https://doi.org/10.3390/ cimb45010032** mój udział polegał na: konsultacji i opiece merytorycznej, ocenie reakcji immunohistochemicznych, interpretacji wyników, zdobywaniu środków finansowych na badania oraz korekcie pracy przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Prof. dr hab. Piotr Dzięgiel

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Dr Mateusz Olbromski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 9.03.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk A, Grzegrzółka J, Piotrowska A, Olbromski M, Smolarz B, Romanowicz H, Rusak A, Mrozowska M, Dziegiel P, Podhorska-Okołów M, Kobierzycki C.
**The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics. Curr.Issues Mol. Biol. 2023, 45, 490–500.https://doi.org/10.3390/ cimb45010032** mój udział polegał na: wykonaniu badań Western-Blot oraz graficznym przedstawieniu wyników (figury: 3,4,5; Tabela 3). Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Mateusz Olbromski

Dr Mateusz Olbromski

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Wrocław, 10.03.2023

Dr Aleksandra Piotrowska

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk A, Grzegrzółka J, Piotrowska A, Olbromski M, Smolarz B, Romanowicz H, Rusak A, Mrozowska M, Dzięgiel P, Podhorska-Okołów M, Kobierzycki C.
**The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics. Curr.Issues Mol. Biol. 2023, 45, 490–500.https://doi.org/10.3390/ cimb45010032** mój udział polegał na: konsultacji w wyborze metodyki badań oraz wykonaniu reakcji immunohistochemicznych. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Dr Aleksandra Piotrowska

Aleksandra Piotrowska

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Dr Agnieszka Rusak

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 9.03.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk A, Grzegrzółka J, Piotrowska A, Olbromski M, Smolarz B, Romanowicz H, Rusak A, Mrozowska M, Dzięgiel P, Podhorska-Okołów M, Kobierzycki C.
**The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics. Curr.Issues Mol. Biol. 2023, 45, 490–500.https://doi.org/10.3390/ cimb45010032** mój udział polegał na: konsultacji w wyborze metodyki badań, przygotowaniu linii komórkowych do badania. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Dr Agnieszka Rusak

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Wrocław, 10.03.2023

Mgr Monika Mrozowska

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk A, Grzegrzółka J, Piotrowska A, Olbromski M, Smolarz B, Romanowicz H, Rusak A, Mrozowska M, Dzięgiel P, Podhorska-Okołów M, Kobierzycki C.
**The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics. Curr.Issues Mol. Biol. 2023, 45, 490–500.https://doi.org/10.3390/ cimb45010032** mój udział polegał na: konsultacji w wyborze metodyki badań, przygotowaniu linii komórkowych do badania. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Mgr Monika Mrozowska

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Prof. dr hab. Marzenna Podhorska-Okołów

Zakład Badań Ultrastrukturalnych

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 9.03.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk A, Grzegrzółka J, Piotrowska A, Olbromski M, Smolarz B, Romanowicz H, Rusak A, Mrozowska M, Dzięgiel P, Podhorska-Okołów M, Kobierzycki C.
**The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics. Curr.Issues Mol. Biol. 2023, 45, 490–500.https://doi.org/10.3390/ cimb45010032** mój udział polegał na: konsultacji i opiece merytorycznej oraz korekcie pracy przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Prof. dr hab. Marzenna Podhorska-Okołów

M. Podhorska-Okołów

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Dr Christopher Kobierzycki

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 9.03.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk A, Grzegrzółka J, Piotrowska A, Olbromski M, Smolarz B, Romanowicz H, Rusak A, Mrozowska M, Dziegiel P, Podhorska-Okołów M, Kobierzycki C.
**The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics. Curr.Issues Mol. Biol. 2023, 45, 490–500.https://doi.org/10.3390/ cimb45010032** mój udział polegał na: konsultacji, opiece merytorycznej, interpretacji wyników, zdobywaniu środków finansowych na badania oraz korekcie pracy przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Dr Christopher Kobierzycki

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Wrocław, 9.03.2023

Dr Jędrzej Grzegrzółka

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk A, Grzegrzółka J, Piotrowska A, Olbromski M, Smolarz B, Romanowicz H, Rusak A, Mrozowska M, Dzięgiel P, Podhorska-Okołów M, Kobierzycki C.
**The Expression of Testin, Ki-67 and p16 in Cervical Cancer Diagnostics. Curr.Issues Mol. Biol. 2023, 45, 490–500.https://doi.org/10.3390/ cimb45010032** mój udział polegał na: analizie statystycznej oraz graficznym przedstawieniu wyników (figury: 1A-1F). Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Dr Jędrzej Grzegrzółka

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Prof. Dr hab. Piotr Dzięgiel

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 8.05.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk, A.; Piotrowska, A.; Sputa-Grzegrzółka, P.; Smolarz, B.; Romanowicz, H.; Dzięgiel, P.; Podhorska-Okołów, M.; Kobierzycki, C. **The Immunohistochemical Expression of Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer**. *Int. J. Mol. Sci. 2023*, 24, 8063. https://doi.org/10.3390/ijms24098063 mój udział polegał na: konsultacji i opiece merytorycznej, ocenie reakcji immunohistochemicznych, interpretacji wyników, zdobywaniu środków finansowych na badania oraz korekcie pracy przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Prof. Dr hab. Piotr Dzięgiel

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Prof. Dr hab. Marzenna Podhorska-Okołów

Zakład Badań Ultrastrukturalnych

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 8.05.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk, A.; Piotrowska, A.; Sputa-Grzegrzółka, P.; Smolarz, B.; Romanowicz, H.; Dzięgiel, P.; Podhorska-Okołów, M.; Kobierzycki, C. **The Immunohistochemical Expression of Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer**. *Int. J. Mol. Sci. 2023*, 24, 8063. https://doi.org/10.3390/ijms24098063 mój udział polegał na: konsultacji, opiece merytorycznej oraz korekcie pracy przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Prof. Dr hab. Marzenna Podhorska-Okołów

M. Podhorska-Okołów

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Dr Aleksandra Piotrowska

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 08.05.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk, A.; Piotrowska, A.; Sputa-Grzegrzółka, P.; Smolarz, B.; Romanowicz, H.; Dzięgiel, P.; Podhorska-Okołów, M.; Kobierzycki, C. **The Immunohistochemical Expression of Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer**. *Int. J. Mol. Sci. 2023*, 24, 8063. https://doi.org/10.3390/ijms24098063 mój udział polegał na: konsultacji w wyborze metodyki badań oraz wykonaniu reakcji immunohistochemicznych. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Dr Aleksandra Piotrowska

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

*Oświadczenie współautorów publikacji stanowiących podstawę Pracy Doktorskiej*

**UNIWERSYTET MEDYCZNY**
**IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU**

Wydział Lekarski

Zakład Histologii i Embriologii

Katedra Morfologii i Embriologii Człowieka

Lek. Patrycja Sputa-Grzegrzółka

Zakład Anatomii Prawidłowej

Katedra Morfologii i Embriologii Człowieka

Uniwersytetu Medycznego im. Piastów Śląskich

we Wrocławiu

Wrocław, 8.05.2023

**Oświadczenie o współautorstwie**

Oświadczam, że w pracy: Popiel-Kopaczyk, A.; Piotrowska, A.; Sputa-Grzegrzółka, P.; Smolarz, B.; Romanowicz, H.; Dzięgiel, P.; Podhorska-Okołów, M.; Kobierzycki, C. **The Immunohistochemical Expression of Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer**. *Int. J. Mol. Sci. 2023*, 24, 8063. https://doi.org/10.3390/ijms24098063 mój udział polegał na: graficznym przedstawieniu danych zastosowanych w badaniu. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Anety Popiel-Kopaczyk pt. „Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych".

Lek. Patrycja Sputa-Grzegrzółka

Ul. T. Chałubińskiego 6a 50-368 Wrocław
T: +48 71 784 1354/55 umw.edu.pl

# 9.2 Opinie Komisji Bioetycznej



KOMISJA BIOETYCZNA
przy
Uniwersytecie Medycznym
we Wrocławiu
ul. Pasteura 1; 50-367 WROCŁAW

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 411/2019

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:

prof. 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, prof. nadzw. (duchowny)
mgr Luiza Műller (prawo)
dr hab. Sławomir Sidorowicz (psychiatria)
dr hab. Leszek Szenborn, prof. nadzw (pediatria, choroby zakaźne)
Danuta Tarkowska (pielęgniarstwo)
prof. dr hab. Anna Wiela-Hojeńska (farmakologia kliniczna)
dr hab. Andrzej Wojnar, prof. nadzw. (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.

„Rola ekspresji testyny w przedinwazyjnym oraz inwazyjnym raku szyjki macicy"

zgłoszonym przez **lek. Anetę Popiel** uczestniczkę studiów doktoranckich w Zakładzie Histologii i Embriologii Katedry Morfologii i Embriologii Człowieka Uniwersytetu Medycznego we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła wyrazić zgodę na przeprowadzenie badania w Zakładzie Histologii i Embriologii Katedry Morfologii i Embriologii Człowieka Uniwersytetu Medycznego we Wrocławiu pod nadzorem prof. dr hab. Piotra Dzięgiela **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 finansowanego z grantu Preludium 15 Narodowego Centrum Nauki

Wrocław, dnia 6 maja 2019 r.

BW

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

KOMISJA BIOETYCZNA
przy
Uniwersytecie Medycznym
we Wrocławiu

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 1003/2022

Komisja Bioetyczna przy Uniwersytecie Medycznym we Wrocławiu, powołana zarządzeniem Rektora Uniwersytetu Medycznego we Wrocławiu nr 278/XVI R/2020 z dnia 21 grudnia 2020 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 514 z 2020 r.) w składzie:

dr Joanna Birecka (psychiatria)
dr Beata Freier (onkologia)
dr hab. Tomasz Fuchs (ginekologia, położnictwo)
prof. dr hab. Dariusz Janczak (chirurgia naczyniowa, transplantologia)
dr hab. Krzysztof Kaliszewski (chirurgia endokrynologiczna)
dr prawa Andrzej Malicki (prawo)
dr hab. Marcin Mączyński, prof.UMW (farmacja)
Urszula Olechowska (pielęgniarstwo)
prof. dr hab. Leszek Szenborn (pediatria, choroby zakaźne)
prof. dr hab. Andrzej Szuba (choroby wewnętrzne, angiologia
ks. prof. Andrzej Tomko (duchowny)
prof. dr hab. Mieszko Więckiewicz (stomatologia)
dr hab. Andrzej Wojnar, prof. nadzw. (histopatologia, dermatologia) przedstawiciel Dolnośląskiej Izby Lekarskiej)
dr hab. Jacek Zieliński (filozofia)

pod przewodnictwem
prof. dr hab. Jerzego Rudnickiego (chirurgia, proktologia)

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

„Ekspresja testyny oraz markerów przejścia epitelialno-mezenchymalnego w rakach szyjki macicy oraz zmianach przedinwazyjnych"

zgłoszonym przez dr Christophera Kobierzyckiego zatrudnionego w Zakładzie Histologii i Embriologii Uniwersytetu Medycznego we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła **wyrazić zgodę** na przeprowadzenie badania w Zakładzie Histologii i Embriologii Uniwersytetu Medycznego we Wrocławiu **pod warunkiem zachowania anonimowości zgromadzonych danych.**

UWAGA: Jeśli projekt/badanie wymaga ubezpieczenia na podstawie Rozporządzenia Ministra Finansów, Funduszy i Polityki Regionalnej z dnia 23.12.2020r. w sprawie obowiązkowego ubezpieczenia odpowiedzialności cywilnej podmiotu przeprowadzającego eksperyment medyczny, Wnioskodawca zobowiązany jest do złożenia wniosku o zawarcie umowy ubezpieczenia odpowiedzialności cywilnej zgodnie z procedurą przyjętą w Uniwersytecie Medycznym we Wrocławiu. W takim przypadku pozytywna opinia Komisji Bioetycznej ma charakter warunkowy i będzie uprawniała do prowadzenia Badania pod warunkiem zawarcia przez Uniwersytet umowy ubezpieczenia OC zgodnie z Rozporządzeniem wskazanym w zdaniu poprzednim.

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 realizowanego poza działalnością statutową.

Przewodniczący Komisji Bioetycznej
przy Uniwersytecie Medycznym

prof. dr hab. Jerzy Rudnicki

Wrocław, dnia 21 12 2022

## 9.3 Dorobek naukowy

- Sumaryczny IF: 33,691 pkt
- Sumaryczne punkty MEiN: 801 pkt
- Indeks Hirscha: 5

**Prace pełnotekstowe:**

1. **Aneta Popiel-Kopaczyk,** Aleksandra Piotrowska, Patrycja Sputa-Grzegrzółka, Beata Smolarz, Hanna Romanowicz, Piotr Dzięgiel, Marzenna Podhorska-Okołów, Christopher Kobierzycki. The Immunohistochemical Expression of Epithelial–Mesenchymal Transition Markers in Precancerous Lesions and Cervical Cancer. *International Journal of Molecular Sciences 2023*, 24, (9): 8063.
   **IF: 6,208; Pkt. MNiSW: 140**
2. Ludwikowska Kamila Maria, **Popiel Aneta**, Matkowska-Kocjan Agnieszka, Biela Mateusz, Wójcik Marta, Szenborn Filip, Wielgos Katarzyna, Pielka-Markiewicz Ewa, Zaryczański Janusz, Kursa Miron, Szenborn Leszek. COVID-19 mRNA BNT162b2 vaccine immunogenicity among children with a history of paediatric multisystem inflammatory syndrome temporally associated with COVID-19 (PIMS-TS). *Vaccine. 2023* Apr 14:S0264-410X(23)00427-9. doi: 10.1016/j.vaccine.2023.04.035. Epub ahead of print. PMID: 37087396; PMCID: PMC10103624.
   **IF: 4,169; Pkt. MNiSW: 100**
3. Ludwikowska Kamila Maria, **Popiel Aneta**, Matkowska-Kocjan Agnieszka, Olbromski Mateusz J., Biela Mateusz, Wójcik Marta, Szenborn Filip, Wielgos Katarzyna, Pielka-Markiewicz Ewa, Zaryczański Janusz, Kursa Miron, Szenborn Leszek: COVID-19 mRNA BNT162b2 vaccine safety and B-cell and T-cell reactogenicity among children with a history of paediatric multisystem inflammatory syndrome temporally associated with COVID-19 (PIMS-TS) - preliminary study, *Vaccine, 2023*, vol. 41, nr 13, s. 2289-2299, DOI:10.1016/j.vaccine.2023.02.072
   **IF: 4,169; Pkt. MNiSW: 100**
4. **Popiel-Kopaczyk Aneta**, Grzegrzółka Jędrzej, Piotrowska Aleksandra, Olbromski Mateusz, Smolarz Beata, Romanowicz Hanna, Rusak Agnieszka, Mrozowska Monika, Dzięgiel Piotr, Podhorska-Okołów Marzenna, Kobierzycki Christopher: The expression

of testin, Ki-67 and p16 in cervical cancer diagnostics , *Current Issues in Molecular Biology, 2023*, s. 490-500, DOI:10.3390/cimb45010032

**IF: 2,976; Pkt. MNiSW: 70**

5. Machałowski Tomasz, Rusak Agnieszka, Wiatrak Benita, Haczkiewicz-Leśniak Katarzyna, **Popiel Aneta**, Jaroszewicz Jakub, Żak Andrzej, Podhorska-Okołów Marzenna, Jesionowski Teofil: Naturally formed chitinous skeleton isolated from the marine demosponge Aplysina fistularis as a 3D scaffold for tissue engineering, *Materials, 2021*, vol. 14, nr 11, art.2992 [21 s.], DOI:10.3390/ma14112992

   **IF: 3,748; Pkt. MNiSW: 140**

6. **Popiel Aneta**, Piotrowska Aleksandra, Sputa-Grzegrzółka Patrycja, Smolarz Beata, Romanowicz Hanna, Dzięgiel Piotr, Podhorska-Okołów Marzenna, Kobierzycki Christopher: Preliminary study on the expression of testin, p16 and Ki-67 in the cervical intraepithelial neoplasia, *Biomedicines, 2021*, vol. 9, nr 8, art.1010 [11 s.], DOI:10.3390/biomedicines9081010

   **IF: 4,757; Pkt. MNiSW: 100**

7. **Popiel Aneta**, Kobierzycki Christopher, Dzięgiel Piotr: The role of testin in human cancers, *Pathology & Oncology Research, 2019*, vol. 25, nr 4, s.1279-1284, DOI:10.1007/s12253-018-0488-3

   **IF: 2,826; Pkt. MNiSW: 70**

8. **Popiel Aneta**, Starostecka Ewa, Szenborn Leszek: Sposoby transmisji czynników infekcyjnych, *Pediatria po Dyplomie, 2018*, vol. 22, nr 5, s. 17-27

   **IF: 0,000; Pkt. MNiSW: 4**

9. Starostecka Ewa, **Popiel Aneta**, Szenborn Leszek: Jak uniknąć zakażenia w pracy? Przydatne wskazówki dla personelu medycznego, *Klinika Pediatryczna, 2018*, vol. 26, nr 1, s. 64-67

   **IF: 0,000; Pkt. MNiSW: 3**

10. **Popiel Aneta**, Szenborn Leszek: Zakaźna pamiątka z podróży - potrzeba gotowości do rozpoznania, *Klinika Pediatryczna, 2018*, vol. 26, nr 1, s. 50-52

    **IF: 0,000; Pkt. MNiSW: 3**

11. **Popiel Aneta**, Szenborn Leszek: Odra - trudności w diagnostyce i zagrożenia dla zdrowia populacji, Analiza Przypadków. *Pediatria, 2017*, nr 3, s. 6-9, [Publikacja w czasopiśmie spoza listy MNiSW]

12. Szylberg Łukasz, Janiczek Marlena, **Popiel Aneta**, Marszałek Andrzej: Expression of COX-2, IL-1β, TNF-α and IL-4 in epithelium of serrated adenoma, adenoma and hyperplastic polyp, *Archives of Medical Science, 2016*, vol. 12, nr 1, s.172-178. DOI:10.5114/aoms.2016.57594

    **IF: 1,969; Pkt. MNiSW: 30**

13. Szylberg Łukasz, Janiczek Marlena, **Popiel Aneta**, Marszałek Andrzej: Large Bowel Genetic Background and Inflammatory Processes in Carcinogenesis-Systematic Review. *Advances in Clinical and Experimental Medicine, 2015*, vol. 24, nr 4, s. 555-563. doi: 10.17219/acem/31239

    **IF: 1,127; Pkt. MNiSW: 15**

14. Szylberg Łukasz, Janiczek Marlena, **Popiel Aneta**, Marszałek Andrzej:Serrated polyps and their alternative pathway to the colorectal cancer: a systematic review. *Gastroenterology Research and Practice, 2015*, vol.2015, art.573814. doi: 10.1155/2015/573814

    **IF: 1,742; Pkt. MNiSW: 15**

15. Ciesielska Natalia, Sokołowski Remigiusz, **Popiel Aneta**, Kozakiewicz Mariusz, Sysakiewicz Martyna, Buda Kamil, Kędziora-Kornatowska Kornelia: Zastosowanie hiperbarycznej terapii tlenowej w leczeniu pacjentów po urazowym uszkodzeniu mózgu. Przegląd badań The use of hyperbaric oxygen therapy in the treatment of patients after traumatic brain injury. *Review of research, Polish Hyperbaric Research,* 2014, vol.46, nr 1, s. 7-26

    **IF: 0,000; Pkt. MNiSW: 5**

16. Lubińska Anna, **Popiel Aneta**, Sokołowski Remigiusz, Ciesielska Natalia, Kurek Katarzyna, Kowalewska Agata, Krzywińska Olga, Zukow Walery: Diagnosis of the frailty syndrome „Diagnostyka zespołu kruchości", *Journal of Health Sciences, 2013*, vol.3 nr 9, s. 217-232

    **IF: 0,000; Pkt. MNiSW: 5**

**Doniesienia zjazdowe:**

1. Janiczek Marlena, **Popiel Aneta**: Influence of inflammatory process in carcinogenesis of colorectal cancer W: 3rd International Student's Conference of Young Medical Researchers; XVIII Ogólnopolska Konferencja Studenckich Kół Naukowych Uczelni Medycznych. Wrocław, 12-13 kwietnia 2013 roku, Wrocław 2013, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, s. 53 poz.14, ISBN 978-83-7055-565-8
2. **Popiel Aneta**, Janiczek Marlena: Expression of COX-2 in polyps of the colorectal W: 3rd International Students' Conference of Young Medical Researchers; XVIII Ogólnopolska Konferencja Studenckich Kół Naukowych Uczelni Medycznych. Wrocław, 12-13 kwietnia 2013 roku, Wrocław 2013, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, s. 225 poz.18, ISBN 978-83-7055-565-8
3. **Popiel Aneta**, Janiczek Marlena, Chorzępa Martyna, Sienkiewicz Anna: Non-specific symptoms as a first signal leading to diagnosis of proliferative disease in preschool and school age groups, W: The 9th International Scientific Conference of Medical Students and Young Doctors. Katowice - Ligota, 22-23 May 2014. Abstract book 2014, s. 256
4. Janiczek Marlena, **Popiel Aneta**: The correlation between pathological staging in colon cancer and clinical picture, W: The 9th International Scientific Conference of Medical Students and Young Doctors. Katowice - Ligota, 22-23 May 2014. Abstract book 2014, s. 498
5. Ciesielska N. , Sokołowski R., **Popiel Aneta**: Zastosowanie hiperbarycznej terapii tlenowej w urazowym uszkodzeniu mózgu. Przegląd badań przedklinicznych i klinicznych, W: III Ogólnopolska Konferencja Doktorantów i Młodych Naukowców "Per scientam ad salutem aegroti". Bydgoszcz, 9-10.05.2014. Książka streszczeń, s.[36-37]
6. **Popiel Aneta**: Kontrowersyjna historia medycyny według Thorwalda, W: Konferencja „Interdyscyplinarność przyszłością nauki". Zieleniec, 4-6.11.2016. Księga abstraktów, s.46
7. **Popiel Aneta**: Wpływ tkanki tłuszczowej na nowotworzenie raka endometrium, W: Konferencja „Interdyscyplinarność przyszłością nauki". Zieleniec, 8-10.04.2016. Księga abstraktów 2016, s. 53

8. **Popiel Aneta**, Sokołowska Natalia, Kopaczyk Mateusz, Sokołowski Remigiusz, Hirnle Lidia: Obesity and cancer. The role of adipocytes in cancer of the corpus uteri, W: iMEDIC - International MEDical Interdisciplinary Congress 2016. Bydgoszcz [11 June 2016]. Abstract book, (red.) Natalia Sokołowska, Wojciech Stemplowski, Bydgoszcz 2016, Dział Wydawnictw Collegium Medicum, s. 14, ISBN 978-83-943177-3-7
9. Cierpikowski Piotr, Gładka Anna, **Popiel Aneta**, Błasiak Piotr: Three case study of patients with pulmonary lymphangioleiomyomatosis, W: iMEDIC - International MEDical Interdisciplinary Congress 2016. Bydgoszcz [11 June 2016]. Abstract book, (red.) Natalia Sokołowska, Wojciech Stemplowski, Bydgoszcz 2016, Dział Wydawnictw Collegium Medicum, s. 21, ISBN 978-83-943177-3-7
10. **Popiel Aneta**, Cierpikowski Piotr: The role of testin in human cancers, W: iMEDIC – International i-MEDical Interdisciplinary Congress 2017. Bydgoszcz, 2017. Abstract book, s.11 7
11. Stemplowski Wojciech, Janowska Justyna, Sokołowski Remigiusz, Sokołowska Natalia, Klimkiewicz-Wszelaki Karolina, Kasperska Paulina, **Popiel Aneta**, Kędziora-Kornatowska Kornelia: Mortality due to Parkinson`s Diseases - Polish population of patients, W: iMEDIC 2017 Bydgoszcz : 2nd International MEDical Interdisciplinary Congress 2017 Bydgoszcz. Abstract Book, 2017, s. 98
12. Gładka Anna, Głowaczewska Amelia, **Popiel Aneta**, Zieliński Jakub: Awareness of the air pollution: changing knowledge and attitudes among inhabitants of Wrocław, W: Juvenes Pro Medicina [55thPolish and 13th International Annual Training & Scientific Medical Congress of Students' Scientific and Junior Doctors]. Łódź, Poland, 19-20 May 2017. Abstract book, [Łódź] 2017, Students' Scientific Association of the Medical University of Lodz, s. 255, ISBN 978-83-947627-0-4
13. **Popiel Aneta**, Gajos Agnieszka, Cierpikowski Piotr, Gładka Anna: Cutaneous manifestations of lung cancer - a case report, W: SIMC - International Medical Congress of Silesia "Our part in science" Katowice-Poland, 27-28th April 2017 2017, s. 21
14. **Popiel Aneta**, Makowska-Donajska Magdalena, Dzięgiel Piotr, Hirnle Lidia: Umbilical cord gases in correlation with clinical data of mothers and newborns, W: 14th International Conference Advances in Pneumology. Opole, Poland, October 12-13, 2018 [online] 2018, poz.ab610_1.pdf, [[Dostęp 09.10.2019]. Dostępny w: http://www.pneumology.pl/archive/opole2018/]

15. **Popiel Aneta**: Objaw Raynaud jako manifestacja zakażenia parwowirusem B19, W: Konferencja „Interdyscyplinarność przyszłością". Zieleniec, 13-15 kwietnia 2018. Księga abstraktów 2018, s. 68
16. **Popiel Aneta**, Grzegrzółka Jędrzej, Piotrowska Aleksandra, Kobierzycki Christopher, Dzięgiel Piotr, Smolarz Beata, Romanowicz Hanna: The expression of testin in cervical intraepithelial neoplasia, W: 53rd Symposium of the Polish Society for Histochemistry and Cytochemistry „From ultrastructure to in vivo imaging: progress in microscopical techniques". Gdańsk, 15-18 September 2019. Program, abstracts, Gdańsk 2019, Polish Society for Histochemistry and Cytochemistry ; Department of Histology Medical University of Gdańsk, 147 poz.P69, ISBN 978-83-61216-07-0

## 9.4 Nagrody i wyróżnienia

- Kierownik projektu: Rola ekspresji testyny w przedinwazyjnym oraz inwazyjnym raku szyjki macicy. Projekt badawczy finansowany ze środków Narodowego Centrum Nauki w ramach konkursu Preludium 15 (2018/29/N/NZ5/01911), okres trwania projektu 05.12.2018-31.01.2024.
- Laureat programu ESPID Research Masterclass 2022, Ateny (Grecja).
- Stypendystka programu Dolnośląscy Liderzy Medycyny 2020-2021.
- Laureat konkursu TransFormation.doc organizowanego przez Ministerstwo Nauki i Szkolnictwa Wyższego na wyjazd szkoleniowy do Western University, Ivey Business School, London (Kanada) 10.08.2015–21.08.2015.
- Stypendystka Rektora CM UMK za najlepsze wyniki w nauce w roku akademickim 2013/2014 oraz 2014/2015.