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Martyna Ewa Parol-Kulczyk

Znaczenie prognostyczne wybranych czynników tkankowych zaangażowanych w przejście epitelialno-mezenchymalne w raku prostaty

Rozprawa na stopień doktora nauk medycznych

Promotor:
Prof. dr hab. n. med. i n. o zdr. Dariusz Grzanka

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Kierownik Katedry: prof. dr hab. n. med. i n. o zdr. Dariusz Grzanka

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SPIS TREŚCI

1. Nota informacyjna wraz z wykazem publikacji składających się na rozprawę doktorską

Na rozprawę doktorską składają się trzy artykuły naukowe, opublikowane w czasopismach latach 2021/2022. **Sumaryczna wartość współczynnika Impact Factor** artykułów składających się na cykl publikacji wynosi **16.126**, a **łączna wartość punktów ministerialnych**, opierając się o wykaz z dnia 21 grudnia 2021r. wynosi **300**. Poniżej zostały wskazane artykuły wchodzące w skład cyklu publikacji:

1) Praca naukowa o wartości **IF=8.448 i MNSiW=100**

   Parol M, Gzil A, Bodnar M, Grzanka D. Systematic review and meta-analysis of the prognostic significance of microRNAs related to metastatic and EMT process among prostate cancer patients. Journal of Translational Medicine. 2021;19:1–24. doi:10.1186/s12967-020-02644-x

2) Praca naukowa o wartości **IF=3.926 i MNSiW=100**

   Parol-Kulczyk M, Gzil A, Ligmanowska J, & Grzanka, D. Prognostic significance of SDF-1 chemokine and its receptors CXCR4 and CXCR7 involved in EMT of prostate cancer. Cytokine. 2022;150:1–11. doi:10.1016/j.cyto.2021.155778

3) Praca naukowa o wartości **IF=3.752 i MNSiW=100**

   Parol-Kulczyk M, Gzil A, Maciejewska J, Bodnar M, Grzanka D. Clinicopathological significance of the EMT-related proteins and their interrelationships in prostate cancer. An immunohistochemical study. PLoS ONE. 2021;16(6):e0253112. doi:10.1371/journal.pone.0253112

2. Wykaz stosowanych skrótów

BMSC – (ang. *Bone Marrow Stromal Cells*) – komórki podścieliska szpiku kostnego

c-Myc - (ang. *Myelocytomatosis*) - protoonkogen, czynnik transkrypcyjny

CXCR4 - (ang. *C-X-C chemokine receptor type 4)* - receptor chemokinowy typu 4 zawierający motyw C-X-C

CXCR7 - (ang. *C-X-C chemokine receptor type 7)* - receptor chemokinowy typu 7 zawierający motyw C-X-C

ECM – (ang. *Extracellular Matrix*) - macierz zewnątrzkomórkowa

EMT – (ang. *Epithelial-Mesenchymal Transition*) - przejście epitelialno-mezenchymalne

E/M - (ang. *Hybrid Epithelial/Mesenchymal Phenotype*) – pośredni fenotyp epitelialno-mezenchymalny

FOXM1 – (ang. *Forkhead Box M1*) – czynnik transkrypcyjny

GPCR - (ang. *G Protein-Coupled Receptor*) - białko G

GSK-3β – (ang. *Glycogen Synthase Kinase-3 Beta*) –kinaza 3β syntazy glikogenu

LEF-1 – (ang. *Lymphoid Enhancer Binding Factor 1*) – limfatyczny wzmacniacz wiążący czynnik 1, czynnik transkrypcyjny

MET – (ang. *Mesenchymal-Epithelial Transition*) - przejście mezenchymalno-epitelialne

MIF —(ang. *Macrophage Migration Inflammatory Factor)* – czynnik hamujący migrację makrofagów

miRISC — (*ang. miRNA containing RNA-Induced Silencing Complex)* - miRNA wbudowane w kompleksy wyciszające translację

miRNA, miR — (ang. *microRNA*) - mikroRNA

NF-κB – (ang. *Nuclear Factor Kappa-Llight-Chain-Enhancer Of Activated B Cells*) – jądrowy czynnik κB, czynnik transkrypcyjny

NR3C2 – (ang. *Nuclear Receptor Subfamily 3 Group C Member 2*) – członek 2 grupy C podrodziny receptorów jądrowych

PSA – (ang. *Prostate Specific Antigen*) - swoisty antygen sterczowy

RMS - (ang. *Rhabdomyosarcoma*) - mięsak mięśni poprzecznie prążkowanych

SDF-1 – (ang. *Stromal Cell Derived Factor-1*) - czynnik pochodzenia zrębowego 1

SNAIL – (ang. *Snail Family Transcriptional Repressor 1* SNAI1, SNAIL1*)*- represor transkrypcyjny 1 należący do rodziny Snail

SLUG – (ang. *Snail Family Transcriptional Repressor 2,* SNAI2, SNAIL2*)*- represor transkrypcyjny 2 należący do rodziny Snail

SOX4 – (ang. *SRY-Box Transcription Factor 4*) - czynnik transkrypcyjny z domeną HMG związany z białkami Sry

TGF-β – (ang. *Transforming Growth Factor Beta*) – transformujący czynnik wzrostu beta

TME – (ang. *Tumor Microenvironment*) - mikrośrodowisko guza

TWIST – (ang. *Twist Family bHLH Transcription Factor 1*) – czynnik transkrypcyjny z rodziny białek helisa-pętla-helisa

Wnt – (ang. *Wingless-Type Like Signaling*) szlak sygnałowy odgrywa istotną rolę w regulacji takich procesów jak embriogeneza, różnicowanie, przeżywalność i proliferacja komórek

ZEB1 – (ang. *Zinc Finger E-box Binding Homeobox 1*) – czynnik transkrypcyjny ZEB1 o domenie palca cynkowego, czynnik transkrypcyjny będący represorem transkrypcyjnym E-kadheryny

ZEB2 - (ang. *Zinc Finger E-box Binding Homeobox 2*) – czynnik transkrypcyjny ZEB2 o domenie palca cynkowego, czynnik transkrypcyjny będący represorem transkrypcyjnym E-kadheryny

3'UTR - (ang. *3' Untranslated Regions*) – region 3' niepodlegający translacji

3. Wprowadzenie

3.1 Znaczenie przejścia epitelialno-mezenchymalnego w raku prostaty

Rak prostaty stanowi drugi pod względem częstości występowania nowotwór złośliwy i jest piątą najczęstszą przyczyną zgonów z powodu raka w populacji mężczyzn na całym świecie [1]. Ze względu na wyjątkową heterogenność, nowotwór ten charakteryzuje różny przebieg kliniczny. Może przybierać formę powolnie rozwijającej się choroby lub w związku z progresją do stadium przerzutowego, zakończyć się śmiercią pacjenta. Z najnowszych statystyk wynika, że wśród mężczyzn, którzy ukończyli co najmniej 50-ty rok życia, nowotwór gruczołu krokowego jest diagnozowany w stadium miejscowym w około 86.8%, a w stadium regionalnym w 1.5% przypadków [2]. Na tym etapie zaawansowania choroby, rak prostaty jest w pełni wyleczalny, a odsetek 5-letnich przeżyć dla obydwu stadiów zaawansowania osiąga prawie 100% [3]. Trudności związane z leczeniem pacjentów chorych na raka prostaty zaczynają się w stadium systemowym. U tych pacjentów odsetek 5-letnich przeżyć wynosi jedynie 30% [2]. Stanowią oni 5.1% populacji mężczyzn ze zdiagnozowanym rakiem prostaty, a program leczenia tych pacjentów opiera się głównie na zastosowaniu ablacji androgenowej, polegającej na zmniejszeniu stymulującego działania androgenów na komórki raka prostaty [3]. Takie postępowanie lecznicze pozwala osiągnąć pozorną poprawę stanu pacjenta, obniżyć stężenie swoistego antygenu sterczowego (ang. *Prostate Specific Antigen*, PSA) w surowicy krwi pacjentów oraz doprowadza do regresji guza pierwotnego i wtórnego u 80% pacjentów. Faza odpowiedzi na hormonoterapię niestety ustępuje po około 18-24 miesiącach leczenia i przechodzi w fazę hormonooporności, w której rokowanie pacjentów jest znacznie gorsze, a w większości przypadków stosuje się leczenie paliatywne.

Dogłębne poznanie biologii, leżącej u podstaw tworzenia się przerzutów nowotworowych stanowi punkt zwrotny w diagnostyce choroby nowotworowej, jej wczesnego wykrywania, prognozowaniu czasu przeżycia i ustaleniu planu leczenia onkologicznego pacjentów chorych na raka prostaty. Kluczową kwestią jest zrozumienie mechanizmów migracji i inwazji komórek rakowych prostaty oraz

wpływu rozregulowanych czynników odpowiedzialnych za modulację procesu przejścia epitelialno-mezenchymalnego (ang. *Epithelial-Mesenchymal Transition*, EMT) jako pierwszego etapu tworzenia się przerzutów. Możliwość szybkiej oceny poziomów ekspresji czynników biorących udział w procesie EMT w warunkach szpitalnych, mogłoby przyczynić się do ulepszenia diagnostyki pacjentów chorych na raka prostaty, a znajomość wpływu deregulacji wspomnianych czynników na proces EMT może stanowić podstawę do opracowania skutecznych terapii celowanych oraz wpłynąć na efekty lecznicze zaawansowanego stadium tej choroby.

3.2 Definicja procesu przejścia epitelialno-mezenchymalnego

Przejście epitelialno-mezenchymalne jest procesem fizjologicznym, które ma fundamentalne znaczenie dla embriogenezy, począwszy od etapu implantacji, poprzez formowanie się zarodka, gastrulację i skończywszy na organogenezie, a co więcej zachodzi również w procesie regeneracji tkanek [4–6]. Zjawisko EMT odgrywa znaczącą rolę również w procesach patologicznych, w szczególności podczas progresji nowotworowej, podczas której obserwuje się ekspresję genów kodujących białka charakterystyczną dla procesu EMT związanego z rozwojem embrionalnym [7,8]. Tworzenie się przerzutów w chorobie nowotworowej polega na oderwaniu się od masy guza pojedynczych komórek, które utraciły połączenia międzykomórkowe oraz właściwości adhezyjne [9,10]. Rozprzestrzenianie się komórek rakowych w organizmie, jest zależne m.in. od zmiany fenotypu komórkowego w przebiegu tranzycji epitelialno-mezenchymalnej. Patologiczna aktywacja procesu EMT powoduje zatem, że komórki nabłonkowe zyskują fenotyp mezenchymalny, charakteryzujący się podwyższonym potencjałem migracyjnym i inwazyjnością, co pozwala komórkom rakowym na przedostanie się do naczyń krwionośnych. Migrujące naczyniami krwionośnymi komórki nowotworowe muszą nabyć zdolności do przeżycia w warunkach braku adhezji, poprzez wykształcenie mechanizmu zapobiegającemu anoikis [11]. Warto również wspomnieć, iż w miejscu pierwotnej zmiany złośliwej komórki mezenchymalne charakteryzują się wyższym poziomem zdolności przenikania do naczyń krwionośnych włosowatych, co dodatkowo sprzyja ich rozprzestrzenianiu się. W

miejscu odległym natomiast, krążące komórki nowotworowe opuszczają naczynia krwionośnie i osiedlają się w nowej lokalizacji, tworząc guzy wtórne (inaczej: ogniska przerzutowe). Zachodzi proces odwrotny do EMT, a mianowicie przejście mezenchymalno-epitelialne (ang. *Mesenchymal-Epithelial Transition*, MET), w przebiegu którego komórki rakowe odzyskują fenotyp epitelialny, kontakt z macierzą zewnątrzkomórkową (ang. *Extracellular Matrix*, ECM) oraz adhezję międzykomórkową [12].

3.3 Zmiany towarzyszące przejściu epitelialno-mezenchymalnemu

3.3.1 Zmiany komórkowe

Cechą charakterystyczną komórek przechodzących proces EMT jest wysoka zdolność do poruszania się i migracji poprzez ECM. Proces transdyferencjacji połączonych i ustabilizowanych komórek nabłonkowych o fenotypie epitelialnym do fenotypu mezenchymalnego, nadającego komórkom unikatowe cechy ułatwiające ich rozprzestrzenianie się w organizmie następuje poprzez serię fizycznych zmian zachodzących w komórkach nabłonkowych, które czynią je zdolnymi do inwazji tkanek, zarówno sąsiadujących, jak i odległych. Zmiany te obejmują przejściową utratę połączeń międzykomórkowych, prowadzącą do zmiany biegunowości wierzchołkowo-podstawnej komórek i reorganizacji cytoszkieletu komórkowego. Zmiana ekspresji markerów epitelialnych (spadek ekspresji keratyny) i mezenchymalnych (wzrost ekspresji wimentyny), towarzysząca procesowi EMT, skutkuje również zmianą kształtu komórek nabłonkowych na kształt fibroblastoidalny, ułatwiający migrację komórkową. Integralność z błoną podstawną zostaje zniesiona w przypadku komórek o wysokim statusie migracyjnym, zdolnych do tworzenia wtórnych ognisk rakowych. Komórki nabłonkowe zaczynają przejawiać ekspresję markerów mezenchymalnych, takich jak: N-kadheryna, wimentyna, fibronektyna oraz aktywność metaloproteinaz macierzy zewnątrzkomórkowej. Klasycznie EMT charakteryzuje się również utratą markerów nabłonkowych, takich jak E-kadheryny oraz β-kateniny, aktywując tym samym specyficzny program transkrypcyjny, prowadzący do zwiększenia poziomu inwazyjności komórek

przechodzących zmiany [13]. Istnieje silny związek pomiędzy ekspresją E-kadheryny i frakcją błonową β-kateniny a progresją raka prostaty do stadium przerzutowego, co stanowi potencjalny marker prognostyczny postępu choroby [14].

3.3.2 Zmiany genetyczne

Czynniki transkrypcyjne, takie jak SNAIL (ang. *Snail Family Transcriptional Repressor 1*), SLUG (ang. *Snail Family Transcriptional Repressor 2)*, ZEB1 (ang. *Zinc Finger E-box Binding Homeobox 1*), ZEB2 (ang. *Zinc Finger E-box Binding Homeobox 2*), czy TWIST (ang. *Twist Family bHLH Transcription Factor 1*) są głównymi modulatorami szlaków sygnałowych procesu EMT. Regulatory transkrypcji hamują ekspresję E-kadheryny, promując jednocześnie ekspresję markerów mezenchymalnych: N-Kadheryny i/lub R-kadheryny oraz wimentyny, a także ekspresję macierzy komórkowej oraz ogniskowych białek adhezyjnych [13]. Niektóre z czynników transkrypcyjnych funkcjonują jako markery niekorzystnego przebiegu choroby [15]. Z kolei, cząsteczki mikroRNA (ang. *microRNA*, miRNA) i niekodujące cząsteczki RNA mogą regulować lub być regulowane przez kluczowe geny procesu EMT i wpływać na przebieg rozprzestrzeniania się komórek rakowych w organizmie. Najbardziej znanymi cząsteczkami niekodującymi RNA, spełniającymi te kryteria jest rodzina cząsteczek mikroRNA-200 oraz rodzina cząsteczek miR-34. Przykładem takiej zależności jest pętla mikroRNA-200c-3p/ZEB2 w raku prostaty, która może mieć istotne znaczenie w projektowaniu strategii leczenia inwazyjnego i przerzutowego raka prostaty w przyszłości [16].

3.4 Aspekt oporności terapeutycznej związanej z przejściem epitelialno-mezenchymalnym

Jednym z ważniejszych wyzwań w leczeniu pacjentów onkologicznych jest aspekt oporności zmian złośliwych na terapie lecznicze, prowadzący do tworzenia się przerzutów nowotworowych. Oporność na terapie lecznicze

obejmuje wrodzoną i nabytą oporność na leki przeciwnowotworowe, ale również oporność na chemioterapię, radioterapię, terapie immunologiczne i ukierunkowane [17–19]. Przejście epitelialno-mezenchymalne jest głównym czynnikiem przyczyniającym się do rozwinięcia oporności terapeutycznej.

Zjawisko EMT jest związane z różnymi etapami rozwoju choroby prostaty, począwszy od inicjacji nowotworowej poprzez progresję nowotworu, stemness, migrację komórek z pierwotnego ogniska rakowego, intrawazacji do krwioobiegu, przerzutowania, ale także pośredniczy w oporności na konwencjonalne terapie i ukierunkowane inhibitory drobnocząsteczkowe [20–22]. Dotychczas przeprowadzone badania z wykorzystaniem linii komórek nowotworowych potwierdzają wpływ procesu EMT na oporność spowodowaną radioterapią lub chemioterapią [23,24]. Warto również wspomnieć, iż w pierwotnych ogniskach rakowych (w guzach) tworzą się subpopulacje komórek o pośrednim fenotypie epitelialno-mezenchymalnym (ang. *Hybrid Epithelial/Mesenchymal Phenotype*, E/M), czyli niepełnym fenotypie epitelialnym lub niepełnym fenotypie mezenchymalnym, co skutkuje występowaniem jednocześnie różnych subpopulacji komórek rakowych, zwiększając poziom plastyczności i agresywności w obrębie guza [22]. Subpopulacje komórek rakowych o pośrednim fenotypie E/M, zwane również quasi-mezenchymalnymi lub hybrydowymi subpopulacjami komórkowymi wykazują cechy morfologiczne, transkrypcyjne, epigenetyczne mieszczące się w zakresie pośrednich stanów fenotypowych, dodatkowo przejawiając niepełną ekspresję markerów epitelialnych i niepełną ekspresję markerów mezenchymalnych [13,25–27].

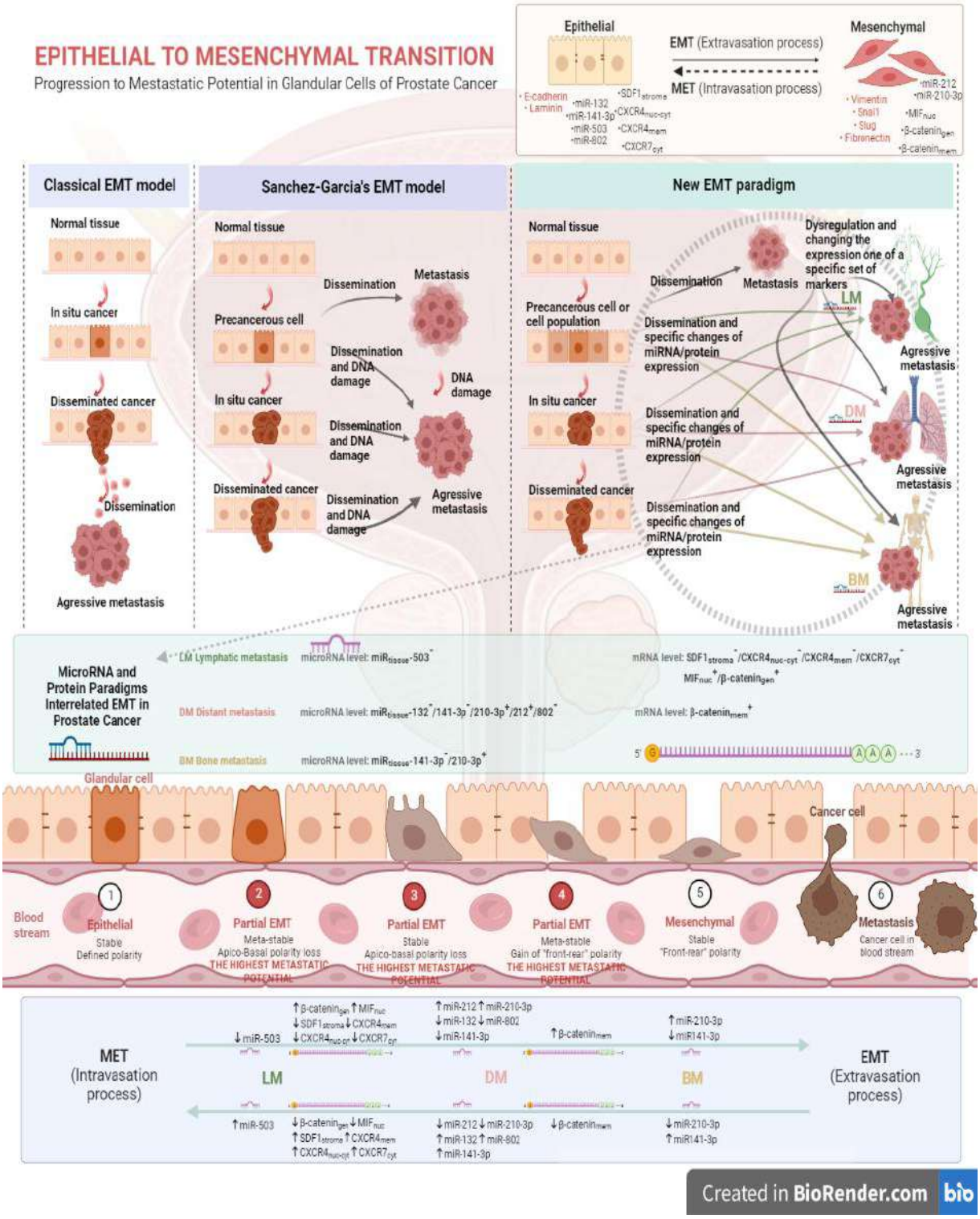
3.5 Model procesu przejścia epitelialno-mezenchymalnego w raku prostaty na podstawie badań własnych

Klasyczny model progresji nowotworowej wskazuje, że kluczową rolę w tym procesie odgrywają czynniki transkrypcyjne, które posiadają zdolność do przeprogramowania komórek raka prostaty, nadając im możliwość tworzenia

się guzów wtórnych nowotworu złośliwego. Według tego modelu zjawisko EMT jest aktywowane jedynie w sporadycznych komórkach nowotworowych. W miarę postępu choroby następuje również zmiana profilu ekspresji białek ECM oraz białek cytoszkieletu. W 2009 roku Sánchez-García zaproponował alternatywny model progresji raka prostaty podając, że patologiczna aktywacja białek związanych z EMT może w sposób ciągły napędzać rozprzestrzenianie się przerzutów w organizmie z pierwotnego ogniska nowotworu [28]. Dziś wiemy, że na proces przejścia epitelialno-mezenchymalnego ma wpływ bezpośrednio lub pośrednio wiele czynników regulujących, które można sklasyfikować jako induktory, regulatory oraz efektory procesu EMT, w konsekwencji prowadząc do rozwoju choroby.

Wyniki przeprowadzonych badań własnych w zakresie wpływu wybranych biomarkerów tkankowych na proces przejścia epitelialno-mezenchymalnego w raku prostaty są w pewnym stopniu zgodne z proponowanym przez Sánchez-García'ę modelem zjawiska EMT. W opublikowanych pracach naukowych wykazano, że zmienione poziomy ekspresji niektórych mediatorów tkankowych na poziomie miRNA i/lub mRNA/białek mogą wpływać na wybrane komórki nowotworowe raka prostaty, prowadząc do patologicznej aktywacji programu EMT. Wiadomo również, że podczas progresji nowotworowej raka prostaty, poziomy ekspresji tych specyficznych czynników tkankowych sukcesywnie zmieniają się, podczas gdy model Sánchez-García'i tego nie uwzględnia. Pacjenci ze zdiagnozowanym pierwotnym rakiem gruczołu krokowego, u których choroba charakteryzowana jest przez plastyczność komórkową, przejawiają zdecydowanie gorsze rokowanie. Zatem do tematu progresji nowotworowej w raku prostaty i innych nowotworach nie można podchodzić z jednoznaczną tezą, mówiącą o tym, że proces EMT w komórce rakowej wiąże się z całkowitym przejściem z fenotypu epitelialnego do czystego fenotypu mezenchymalnego, nie wykazując stanów pośrednich, jak to się ma w przypadku rozwoju osobniczego. Ten meta-stan EMT, znany również jako częściowy EMT, charakteryzuje się współistnieniem zarówno komórek rakowych o fenotypie epitelialnym niecałkowicie stłumionym, jak i fenotypie

mezenchymalnym, nie w pełni osiągniętym. Dieter i inni współautorzy, w 2011 roku wykazali, że komórki nowotworowe z ksenograftu ludzkiego raka okrężnicy, wyłącznie w warunkach obecności komórek macierzystych i wtedy, gdy wykazują zdolność do samoodnowy, mogą tworzyć przerzuty do wątroby [29]. Kolejnym przykładem plastyczności fenotypowej jest pączkujący fenotyp (ang. *budding-type*) wczesnego stadium raka jelita grubego, który koreluje dodatnio z gorszym rokowaniem, w stosunku do raka nie wykazującego fenotypu pączkującego (ang. *non-budding type*) ze względu na fakt, iż komórki rakowe prezentują fenotyp EMT i posiadają cechy komórek macierzystych [30]. Ponadto, wykazano, że gorsze rokowanie wśród pacjentów ze zdiagnozowanym pączkującym rakiem jelita grubego jest wynikiem tworzenia się przerzutów do węzłów chłonnych, oraz odległych do wątroby i płuc [30]. Wyniki uzyskane w toku badań naukowych własnych są również zgodne z zasadą plastyczności fenotypowej komórek nowotworowych przechodzących proces EMT. Inicjujące nowotwór komórki rakowe wczesnego stadium raka prostaty mogą tworzyć przerzuty do węzłów chłonnych lub przerzuty odległe do płuc, czy układu kostnego, jednakże na każdym etapie progresji nowotworowej zadziała specyficzna grupa czynników tkankowych o symptomatycznej ekspresji, na podstawie której możliwe będzie określenie rodzaju tworzącego się przerzutu nowotworowego.



Rycina 1. Modele przejścia epitelialno-mezenchymalnego w raku prostaty opracowane na podstawie dotychczas przeprowadzonych badań naukowych. Schemat pokazuje wpływ istotnych czynników zaangażowanych w przejście epitelialno-mezenchymalne w raku prostaty na proces przerzutowania. Zastosowane skróty: EMT=przejście epitelialno-mezenchymalne; MET=przejście mezenchymalno-epitelialne; LM=przerzuty do węzłów chłonnych; DM=przerzuty odległe; BM=przerzuty do kości; Schemat przygotowany w BioRender.com

3.6 Czynniki związane z przejściem epitelialno-mezenchymalnym i progresją do stadium letalnego w raku prostaty na podstawie badań własnych

W ciągu ostatniej dekady badania naukowe w dziedzinie progresji chorób nowotworowych zostały ukierunkowane na poszukiwanie tzw. białek targetowych, które pośredniczą zarówno w procesie transformacji nowotworowej, jak i procesie przerzutowania u pacjentów chorych na raka prostaty i inne nowotwory złośliwe. Istotnie, największy potencjał diagnostyczny, prognostyczny i terapeutyczny będą zatem stanowiły czynniki stymulujące progresję choroby do stadium systemowego już na wczesnym jej etapie.

3.6.1 Czynniki osi zapalenie-rak-przejście epitelialno-mezenchymalne

Przewlekły stan zapalny może aktywować proces kancerogenezy. Komórki odpowiedzi zapalnej mogą wydzielać szereg czynników, które wspierają zarówno inicjację, jak i progresję nowotworu, a także mogą indukować przejście nabłonkowo-mezenchymalne. Rozwinięte nowotwory pobudzają odpowiedź zapalną poprzez wydzielanie cytokin, chemokin i czynników wzrostu, które rekrutują populacje naciekających komórek odpornościowych bezpośrednio do mikrośrodowiska guza (ang. *Tumor Microenvironment*, TME). Reakcja zapalna potencjalnie wywiera kontrolę nad guzem, ale zamiast tego może zostać przechwycona przez guz, aby stymulować jego własny rozwój w kierunku postaci przerzutowej.

3.6.1.1 Czynnik hamujący migrację makrofagów

Jedną z cytokin prozapalnych, uważaną za łącznik pomiędzy chronicznym stanem zapalnym a procesem nowotworowym jest czynnik hamujący migrację makrofagów (ang. *Macrophage Migration Inflammatory Factor*, MIF). MIF prowadzi do aktywacji procesu przerzutowego komórek nowotworowych poprzez wpływ na zmniejszenie ekspresji E-kadheryny i zwiększenie poziomu N-kadheryny [31]. Ponadto, badania własne wykazały, że istotną rolę

w procesie progresji nowotworowej raka prostaty wykazuje frakcja jądrowa białka MIF. Dowiedziono, że MIF jądrowy ujemnie koreluje z ogólną ekspresją β-kateniny, zatem istnieje prawdopodobieństwo, że czynnik MIF wpływa na nieprawidłową aktywację szlaku sygnałowego Wnt/β-katenina, pobudzając translokację aktywnej formy β-kateniny do jądra komórkowego, aby zadziałać jako aktywator transkrypcji [32]. Co więcej, frakcja jądrowa białka MIF jest odpowiedzialna za udział w procesie tworzenia się przerzutów do węzłów chłonnych w raku prostaty. Badania własne potwierdziły istotnie wyższą ekspresję jądrowej frakcji białka MIF u pacjentów z przerzutami do węzłów chłonnych, w porównaniu do pacjentów z rakiem prostaty, nie wykazujących przerzutów do węzłów chłonnych ($p<0.05$). Te same badania dowiodły również, że patologiczna aktywacja szlaku Wnt/β-katenina wpływa na progresję raka prostaty do stadium systemowego, poprzez tworzenie się przerzutów zarówno do węzłów chłonnych, jak i przerzutów odległych [33]. Wpływ czynnika MIF na przyspieszenie wzrostu i przerzutów guzów w raku trzustki potwierdził również Funamizu i jego współautorzy. Przeprowadzając badania na modelach myszy wykazujących nadekspresję MIF dowiedli, znaczący wzrost guza ($p<0.001$) u myszy z nadekspresją MIF, w porównaniu z kontrolnymi komórkami myszy [34].

3.6.1.2 Czynnik pochodzenia zrębowego-1 oraz jego receptory wiążące

Oddziaływanie czynnika pochodzenia zrębowego (ang. *Stromal Cell Derived Factor-1*, SDF-1) z receptorami chemokinowymi typu 4 zawierającymi motyw C-X-C (ang. *C-X-C Chemokine Receptor Type 4*, CXCR4) lub receptorami chemokinowymi typu 7 zawierającymi motyw C-X-C (ang. *C-X-C Chemokine Receptor Type 7*, CXCR7) wpływa na regulację wielu procesów fizjologicznych i patologicznych, takich jak proliferacja i różnicowanie komórek, adhezja, migracja, czy też przerzutowanie komórek

nowotworowych. Receptory wiążące, CXCR4 i CXCR7, to receptory metabotropowe obecne na powierzchni ludzkich komórek nowotworowych [35], które współpracują z białkiem G (ang. *G Protein-Coupled Receptor*, GPCR) i mogą być aktywowane przez wspólny ligand SDF-1. Cząsteczki te odgrywają kluczową rolę w TME poprzez promowanie progresji nowotworu, zarządzają również proliferacją i migracją komórek nowotworowych oraz rekrutacją komórek odpornościowych i zrębowych w TME. SDF-1 eliminuje komórki T z TME poprzez gradient stężeń, który hamuje dostęp komórkom immunoaktywnym i promuje powstawanie naczyń krwionośnych w obrębie guza [36]. Obecność receptorów wiążących CXCR4 oraz CXCR7 wykryto między innymi na komórkach ludzkiego mięsaka mięśni poprzecznie prążkowanych (ang. Rhabdomyosarcoma, RMS). W badaniu tym udowodniono, że receptory wiążące uczestniczą w nadawaniu komórkom RMS potencjału do przerzutowania poprzez wpływ CXCR7 na adhezję komórek nowotworowych oraz pośrednictwo CXCR4 w przekazywaniu sygnałów związanych z migracją [35,37]. Blokowanie ścieżek sygnałowych CXCR4 i CXCR7 może zatem zapobiegać progresji raka prostaty do stadium przerzutowego w dualistyczny sposób, poprzez zahamowanie wzrostu, migracji i chemotaksji komórek nowotworowych i poprzez sprzyjanie występowania komórek T w TME. W badaniach własnych na tkankach ludzkiego raka prostaty dowiedziono, że zarówno czynnik SDF-1, jak i jego receptory wiążące CXCR4 oraz CXCR7 biorą udział w powstawaniu przerzutów do węzłów chłonnych. W przypadku frakcji podścieliskowej białka SDF-1 odnotowano spadek ekspresji owego białka u pacjentów z obecnymi przerzutami do węzłów chłonnych, w porównaniu do pacjentów bez przerzutów. Podobne zjawisko zauważono w przypadku receptorów wiążących: frakcji jądrowo-cytoplazmatycznej białka CXCR4 oraz frakcji cytoplazmatycznej białka CXCR7 – spadek ekspresji tych białek był

widoczny w tkankach u pacjentów z obecnymi przerzutami do węzłów chłonnych [38]. Dotychczasowe badania wykazały, że ludzkie komórki raka prostaty produkujące CXCR7 mają zdolność do szybszej proliferacji, tworzenia przerzutów oraz rozwinięcia naczyń krwionośnych [35]. Nowoczesne podejście do terapii celowanej raka prostaty wskazuje ukierunkowanie podejścia jednocześnie na immunologiczne punkty kontrolne i oś SDF-1/CXCR4/CXC7.

3.6.2. MicroRNA modulujące proces przejścia epitelialno-mezenchymalnego

Ważnym mechanizmem regulującym ekspresję genów związanych z progresją nowotworową raka prostaty są cząsteczki miRNA. Stanowią one grupę krótkich, jednoniciowych, niekodujących cząsteczek RNA. Dojrzałe cząsteczki miRNA długości około 19-23 nukleotydów, syntetyzowane są z dwuniciowych prekursorów, przy udziale polimerazy II i wbudowywane w kompleksy wyciszające translację (ang. *miRNA Containing RNA-Induced Silencing Complex*, miRISC). Posiadają zatem zdolność do potranskrypcyjnego wyciszania genów docelowych poprzez przyłączenie do regionów 3'UTR (ang. *3' Untranslated Regions*) mRNA genu docelowego, tym samym powodując degradację mRNA lub zahamowanie procesu translacji. Cząsteczki miRNA uczestniczą również w kontrolowaniu istotnych procesów komórkowych, takich jak procesy różnicowania, czy proliferacji komórek oraz modulują epigenetyczne procesy regulatorowe. W zależności od genów docelowych, na które oddziałują cząsteczki miRNA, mogą one funkcjonować jako onkogeny lub geny supresorowe.

Istotną rolę regulacyjną w procesie EMT przypisuje się cząsteczkom miRNA-200 oraz miRNA-205, które posiadają zdolność represji czynników transkrypcyjnych związanych z aktywnością E-kadheryny, czyli ZEB1 i ZEB2 oraz hamują aktywność innych czynników transkrypcyjnych związanych z procesem EMT, takich jak TWIST1. Z kolei cząsteczki miRNA-132 oraz miRNA-212 hamują aktywność białka TGF-β (ang. *Transforming Growth Factor Beta*),

które poprzez blokowanie czynnika SOX4 moduluje przebieg procesu EMT, a ponadto miRNA-132 wpływa na wyciszenie aktywności białka ZEB2 [39].

MiRNA wykazują wysoce specyficzną tkankową ekspresję, dzięki czemu na podstawie zmian w poziomie ich ekspresji można różnicować tkanki prawidłowe od zmienionych chorobowo, ale również rozróżniać poszczególne rodzaje nowotworów. Okazuje się, że posługując się profilami cząsteczek miRNA specyficznych dla nowotworu prostaty, na podstawie zmian poziomu ekspresji tych cząsteczek można przewidzieć poziom jego agresywności, prognozować progresję choroby oraz klasyfikować stadium zaawansowania nowotworu. Badania własne wyszczególniły 9 cząsteczek miRNA ściśle związanych z procesem przerzutowania u pacjentów z rakiem prostaty. Wykazano między innymi, że nadekspresja miR-210-3p oraz deregulacja miR-141-3p jest związana z obecnością przerzutów u pacjentów z rakiem prostaty, w szczególności przerzutów odległych i przerzutów do kości [40]. Ponadto, na podstawie przeprowadzonej meta-analizy wyodrębniono charakterystyczne sygnatury miRNA dla poszczególnych rodzajów przerzutów u pacjentów z rakiem prostaty. Wyniki przeprowadzonych badań własnych zsyntetyzowano i przedstawiono na rycinie 1.

Nieprawidłowy profil ekspresji cząsteczek miRNA może stanowić wiarygodny biomarker diagnostyczno-prognostyczny w przebiegu choroby nowotworowej prostaty, w tym posłużyć do oceny stopnia zaawansowania choroby, szacowania czasu przeżycia, prognozowania progresji choroby, typowania rodzaju przerzutowania. Co więcej, ocena zaawansowania choroby na podstawie nieprawidłowego profilu cząsteczek miRNA może okazać się pomocnym narzędziem przy wyborze leczenia celowanego, czy dostosowanego do indywidualnych potrzeb pacjenta.

3.6.3 Powiązania pomiędzy poszczególnymi cząsteczkami miRNA oraz białkami w procesie przejścia epitelialno-mezenchymalnego

Istotnie, niewyjaśnioną kwestią w niniejszej dysertacji pozostaje określenie stopnia zależności pomiędzy poszczególnymi czynnikami zaangażowanymi w proces EMT, w wyodrębnionych, w toku badań własnych, profilach miRNA-białkowych, charakteryzujących poszczególne rodzaje przerzutów u pacjentów z rakiem prostaty (rycina 1). Informacji na temat takowych korelacji, w niepełnym zakresie, dostarczają wyniki dotychczas przeprowadzonych badań naukowych. Wenjing i jego współautorzy wykazali, że cząsteczka mir-503 jest odpowiedzialna za tłumienie szlaku sygnałowego Wnt/β-katenina poprzez doprowadzenie do wzrostu ekspresji GSK-3β (ang. *Glycogen Synthase Kinase-3 Beta*) i p-β-kateniny, jednocześnie zmniejszając poziom obydwu czynników w komórkach raka żołądka [41]. Z kolei, Pengbo i współpracownicy wykazali, że inhibicja mir-212 prowadzi do aktywacji czynnika FOXM1 (ang. *Forkhead Box M1*), ale wpływa hamująco na kanoniczną ścieżkę Wnt/β-katenina poprzez tłumienie takich czynników jak: Wnt (ang. *Wingless-Type Like Signaling*), LEF-1 (ang. *Lymphoid Enhancer Binding Factor 1*), c-Myc (ang. *Myelocytomatosis*) i jądrowej frakcji β-kateniny w raku wątrobowokomórkowym [42]. Natomiast Lun-Qing i pozostali współtwórcy pracy naukowej udowodnili, że nadekspresja miR-802 prowadzi do aktywacji szlaku sygnalizacji Wnt/β-katenina oraz NF-κβ/p65 (ang. *Nuclear Factor Kappa-Llight-Chain-Enhancer Of Activated B Cells/p65*) w komórkach raka płuc, wykazując wzrost poziomu jądrowej frakcji β-kateniny oraz białka p65 [43].

Określenie wzajemnego wpływu zbadanych cząsteczek miRNA oraz białek może doprowadzić do wytyczenia nowych szlaków sygnałowych związanych z progresją nowotworową w raku prostaty. Istnieją bowiem doniesienia literaturowe o możliwym wpływie regulacyjnym chemokiny SDF-1 na wybrane cząsteczki miRNA. Potter i jego współautorzy w swoim badaniu nad BMSC (ang. *Bone Marrow Stromal Cells*) wskazali, że SDF-1, oddziałując na niektóre cząsteczki miRNA, moduluje proces migracji BMSC [44]. Cytokiny i mediatory procesu zapalnego mogą regulować ekspresję cząsteczek miRNA,

przez co pośrednio wpływają również na regulację wielu genów [45,46]. Yang i pozostali współautorzy pracy udowodnili, że czynnik MIF stymulował wzrost ekspresji miR-301b wpływając tym samym hamująco na NR3C2 (ang. *Nuclear Receptor Subfamily 3 Group C Member 2*) u pacjentów chorych na raka trzustki. Badania funkcjonalne wykazały, że obniżony poziom ekspresji NR3C2 korelował z gorszym rokowaniem u tych pacjentów. Ponadto, w tym samym badaniu wykazano, że genetyczna delecja MIF zakłóciła oś sygnałową MIF-miR-301b-NR3C2, hamując rozprzestrzenianie się i tworzenie przerzutów oraz wydłużając przeżycie w modelu genetycznie zmodyfikowanej myszy [47]. Potwierdzenie takich zależności między białkami a cząsteczkami miRNA u pacjentów chorych na raka prostaty, mogłoby sugerować, że stan zapalny odgrywa kluczową rolę w inicjacji i progresji nowotworowej u pacjentów chorych na raka prostaty**,** a chemokina SDF-1 oraz cytokina prozapalna MIF mogłyby stanowić wczesne markery postępu choroby oraz punkt wyjścia do indywidualnego doboru metod leczenia. Zastosowanie profili miRNA-białkowych w diagnostyce onkologicznej pacjentów chorych na raka prostaty jako narzędzia pomocniczego w klasyfikacji stopnia zaawansowania pacjentów, u których zdiagnozowano ten nowotwór, wydaje się być przyszłościowe.

4. Cele pracy doktorskiej

1. Określenie zależności pomiędzy danymi kliniczno-patologicznymi, a ekspresją szeregu cząsteczek miRNA, na podstawie meta-analizy opublikowanych danych, ze szczególnym uwzględnieniem procesu przejścia epitelialno-mezenchymalnego.

2. Określenie wpływu procesu przejścia epitelialno-mezenchymalnego na przebieg kliniczny raka prostaty poprzez analizę ekspresji szeregu białek, takich jak: MIF, SOX-4, β-katenina, E-kadheryna.

3. Próba oceny zależności pomiędzy procesem przejścia epitelialno-mezenchymalnego, a przebiegiem klinicznym raka prostaty poprzez ocenę ekspresji szeregu białek, takich jak: SDF-1, CXCR4 oraz CXCR7.

5. Artykuły wchodzące w skład rozprawy doktorskiej

1. **Parol M**, Gzil A, Bodnar M, Grzanka D. Systematic review and meta-analysis of the prognostic significance of microRNAs related to metastatic and EMT process among prostate cancer patients. Journal of Translational Medicine. 2021;19:1–24. doi:10.1186/s12967-020-02644-x
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Systematic review and meta-analysis of the prognostic significance of microRNAs related to metastatic and EMT process among prostate cancer patients

Martyna Parol*, Arkadiusz Gzil, Magdalena Bodnar and Dariusz Grzanka

Abstract

The ability of tumor cells to spread from their origin place and form secondary tumor foci is determined by the epithelial–mesenchymal transition process. In epithelial tumors such as prostate cancer (PCa), the loss of intercellular interactions can be observed as a change in expression of polarity proteins. Epithelial cells acquire ability to migrate, what leads to the formation of distal metastases. In recent years, the interest in miRNA molecules as potential future treatment options has increased. In tumor microenvironment, miRNAs have the ability to regulate signal transduction pathways, where they can act as suppressors or oncogenes. MiRNAs are secreted by cancer cells, and the changes in their expression levels are closely related to a cancer progression, including epithelial–mesenchymal transition. These molecules offer new diagnostic and therapeutic possibilities. Therapeutics which make use of synthesized RNA fragments and mimic or block miRNAs affected in PCa, may lead to inhibition of tumor progression and even disease re-emission. Based on appropriate qualification criteria, we conducted a selection process to identify scientific articles describing miRNAs and their relation to epithelial–mesenchymal transition in PCa patients. The studies were published in English on Pubmed, Scopus and the Web of Science before August 08, 2019. Hazard ratios (HRs) and 95% confidence intervals (CI) as well as total Gleason score were used to assess the concordance between miRNAs and presence of metastases. A total of 13 studies were included in our meta-analysis, representing 1608 PCa patients and 15 miRNA molecules. Our study clarifies a relationship between the clinicopathological features of PCa and the aberrant expression of several miRNA as well as the complex mechanism of miRNA molecules involvement in the induction and promotion of the metastatic mechanism in PCa.

Keywords: Epithelial–mesenchymal transition, EMT, Metastases, microRNA, miRNA, Prostate cancer, Meta-analysis

Introduction

Prostate cancer (PCa) is the second most common diagnosed cancer and is considered the fifth leading cause of cancer-related deaths among men worldwide [1]. According to American Cancer Society statistics, the PCa incidence rates between 2011 and 2015 amounted 109.2 per year and the PCa death rates between 2012 and 2016 reached 19.2, showing decrease tendency [2] with the peak incidence (237.5 per year) and death rates (39.2) in 1992 [3]. In general, PCa has outstanding survival rates [3]. Surveillance, Epidemiology, and End Results Program (SEER 18 2009–2015) data shows the relative 5-year survival rate of local and regional stage PCa is almost 100%, for all races [2, 3]. However, for late stage PCa, the relative 5-year survival rate is only 30.5% [2, 3] Moreover, it is estimated that about 30% of men with diagnosed PCa have bone metastases [4]. In the U.S., the estimations for

*Correspondence: mparol@cm.umk.pl
Department of Clinical Pathomorphology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, 9 Curie-Sklodowskiej Street, 85-094 Bydgoszcz, Poland

PCa with bone metastases amount to 6.5% (47,607 PCa with bone metastases in 735,812 of all PCa cases) of all PCa cases in 2004 [5]. Since 2007, the low-risk PCa incidence rate has decreased, however, the metastatic PCa incidence rate has notable increased [6].

The metastatic PCa involves multistep processes, including invasion, migration and survival of migrating cells, also location and proliferation in distant sites [7]. A critical point in metastases formation is attributed to the epithelial–mesenchymal transition (EMT) process [8]. EMT is a unique process by which tumor epithelial cells undergo molecular reprogramming and cytoskeleton remodeling through their transition to mesenchymal cell phenotype, The cells lose their adhesion capability and acquire the ability to move and to invade other tissues [9]. It is well known that the EMT process constitutes the early stage of metastases [10]. More specifically, the EMT transition is characterized by E-cadherin suppression associated with loss of cell adhesion properties and acquisition of mesenchymal markers, such as N-cadherin, Vimentin, Fibronectin, which enable cell mobility [11].

In the last decade, microRNAs have been shown to play an important role in managing single or numerous complex steps of metastases leading to tumor outgrowth by deregulation of target genes expression. MicroRNAs (miRNAs) are approximately 18–22 nucleotides long, single-stranded and non-coding regulatory RNAs, habitually aberrant in PCa [12, 13]. In normal tissue, microRNAs have the ability to regulate many biological processes such as cell proliferation, cell cycle arrest, aging and apoptosis. A number of studies have confirmed their presence and participation in metastases. They are responsible for controlling the expression level of pro-metastatic genes through base-paring with messenger RNAs (mRNAs) at the post-transcriptional level and lead to enhancement or suppression of the metastatic process.

Single miRNA has the ability to target multiple mRNA genes simultaneously. Many previous studies have revealed that above 50% of miRNA genes are situated in cancer-associated genomic regions and they form a central nodal points due to cancer progression [14]. They constitute crucial modulators in human cellular signaling pathways and are engaged in controlling gene expression of their targets. The abnormal expression level of miRNAs is heavily related to disease progression [15]. Owing to changes in expression levels, miRNAs have been classified as oncogenic molecules and tumor suppressors. Oncomirs (Onc) promote cancerogenesis through damping the mRNAs encoding tumor suppressor proteins at translational level (miR-301a, miR-409-3p, miR-210-3p, miR-543, miR-409-3p, miR-410-3p in PCa). While, tumor suppressors (TS) miRNAs are responsible for repression of mRNAs encoding onco-proteins at translational level (miR-573, miR-802, miR-3622a, miR-466, miR-203, miR-132/212, miR-141-3p in PCa).

MicroRNAs show strong promise as molecular biomarkers and treatment opportunity due to several aspects. Firstly, they manifest tissue-specific expression patterns in malignancies and exhibit a highly stable form in formalin-fixed tissues [16], as well as in blood, plasma and other bodily fluids [17, 18]. Circulating microRNAs are resistant to endogenous RNases activity, therefore their expression level can be determined in bodily fluids. Secondly, miRNA expression levels are varied in individual cancers, so they can specify different types of cancer. Thirdly, there has been observed a clear dissimilarity of miRNA regulation between different kinds of cancer. Fourthly, the efficiency and accuracy of miRNAs oriented to the target cells, make them attractive molecules in miRNA-based therapy. Fifthly, various microRNA profiles could be used for distinguish pathological and normal tissue as well as accurately recognize cancerous subtypes [19].

To the best of our knowledge there is no meta-analysis which describes the prognostic significance of miRNAs-related to the EMT process in PCa. In this study, we revealed the importance of fifteen deregulated miRNAs that constitute therapeutic potential and could be utilized as measurable indicators of PCa metastatic potential. To investigate their significance in EMT transition, we performed a meta-analysis, based on patient data extracted from scientific articles, where studies were conducted on formalin-fixed paraffin-embedded or fresh/snap-frozen tissues.

Materials and methods

Search strategy and eligibility criteria

The selected publications were identified by using up-to-date electronic databases, including PUBMED, Scopus and Web of Science online databases. The following keywords were used for the search: miRNA, EMT and prostate cancer. The inclusion criteria were as follows: (1) type of microRNA (miRNA was related to EMT process); (2) the articles were published between 2010 and 2019 year; (3) the study was conducted on formalin-fixed paraffin embedded tissue samples (FFPE) or fresh/snap-frozen prostate cancer tissues; (4) the study contained the following clinicopathological descriptions: TNM stages, according to TNM Classification of Malignant Tumors system (TNM) as well as Gleason score (GS) which were grouped by both low and high level of microRNAs expression. Exclusion criteria were as follows: (1) review articles; (2) studies based on PC cell lines; (3) non-clinical samples; (4) lacked sufficient data for estimating Hazard Ratios (HRs) and their 95% confidence intervals (CIs); (5) duplicate articles; (6) non-English paper; (7) case reports,

letters, commentaries, conference abstract. The publication list was last updated on August 08, 2019.

Quality assurance

Methodology quality evaluation of incorporated studies was performed by two reviewers, working independently with the Newcastle–Ottawa Scale (NOS) for case–control quality assessment [20]. The evaluation process was divided into three steps: selection with a maximum score of 4, comparability with a maximum score of 2 and exposure/outcome with a maximum score of 3. A total score of 6–9 points ensured the highest quality.

Data extraction

The data extraction process was provided irrespectively two times by one author (MP) and was supervised by the senior reviewers in case of discrepancies (MB, DG). We collected the following items extracted from all included publications: (1) basic details: the title, first author's last name, year of publication, country/continent of origin; (2) general details of the study: the name of microRNAs, sample size and kind of sample, microRNA detection method, size of both the low and high group of microRNAs expression level; (3) clinicopathological details: the TNM Classification of Malignant Tumors and Gleason score.

Statistical analysis

All statistical analyses were completed with the PQStat package. P value < 0.05 was considered as statistically significant. Estimation of miRNAs expression in PCa patients and their prognostic effects was presented using forest plots. We used the fixed-effects model to calculate Relative Risks (RRs) and 95% CIs in all included studies. In general, a RR > 1 demonstrated a poor patient outcome in cases with higher miRNA expression. The Cochrane's Q statistic and Inconsistency index (I^2) test was used to assess of heterogeneities. An $I^2 \geq 50\%$ is considered as a high heterogeneity level. Funnel plots were used to visualize of publication bias.

Results

Study characteristics

Study selection process identified 13 articles, which were included in the meta-analysis. Our study worked with articles published between 2010 and 2019, focusing on data of following miRNA: miR-410-3p [21], miR-573 [22], miR-3622a [23], miR-301a [24], miR-466 [25], miR-503 [26], miR-203 [27], miR-210-3p [28], miR-802 [29], miR-543 [30], miR-132 and miR-212 [31], miR-409-3p and miR-409-5p [32] and miR-141-3p [33].

The total of 289 primary articles were searched in PubMed, Scopus and Web of Science databases. The flow chart diagram of the study selection process in our meta-analysis is presented below (Fig. 1). In the first step, we excluded 115 duplicated literatures. After examination of abstracts and full-text articles, we removed 161 publications and included 13 articles, finally. The main characteristics of involved studies was recapitulated in Table 1. The miRNAs in Table 1 were arranged in alphabetical order and some articles were divided into two parts because of more described miRNAs. The total group of the meta-analysis incorporated 1608 patients. The enrolled participants were divided into clinical and pathological stages, according to the TNM Classification of Malignant Tumors (TNM) standard. The participants of included studies mainly derived from China and the USA, but the dominant ethnicity was Chinese in approximately 60% of all studies. Detection of miRNAs deregulation was accomplished using real-time polymerase chain reaction (RT-PCR). The miRNAs fraction was isolated from fresh/snap-frozen or formalin-fixed paraffin embedded sections of tissue.

Associations between miRNAs and the clinicopathological features of prostate cancer

In the current meta-analysis, we checked the association between miRNAs and clinicopathological features of prostate cancer presented in Table 2. For these compilations the Relative Risk (RR) and corresponding 95% Confidence Intervals (95% CI) were calculated.

Association between miRNAs and the metastasis generally

A total of eight articles, involving ten miRNA molecules, were included to analysis of the association between miRNAs expression and the presence of metastasis. The total number of included patients was 881 individuals. The heterogeneity of this analysis was at high level ($p < 0.000001$, $I^2 = 87\%$). Patients with high expression of miR-410-3p, miR-212, miR-210-3p and miR-543 had significantly poorer prognosis compared to their low expression level respectively (RR = 1.85, 95% CI 1.25–2.74, $p = 0.002$; RR = 11.59, 95% CI 1.61–83.31, $p = 0.014$; RR = 2.43, 95% CI 1.61–3.67, $p = 0.000$; RR = 6.60, 95% CI RR = 1.68–25.95, $p = 0.005$) (Fig. 2a). Furthermore, low expression of other four miRNAs, miR-132, miR-802, miR-141-3p, miR-503 were predicted significantly to tumor progression, compared to high expression of them (RR = 0.36, 95% CI 0.14–0.92, $p = 0.033$; RR = 0.35, 95% CI 0.18–0.69, $p = 0.002$; RR = 0.45, 95% CI: 0.28–0.73, $p = 0.001$; RR = 0.23, 95% CI 0.05–0.99, $p = 0.05$). In the one analysis with miR-301a no statistically significant association was noticed (Fig. 2a). The clinicopathological details of the group, concerning information about metastases were presented in Table 3.

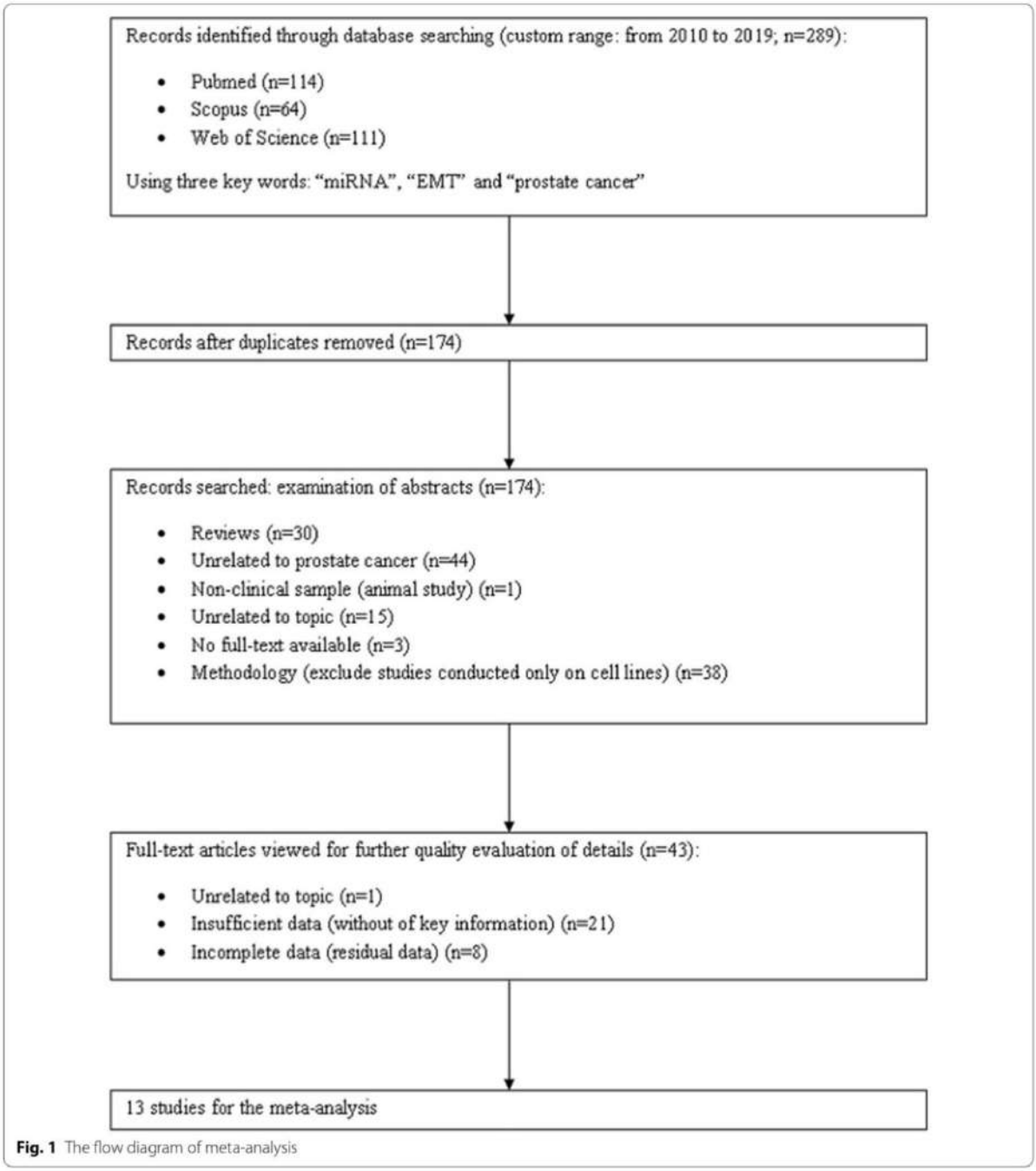

Fig. 1 The flow diagram of meta-analysis

Sub-analysis 1. Association between miRNAs and the lymph node metastasis

Out of eight published papers with the metastasis patients data the analyses selected three articles, describing miRNAs expression among prostate cancer patients with lymph node metastasis. The total number of engaged patients was 353 individuals, constituting approximately 43% of all PCa patients with lymph node metastasis. The heterogeneity of this sub-analysis was at low level and the heterogeneity was not statistically

Table 1 The main characteristic of included studies

Author	Publication year	Country	miRNA type	General details of the study		
				Sample type	Sample size	Detection method
Bucay	2017	USA	miR-3622a	FFPE	138	RT-PCR
Colden	2017	USA	miR-466	FFPE	96	RT-PCR
Du	2017	China	miR-543	Fresh	42	RT-PCR
Fu	2016	China	miR-132	FFPE	57	RT-PCR
Fu	2016	China	miR-212	FFPE	57	RT-PCR
Huang	2017	China	miR-141-3p	Snap-frozen	141	RT-PCR
Jiang	2016	China	miR-503	FFPE	82	RT-PCR
Josson	2017	USA	miR-409-3p	Tissue microarray	61	RT-PCR
Josson	2017	USA	miR-409-5p	Tissue microarray	61	RT-PCR
Nam	2016	Canada	miR-301a	FFPE	585	RT-PCR
Ren	2017	China	miR-210-3p	Snap-frozen	149	RT-PCR
Saini	2010	USA	miR-203	FFPE	22	RT-PCR
Wang	2015	China	miR-573	FFPE	80	RT-PCR
Wang	2017	China	miR-802	Fresh	73	RT-PCR
Zhang	2018	China	miR-410-3p	Fresh	82	RT-PCR

Table 2 Associations between miRNAs and clinicopathological features studied in current meta-analysis

Clinicopathological features	miRNAs	Figures
Presence of metastasis		
GM	miR-410-3p, miR-132, miR-212, miR-802, miR-141-3p, miR-210-3p, miR-301a, miR-503, miR-543	Figure 2a
LNM	miR-802, miR-301a, miR-503	Figure 2b
DM	miR-132, miR-212, miR-802, miR-141-3p, miR-210-3p	Figure 2c
BM	miR-141-3p, miR-210-3p	Figure 2d
Total Gleasone scor		
GS > 6	miR-410-3p, miR-132, miR-212, miR-203, miR-409-3p, miR-409-5p, miR-301a, miR-466, miR-503, miR-543, miR-573, miR-3622a	Figure 2e
GS > 7	miR-132, miR-212, miR-802, miR-141-3p, miR-210-3p, miR-203, miR-409-3p, miR-409-5p, miR-301a, miR-466, miR-503, miR-543, miR-573, miR-3622a	Figure 2f
pT stage according to TNM	miR-410-3p, miR-132, miR-212, miR-802, miR-203, miR-301a, miR-466, miR-503, miR-543, miR-573, miR-3622a	Figure 2g

GM general metastasis, *LNM* lymph node metastasis, *DM* distant metastasis, *BM* bone metastasis, *GS* Gleason score, *pT* stage-pathological tumor stage

significant ($p = 0.330$, $I^2 = 9.71\%$). The low expression of miR-503 was associated with the presence of lymph node metastasis (RR = 0.22, 95% CI 0.052–0.989, $p = 0.047$), compared to high expression of miR-503 (Fig. 2b). The results of others two of three miRNAs were not statistically significant (miR-802 and miR-301a).

Sub-analysis 2. Association between miRNAs and the distant metastasis

From the total eight articles, describing data of metastasis presence, we distinguished four articles, including five miRNAs related to the distant metastasis. This investigated group consisted of 477 individuals, constituting 51% of all patients with metastasis. The heterogeneity analysis for these five miRNAs was at high level ($p < 0.000$, $I^2 = 91.59\%$). The overexpression of miR-212 and miR-210-3p were found to associate with the higher risk of distant metastasis (RR = 11.59, 95% CI 1.61–83.31, $p = 0.015$; RR = 2.43, 95% CI 1.61–3.67, $p = 0.000$, respectively), compared to loss of miR-212 and miR-210-3p expression. (Fig. 2c). On the other hand, the downregulation of miR-132, miR-802, miR-141-3p were associated with higher distant metastasis risk (RR = 0.36, 95% CI

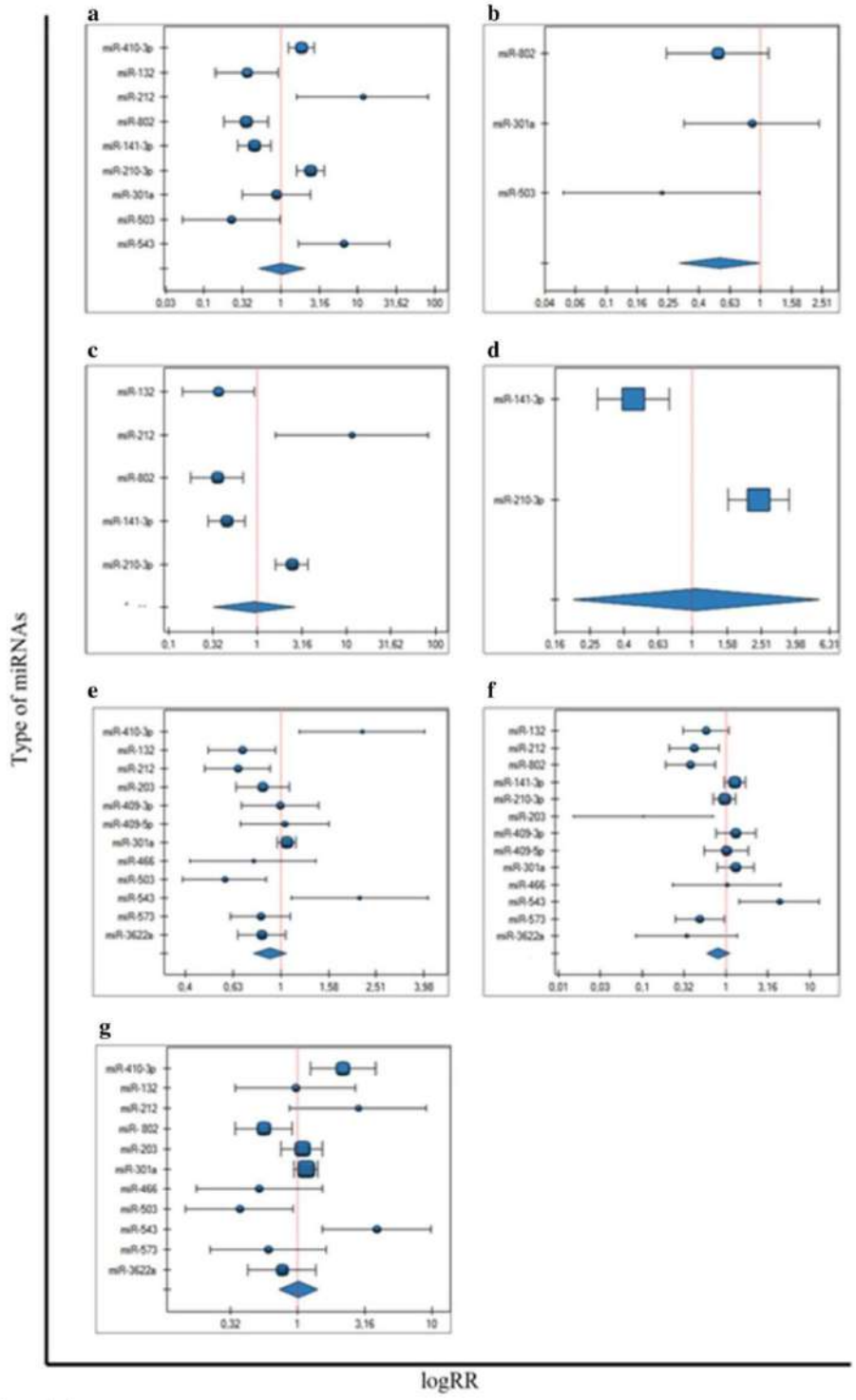

Fig. 2 Forest plot analysis

Table 3 The metastasis presence of included studies

Author (year)	miRNA	Expression level	Clinicopathological details	
			Presence of metastasis	p-value
Du (2017)	miR-543	Low	2	0.005
		High	12	
Fu (2016)	miR-132	Low	7	0.043
		High	6	
Fu (2016)	miR-212	Low	1	0.001
		High	12	
Huang (2017)	miR-141-3p	Low	36	< 0.001
		High	16	
Jiang (2016)	miR-503	Low	9	0.04
		High	2	
Nam (2016)	miR-301a	Low	7	0.5260
		High	7	
Ren (2017)	miR-210-3p	Low	20	< 0.001
		High	48	
Wang (2017)	miR-802	Low	15	0.002
		High	9	
Zhang (2018)	miR-410-3p	Low	17	0.001
		High	33	

0.144–0.924, $p=0.034$; RR = 0.35, 95% CI 0.179–0.692, $p=0.002$; RR = 0.45, 95% CI 0.277–0.734, $p=0.001$) in comparison to the up regulated miR-132, miR-802 and miR-141-3p (Fig. 2c).

Sub-analysis 3. Association between miRNAs and the bone metastasis

Out of eight articles analyzing data of patients with metastases, we distinguished two articles analyzing patients with bone metastases and performed a separate sub-analysis. We decided to include these two articles to sub-meta-analysis apart from the rest types of metastasis. The total number of included patients was 290, which constitutes 35% of all metastatic patients. The heterogeneity test was at high level ($p<0.000$, $I^2=96.27\%$). The results indicated that high miR-210-3p expression level in prostate cancer tissue was significantly associated with the frequent occurrence of bone metastasis (RR = 2.43, 95% CI 1.61–3.67, $p=0.000$), compared to low miR-210-3p expression. (Fig. 2d). On the other hand low expression level of miR-141-3p among patients with prostate cancer was associated with the higher risk of bone metastasis occurrence (RR = 0.45, 95% CI 0.27–0.73, $p=0.001$) in comparison to the high miR-141-3p expression (Fig. 2d).

Association between miRNAs and the Gleason score

Out of ten articles, we included twelve miRNA molecules to analyze the association between each miRNAs expression level and the total Gleason score > 6. The number of patients was 1448 patients. The heterogeneity test was at middle-high level ($p=0.0001$, $I^2=70\%$). We distinguished five miRNA molecules with RR value statistically significant. We found that elevated expression of miR-410-3p was significantly associated with Gleason score > 6 (RR = 2.19, 95% CI 1.20–4.00, $p=0.01$), compared to the loss of miR-410-3p expression level (Fig. 2e). On the other hand, we noticed that decreasing expression of the miR-132, miR-212, mir-503, miR-543 was significantly associated with a total Gleason score > 6 (RR = 0.69, 95% CI 0.49–0.95, $p=0.02$; RR = 0.66, 95% CI 0.48–0.90, $p=0.0007$; RR = 0.58, 95% CI 0.39–0.81, $p=0.009$; RR = 2.14, 95% CI 1.10–4.16, $p=0.02$), compared to the higher miR-132, miR-212, miR-503, miR-543 expression level (Fig. 2e).

Among eleven publications, we found 13 miRNA molecules, which were included in the analysis of association with the Gleason score > 7. The number of patients was 1630 individuals. The heterogeneity test was middle-high level ($p=0.00007$, $I^2=69.68\%$). The five of mentioned miRNA molecules were found statistically significant. The analysis showed that low expression level of the miR-212, miR-802, miR-203, miR-543 and miR-573 was significantly associated with the > 7 Gleason score (RR = 0.42, 95% CI 0.21–0.83, $p=0.01$; RR = 0.38, 95% CI 0.19–0.75, $p=0.005$; RR = 0.1, 95% CI 0.01–071, $p=0.02$; RR = 4.33, 95% CI 1.44–13.02, $p=0.009$; RR = 0.49, 95% CI 0.25–0.96, $p=0.04$) in comparison to the high miR-212, miR-802, miR-203, miR-543 and miR-573 expression (Fig. 2f). The Gleason score details of studied samples were demonstrated in Table 4.

Association between miRNAs expression level and pT stage

The eleven miRNA molecules from ten publications have been included to analyze of the association of miRNAs expression and pT stage. The patients' number was 1389 individuals. The heterogeneity test was at middle-high level ($p=0.0003$, $I^2=69.53\%$). The higher risk of the advanced prostate cancer stage was related to the high expression level of miR-410-3p (RR = 2.16, 95% CI 1.23–3.79, p = 0.007) and miR-543 (RR = 3.85, 95% CI 1.52–9.77, $p=0.004$), compared to the low miR-210-3p and miR-410-3p expression levels (Fig. 2g). On the contrary, the higher risk of the advanced prostate cancer stage was connected to the miR-802 (RR = 0.55, 95% CI 0.34–0.9, $p=0.02$) and miR-503 (RR = 0.37, 95% CI 0.14–0.92, $p=0.03$) low expression levels, compared to the high expression level of miR-802 and miR-503 molecules

Table 4 The Gleason score presence of included studies

Author (year)	miRNA	Expression level	Clinicopathological details		
			Gleason score		p-value
			≤7	>7	
Bucay (2017)	miR-3622a	Low	89	9	0.0223
		High	22	1	
Colden (2017)	miR-466	Low	57	7	<0.0001
		High	14	2	
Du (2017)	miR-543	Low	18	3	0.006
		High	8	13	
Fu (2016)	miR-132	Low	14	19	0.028
		High	16	8	
Fu (2016)	miR-212	Low	11	20	0.004
		High	19	7	
Huang (2017)	miR-141-3p	Low	23	48	<0.001
		High	54	16	
Jiang (2016)	miR-503	Low	11	30	<0.01
		High	23	17	
Josson (2017)	miR-409-3p	Low			
		High			
Josson (2017)	miR-409-5p	Low			
		High			
Nam (2016)	miR-301a	Low	268	24	0.3634
		High	262	31	
Ren (2017)	miR-210-3p	Low	0	22	<0.001
		High	0	49	
Saini (2010)	miR-203	Low	10	1	
		High	11	0	
Wang (2015)	miR-573	Low	16	18	0.044
		High	23	8	
Wang (2017)	miR-802	Low			0.004
		High			
Zhang (2018)	miR-410-3p	Low	30 (<7)	10 (≥7)	0.006
		High	19 (<7)	23 (≥7)	

Table 5 The pathological T stage for tumors of included studies

Author (year)	miRNA	Expression level	Clinicopathological details		
			Pathological/clinical Stage[a]		p-value
			≤pT2	≥pT3	
Bucay (2017)	miR-3622a	Low	68	22	1.000
		High	16	1	
Colden (2017)	miR-466	Low	40	3	<0.0001
		High	13	22	
Du (2017)	miR-543	Low	18[a]	4[a]	<0.001
		High	6[a]	14[a]	
Fu (2016)	miR-132	Low	13	4	1.000
		High	31	9	
Fu (2016)	miR-212	Low	23	3	0.063
		High	21	10	
Jiang (2016)	miR-503	Low	27	14	0.03
		High	35	5	
Nam (2016)	miR-301a	Low	189	103	0.2968
		High	175	118	
Saini (2010)	miR-203	Low	9	2	
		High	10	1	
Wang (2015)	miR-573	Low	30[a]	11[a]	0.003
		High	18[a]	23[a]	
Wang (2017)	miR-802	Low	10	17	0.02
		High	30	16	
Zhang (2018)	miR-410-3p	Low	29[a]	11[a]	0.003
		High	17[a]	23[a]	

[a] clinical tumor stage

(Fig. 2g). The pathologic tumor stage (pT stage) and details of included samples were shown in Table 5.

Regulatory pathways for miRNA-related to EMT program in prostate cancer

In the current meta-analysis, we obtained the important results for 15 miRNAs, based on primary data from 13 published studies. For 7 miRNAs (miR-410-3p, miR-301a, miR-802, miR-543, miR-409-3p, miR-409-5p, miR-210-3p) of 13 articles (54%), the deregulation was expressed as an overexpression in prostate cancer samples, while for 8 (62%) of 13 published papers the expression was down regulated (miR-573, miR-3622a, miR-466, miR-503, miR-203, miR-141-3p, miR-212, miR-132). The direct targets of specific miRNAs and their expression alterations in a prostate cancer were presented in the Fig. 3. The associations between specific deregulation of miRNAs expression and following parameters: clinicopathological features, including general, lymph node, distant or bone metastasis, the both Gleason scores > 6 and > 7 as well as pT stage were demonstrated in Table 6. For better understanding aforementioned dependencies, the expression signatures for individual clinicopathological features were demonstrated in Fig. 4. The various mRNA targets of dysregulated miRNAs and their signal transduction pathways in prostate tumor cells were demonstrated in Fig. 5. The presentation of key regulatory molecular mechanisms of analyzed miRNAs involved in progression to metastatic disease supports the understanding of the underlying miRNA functions in prostate cancer cells.

Many previous studies have indicated that miRNAs play a crucial role in various biological processes,

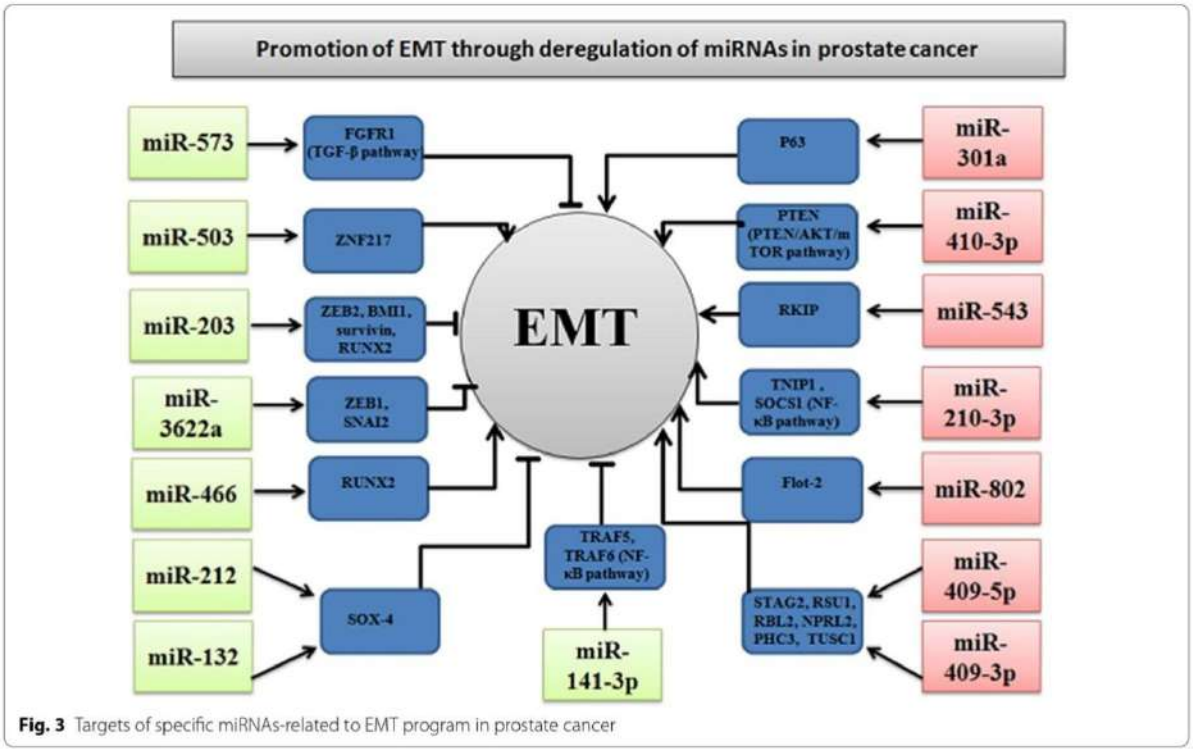

Fig. 3 Targets of specific miRNAs-related to EMT program in prostate cancer

Table 6 Association between miRNAs expression and clinicopathological features

	GM	LNM	DM	BM	GS > 6	GS > 7	pT stage
miR-132	↓		↓		↓		
miR-141p	↓		↓	↓			
miR-503	↓	↓			↓		↓
miR-212					↓	↓	
miR-573						↓	
miR-210-3p	↑		↑	↑			
miR-410-3p	↑				↑		↑
miR-543	↑						↑
miR-203						↓	

GM general metastasis, *LNM* lymph node metastasis, *BM* bone metastasis, *GS > 6* Gleason score > 6, *GS > 7* Gleason score > 7, *pT stage* pT stage according to the TNM system, ↑ overexpressed miRNA, ↓ downregulated miRNA

encompassing development, proliferation, differentiation, cell fate determination, apoptosis, angiogenesis, adhesion, metastasis, signal transduction, organ development, hematopoietic lineage differentiation, host-viral interaction and tumor genesis [34–40]. The known biological functions of analyzed miRNAs in the cancerous tissues have been presented in Table 7.

Publication bias

The high heterogeneity level of data of assembled miRNAs could be related to the variables selected in each study. The most frequently heterogeneity sources of variance in meta-analyses are different research methodologies, various patient races, various research samples and methods of sample collection or storage, insufficient data, the small study groups, different analysis periods

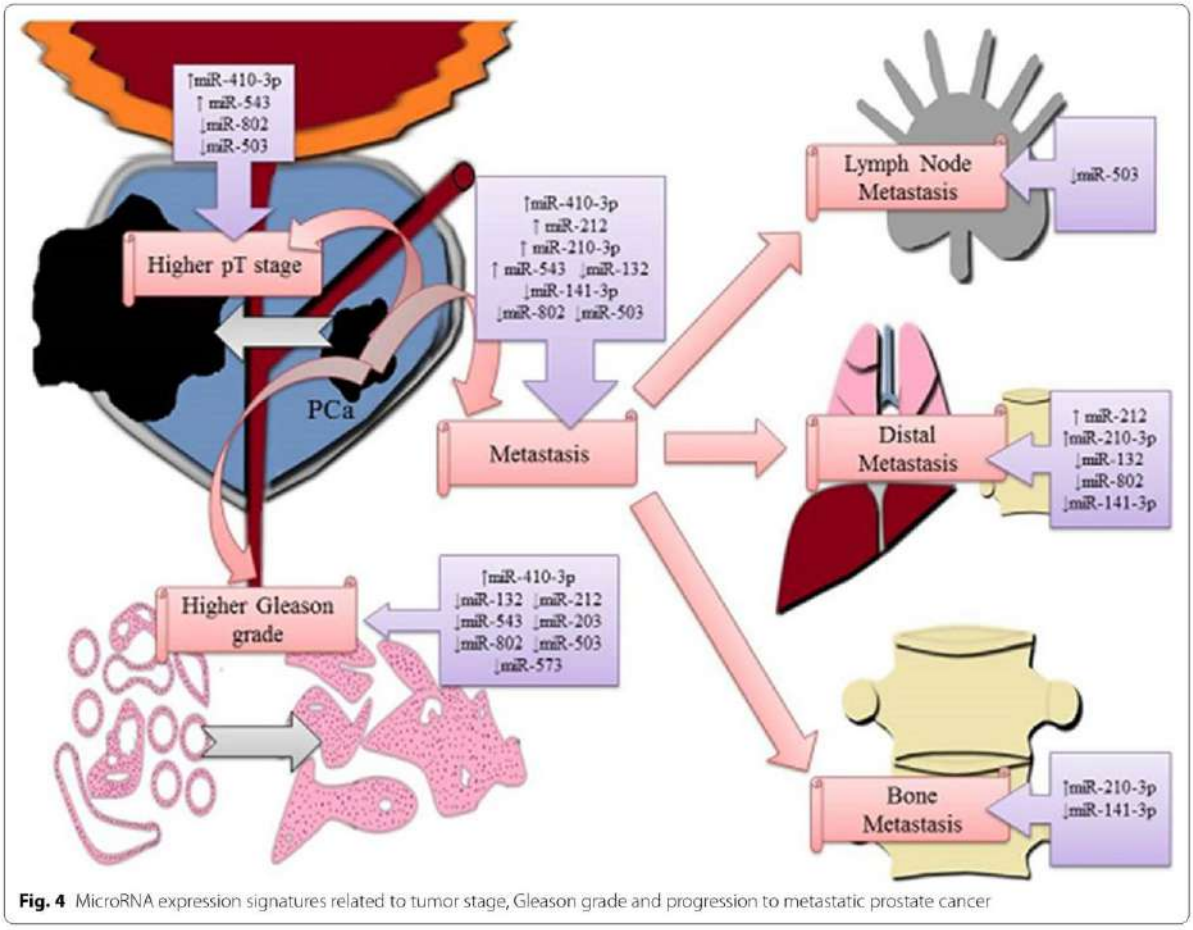

Fig. 4 MicroRNA expression signatures related to tumor stage, Gleason grade and progression to metastatic prostate cancer

(follow-up time), various indicators and methods of estimating results. In order to evaluate disturbing factors we performed sensitivity analysis.

We performed Begg's funnel plot and Egger's test to judge potential publication bias of completed analysis. Significant heterogeneities ($p < 0.05$, $I^2 > 50\%$) were found in a miRNAs and metastasis analysis. The heterogeneity test performed for the analysis of the association of miRNAs and the general metastasis indicated high level ($I^2 = 87.99\%$) and was statistically significant ($p < 0.00002$). $I^2 > 75\%$ indicates a high heterogeneity of the study, which could be disturbed by different types of metastasis. The main results for pooled RRs for meta-analysis were recapitulated in Table 8. It was right to consider a more homogenous group to meta-analysis in the way of excluding miRNAs, which lead to perturb a heterogeneity of study. In order to reduce the risk of heterogeneity and better presenting of the results, we decided to repeat the analysis formed subgroups based on metastasis type: lymph node metastasis, distant metastasis and bone metastasis. The associations of miRNAs and both presence of distant metastasis and bone metastasis revealed the high level of heterogeneity test and the results was statistically significant ($I^2 = 91.59\%$, $p < 0.000001$; $I^2 = 96.27\%$, $p < 0.000001$, respectively). However, the association between miRNAs expression and lymph node metastasis noticed a low level of heterogeneity test at $I^2 = 9.71\%$ and was statistically insignificant. Others compilations indicated a moderate heterogeneity level: $I^2 = 70.09\%$, $p = 0.000125$ for GS.6; $I^2 = 69.68\%$, $p = 0.000085$ for GS > 7; $I^2 = 69.53\%$, $p = 0.000293$ for pT stage. The Egger's test, counting the asymmetry of conducted analysis, showed statistically non-significant results for all compilations. The funnel plots presenting publication bias were shown in Fig. 6.

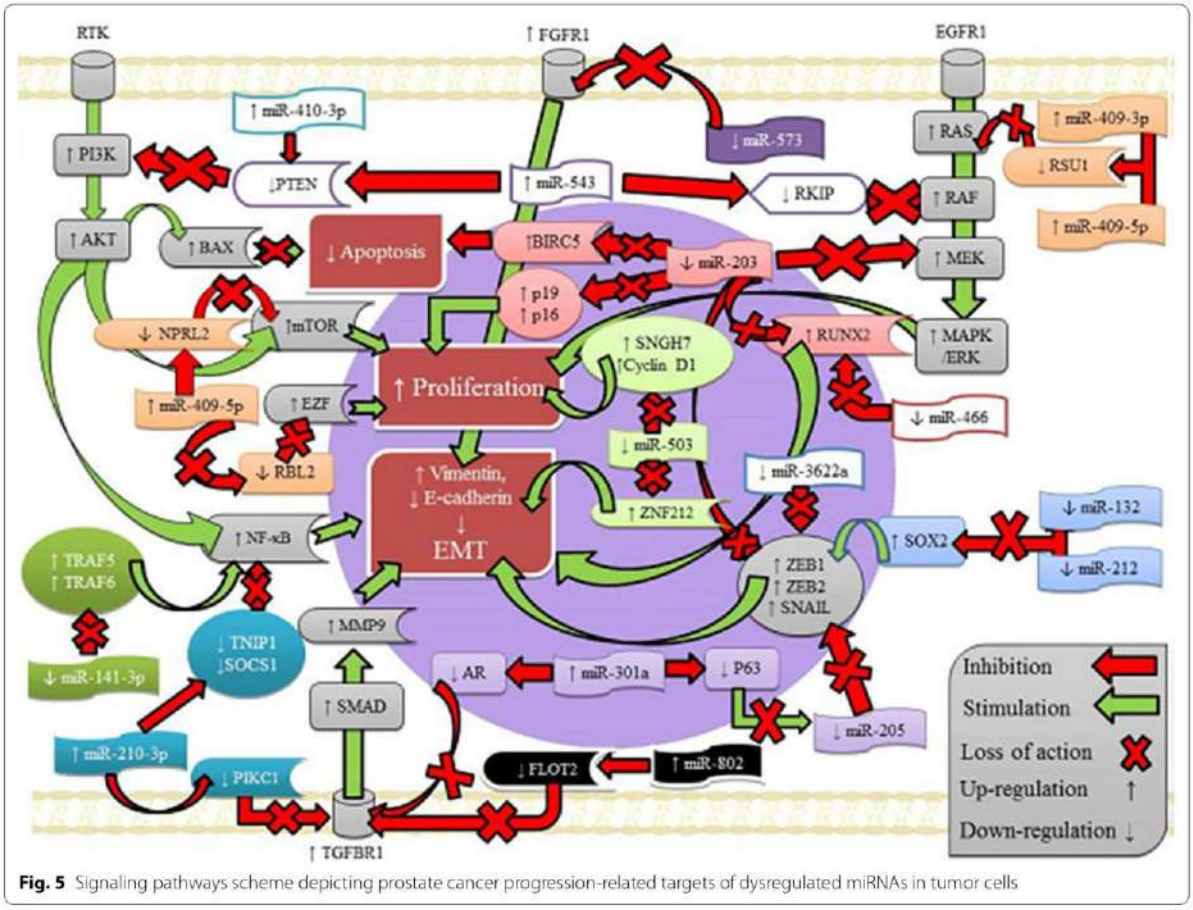

Fig. 5 Signaling pathways scheme depicting prostate cancer progression-related targets of dysregulated miRNAs in tumor cells

Sensitivity analysis

We performed sensitivity analysis to establish the impact of single miRNA studies on the stability of achieved results through removing individual miRNA study, in a sequential manner. For the analysis of association between miRNAs expression level and the general metastasis, we noticed that three studies with miR-212, miR-210-3p and miR-543 had the largest effect on the results, but each p-value was statistically non-significant. The rest of the studies showed the RR value close to one or greater than one and constituted stable and robust results. For the comprehensive analysis of individual miRNAs expression and specific metastasis types, the sensitivity analysis demonstrated disorder results for miR-301a, concerning the lymph node metastasis, with the statistically significant p-value ($p = 0.018181$) as well as miR-212 and miR-210-3p, concerning the distant metastasis, but the p-value was not statistically significant. The RR values of the rest engaged studies of mentioned two analyses were close to each other. For GS > 6, the sensitivity analysis showed two studies with miR-410-3p and miR-543, disturbing the association analysis, but the p-value indicated statistically non-significant results. The rest of RR value of miRNA studies was close to one and stable. Similarly, for pT stage, the sensitivity analysis showed three studies (with miR-410-3p, miR-212, miR-543) with RR value, deviating from the rest studies, but the p-value revealed statistically non-significant results. The diagrams illustrating sensitivity analysis were included in Fig. 7.

Discussion

MicroRNAs are molecules which show promise as potential markers of poor prognosis among patients with malignancies. The latest advances in prostate cancer research showed diagnostic, prognostic and treatment utility of variety miRNA molecules measured in serum, plasma, ejaculate, tissue or urine [104–108]. Nonetheless, our study describes the significance of miRNAs involved in the EMT program for the first time, incorporating 1608 prostate cancer patients.

Table 7 Biological functions of discussed miRs in cancer

Name of microRNA	Genomic locations	Biological functions in cancer	Ref.
miR-3622a	8p21.1	miR-3622 regulates cancer cells proliferation, progression, apoptosis, G0/G1 cell cycle arrest, migration and invasion abilities of cancer, EMT, metastasis	[23, 41–44]
miR-466	3p23	miR-466 induces proliferation, migration, invasion, angiogenesis cell cycle arrest, apoptosis in cancer cells, EMT and metastasis	[25, 45, 46]
miR-543	14q32.31	mir-543 mediates cell proliferation and tumor growth, apoptosis, the cell cycle, migration, invasion, EMT and metastasis	[30, 47–53]
miR-132	17p13.3	miR-132 regulates proliferation of cancer cells, colony formation, tumor growth, migration and invasion of cancer cells, the G1/S phase transition of the cell cycle, EMT in cancer cells, apoptosis in cancer cells	[54–58]
miR-212	17p13.3	miR-212 mediates the proliferation and invasion of cancer cells, EMT and metastasis	[59–62]
miR-141-3p	12p13.31	miR-141 regulates proliferation, tumor growth, migration, EMT, chemosensitivity, apoptosis, angiogenesis, oxidative stress response, cellular motility and control "stemness" miR-141–3p regulates proliferation, tumor growth, migration, and invasion of cancer cells, cancer cell progression, EMT and metastasis	[33, 63–70]
miR-503	Xq26.3	miR-503 regulates cell proliferation, tumor angiogenesis, cell growth and EMT in cancer cells	[71–77]
miR-409-3p	14q32.31	miR-409-3p mediates tumorigenesis, tumor growth, invasion, EMT and metastasis of tumor cells	[32, 78–81]
miR-409-5p	14q32.31	miR-409-5p mediates tumorigenesis, EMT and bone metastasis	[32, 81]
miR-301a	17q22	miRNA-301a regulates tumor cell invasion, migration, cell proliferation, apoptosis and enhancing chemosensitivity, EMT and metastasis	[82–84]
miR-210-3p	11p15.5	miR-210-3p regulates cell survival, stem cell differentiation, angiogenesis, DNA damage repair, mitochondrial metabolism, immune response, EMT and metastasis	[28, 63, 85–89]
miR-203	14q32.33	miR-203 regulates tumor proliferation, invasion, migration, apoptosis, EMT and metastasis	[90–93]
miR-573	4p15.2	miR-573 mediates cell proliferation, tumor growth, apoptosis, angiogenesis, EMT and invasion of cancer cells	[94–96]
miR-802	21q22.12	miR-802 modulates proliferation, tumor growth, apoptosis, migration, invasion, EMT and metastasis of cancer cells	[29, 97–100]
miR-410-3p	14q32.31	miR-410-3p mediates cell proliferation, invasion, migration, EMT, apoptosis, chemosensitivity and autophagy	[21, 101–103]

Table 8 Main results of pooled RRs in the meta-analysis

Comparisions (miRNA groups)	Heterogeneity test			Summary RR (95% CI)	Asymmetrical distribution		Studies
	Q	p-value	I^2 (%)		b Egger coefficient	p-value	
General metastasis	66.60	< 0.000001	87.99	1.05 (0.52, 2.09)	− 0.86	ns (0.70)	9
Distant metastasis	47.58	< 0.000001	91.59	0.92 (0.32, 2.61)	− 0.82	ns (0.86)	5
Lymph node metastasis	2.21	ns (0.33)	9.71	0.54 (0.30, 1.00)	− 1.80	ns (0.65)	3
Bone metastasis	26.83	< 0.000001	96.27	1.05 (0.84, 5.49)	NA	NA	2
Gleason score > 6	36.78	0.000125	70.09	0.90 (0.77, 1.06)	− 0.71	ns (0.46)	12
Gleason score > 7	39.58	0.000085	69.68	0.82 (0.60, 1.13)	− 1.55	ns (0.15)	13
pT stage	32.81	0.000293	69.53	1.02 (0.73, 1.41)	− 0.31	ns (0.77)	11

Cancer metastases are a leading cause of cancer mortality and accounts for approximately 90% of deaths due to cancer [109]. However, appropriate diagnostic techniques for predicting progression potential have still not been discovered. Our results provided by 13 tissue-based studies for 7 overexpressed and 8 down regulated miRNAs revealed 9 miRNAs with the highest association with metastatic stage of PCa. The current meta-analysis suggested that miR-210-3p and miR-141-3p had particularly notable significance in a formation of metastasis. Overexpressed miR-210-3p and down regulated miR-141-3p have been found to be associated with the presence of general metastasis, distant metastasis as well as bone metastasis.

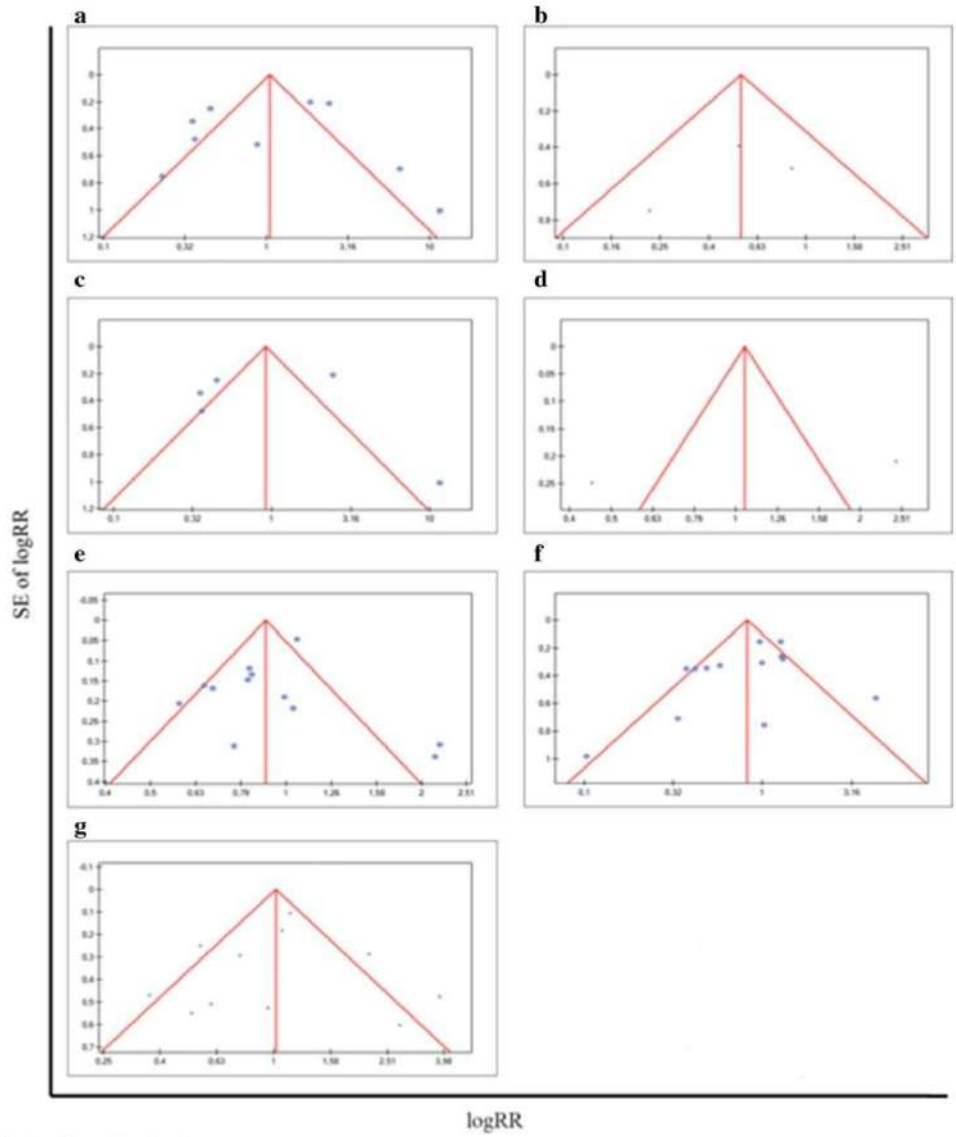

Fig. 6 Funnel plots for publication bias

MiR-210-3p has been widely recognized as an oncogene, engaged in tumor progression process. MiR-210-3p was documented as up regulated in many cancers: breast cancer [110], lung cancer [111], pancreatic cancer [112], head and neck cancer [113]. In breast cancer, miR-210-3p was recognized in rapid formation of distant metastases [114–116]. Similarly, miR-210-3p expression was found to be elevated in bone metastatic PCa patients compared to non-metastatic PCa patients. Additionally, it was found that miR-210-3p silencing has significantly attenuated bone metastasis formation in PC-3 cells in vivo [28]. As a consequence, it is likely that through the assessment of miRNA levels, metastasis types could be differentiated. Our meta-analysis revealed characteristic miRNAs signatures for each metastasis type. Based on presented results a downregulation of miR-503 seems to be specific for lymph node metastasis while a upregulation of miR-210-3p as well as a downregulation of miR-132 and miR-141-3p are associated to presence of distant metastasis. The signature of aforementioned miRNAs

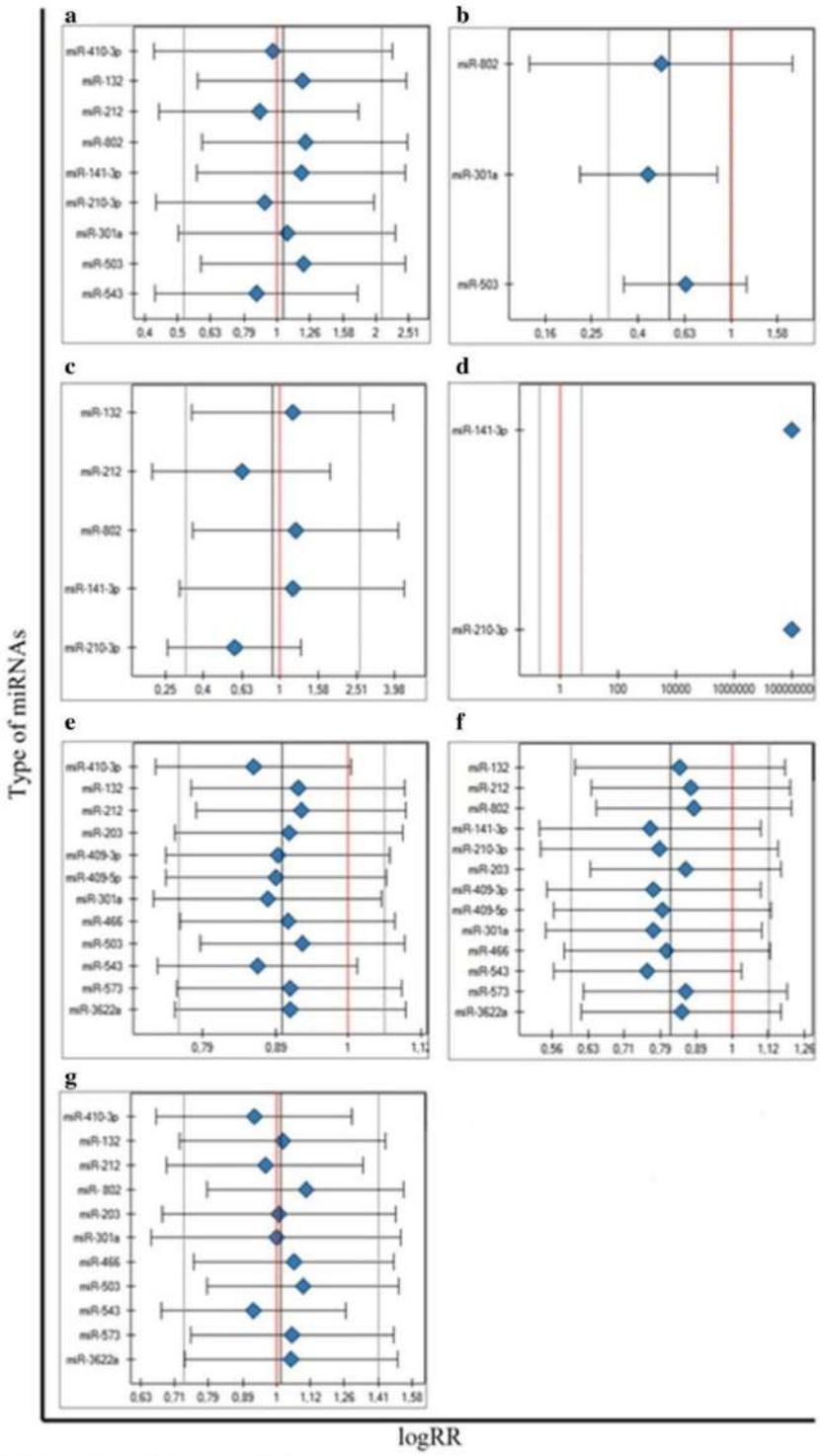

Fig. 7 Sensitivity analysis for meta-analysis groups and subgroups

could be potentially used to estimate a risk of metastasis development at an early stage of PCa followed by a more radical treatment procedure in selected cases. On the other hand, the miR-141-3p has been found acting as a tumor-suppressing miRNA, inhibiting the cancer cells progression via directly targeting of tumor necrosis factor receptor-associated factor 5 (TRAF5) in colorectal cancer tissues and cell lines [117]. Similarly, at PCa study, miR-141-3p has been found down regulated, acting as a tumor suppressor that targets TRAF5 and TRAF6 [33].

In the current meta-analysis it was shown that the formation of metastasis overall has been significantly associated with increased expression of miR-410-3p and miR-543. The recent study of Zhang et al. has shown that the elevated expression of miR-410-3p affects a downregulation of phosphatase and tensin homolog deleted on chromosome ten (PTEN) and results in an activation of AKT/mTOR signaling pathway. They have also shown that miR-410-3p plays a crucial role as an oncogene in PTEN/AKT/mTOR pathway [21]. It is possible that its upregulation could promote a disease progression and a metastatic ability of prostate cancer due to deactivation of PTEN protein, a natural inhibitor of PI3K/AKT/mTOR pathway. Similar mechanism may be associated with increased miR-543 level. In colorectal cancer (CRC), miR-543 has been found acting as an oncogene and promotes tumor progression [47]. Moreover, MiR-543 has been significantly up regulated in metastatic prostate cancer tissues [30]. Sun et al. have revealed that PTEN is a direct target of miR-543 [47]. In turn, PTEN has been considered as a tumor suppressing gene, which is strongly involved in the apoptosis process [118]. In the another study, Liu and co-workers have noticed that rising expression of miR-543 may lead to a downregulation of PTEN and inversely, the inhibition of miR-543 could activate PTEN in CRC cells [51]. In many previous studies have been shown that the loss of PTEN gene can increase the PI3K/AKT signal transduction pathway, leading to the tumor progression and formation of metastases [119, 120].

Furthermore, MiR-543 has been also described as a pivotal modulating factor of tumor growth and metastasis through binding to Raf kinase inhibitor protein (RKIP) in PCa. Previous studies have suggested that RKIP is responsible for inhibition of PCa progression by blocking MAP kinase (MAPK) signaling pathway and activation of NF-ŘB factor through G-protein regulating [121, 122]. Li and colleagues in their study at gastric cancer (GC) have demonstrated that miR-543 enhances ability to cell proliferation and formation of colony, what in consequence lead to a progression to the S phase, by directly targeting histone deacetylase sirtuin 1 (SIRT1) [49]. In the same study, the authors have shown that miR-543 molecule negatively modulates the SIRT1 expression in GC cells. Moreover, themiR-543 was positively associated with such clinicopathological parameters as size of the tumor, clinical grade, TNM stage and metastasis to the lymph nodes in gastric cancer patients [49]. Additionally, Haga and co-workers have presented seven miRNAs: miR-300, miR-382, miR-494, miR-495, miR-539, miR-544 and miR-543 in the imprinted DLK1-DIO3 region, which cooperatively regulates the EMT process, by repressing signal transduction network, including TWIST1, BMI1, ZEB1/2, and miR-200 family miRNAs [123]. The epigenetic silencing of miRNAs cluster that appears through hypermethylation of upstream CpG islands in human ductal carcinomas lead to initiate the EMT process [123].

On the other hand, we observed also in current meta-analysis that microRNA molecules might be closely related to a specific type of metastases. The decreased miR-503 expression could correspond with a formation of metastasis generally and lymph node metastasis specifically, while the decreased expression of the miR-132 seems to be associated with the development of metastasis overall as well as with the presence of distal metastasis. These observations may suggest a possibility of determining a risk of lymph node metastasis or distant metastasis development via changes in miR-503 and miR-132 level. MiR-503 has been found to be involved in formation of a malignant phenotype in a wide spectrum of cancers. Using the RT-qPCR, it has been demonstrated the elevated expression level of miR-503 in esophageal squamous cell carcinoma (ESCC). Also, the overexpression of miR-503 was found in correlation with following clinicopathological features: TNM stage, tumor differentiation and lymph node metastasis. Summarizing this study, the authors revealed that miR-503 represses ESCC progression by induction of autophagy, regulated in the PKA/mTOR signaling pathway [124]. Therefore, a decrease in miR-503 level leads to tumor proliferation and metastasize. In human non-small cell lung carcinoma (NSCLC), miR-503 was observed as a tumor-suppressive miRNA, which was inversely associated with patient overall survival. In turn, upregulation of either PI3K p85 or IKK-β, direct targets of miR-503, guaranteed fractionally restoring of malignant phenotype of cancer cells [125]. Interestingly, in A549/CDDP non-small cell lung cancer cells in comparison to the parental A549 cells, deregulation of miR-503 expression was found to decrease the resistance on cisplatin of NSCLC cells [126]. These findings may have important significance in case of chemotherapy failure due to cancer drug resistance. However, in current work we point out a possibility of miR-503 implementation to predict progression to the lymph node metastasis stage, specifying their potential between miR-503, miR-802 and 301a.

The presence of lymphovascular metastasis constitutes a formidable foundation for the possibility of further extending metastases and forming the tumor cells foci, located at distant body parts. Hence, in the future perspective, a prediction of metastasis development and a clinical classification of metastasis types via fluctuations in microRNA molecules expression levels is justified. Predicting lymph node metastasis by evaluation of miR-503 expression possess a valuable potential in particular for the possibility to inhibit spreading of tumor cells to distant body parts.

Contrarily, miR-132 can play a dualistic role in cancers [127]. In this meta-analysis, miR-132 was found to be associated with both general metastasis and distant metastasis The results of latest studies may confirm an oncogenic role of miR-132 in cancer. Wong and co-workers have found 24-upregulated miRNAs, including miR-132, in tongue squamous cell carcinoma [128]. Liu et al. have revealed that miR-132 was down-regulated in both hepatocellular cancer (HCC) tissues and HCC cell lines and the same miR-132 was responsible for a suppression of tumor development and blocking of invasiveness. Additionally, miR-132 can directly target PIK3R3 and controls the AKT/mTOR signal transduction pathway in HCC [58]. MiR-132 may act as a tumor suppressing miRNA in non-small lung cancer [56] and ovarian cancer [129]. In PCa the loss of miR-132 was found to be in correlation with poor clinical outcome. MiR-132 deregulation leads to decreased a cell-to-cell adhesion and to cell death inhibition, which is necessary for circulating tumor cells to adhere to foreign organ areas. Formosa et al. have observed that the CpC island hypermethylation seems to be responsible for silencing of miR-132 [130].

Such highly heterogeneous tumor as PCa creates significant diagnostic challenges and hinders the ability to predict disease progression. Exploiting the expression alterations of miRNAs specific for kind of metastasis may provide opportunity to early prediction of prostate cancer progression. In this meta-analysis, we want to point out the potential of panel with 6 miRNAs for monitoring disease progression, directed to metastasis occurrence. Our findings revealed that the increased expression of miR-410-3p, miR-210-3p, miR-543 and the decreased expression of miR-132, miR-141-3p and miR-503 have been significantly associated with the appearance of metastases in progress of PCa.

The second aim of our study was to analyze a relationship between miRNAs in PCa and a Gleason score. The Gleason scoring system constitutes an especially important prognostic factor associated with the growing aggressiveness of neoplastic changes in the prostate. The increased Gleason grade has been connected inter alia with pathologic stage, tumor size, margin status, progression to metastatic form and patient survival [131]. Total Gleason score is one of the main factors involved in making treatment decisions, together with pathological stage and pre-PSA level [132].

In our meta-analysis we decided to compare the miRNAs importance in the set of "Gleason score above 6" and in the set of "Gleason score above 7". Aberrant miR-410-3p, miR-132, miR-212, miR-503 are separately the most associated with total Gleason score > 6 and abnormal expression of miR-212, miR-203 and miR-573 are individually the most associated with Gleason score > 7 in PCa tissue. MiRNAs molecules most integrated with both Gleason scores above 6 and 7 is miR-212. The future implementation of diagnostic tests to capture the variations in miRNAs expression among prostate cancer patients limiting to Gleason score, suggests the opportunity of monitoring the patient's condition and disease progression at early cancer stage, excluding need to resection of prostate gland or radiotherapy.

The miR-212 was found to be upregulated in pancreatic ductal adenocarcinoma in comparison to the health tissue. In the current study, it has been shown the positive correlation between deregulation of miR-212 and TNM stage, lymph node metastasis, vessel invasion, size of the tumor and the overall survival time. Latest, it has been found that in hypoxic conditions miR-212 aberration was correlated with the hypoxia-inducible factor (HIF-1α) in vivo and in vitro [133]. In turn, in cervical cancer tissues and cell lines, miR-212 was found outstandingly down regulated and promoted proliferation and invasion of cancer cells. Using the Western blot tool the authors have indicated that miR-212 upregulation notably blocked the transcription factor 7-like 2 (TCF7L2) protein expression level [134].

Furthermore, miR-212 contributes in the miR-212/132 cluster in human lung cancer [135]. Interestingly, the current study also showed an association between miR-132 level and Gleason score > 6. Jiang et al. have suggested that overexpression of miR-212/132 cluster remarkably blocks the growth and invasiveness of tumor cells in A549 and H1299 lung cancer cell lines [135]. Additionally, miR-212/132 upregulation leads to activation of cell cycle arrest at the G1/S phase transition via regulation of p21 and cyclin D1 expression [135]. Upregulation of miR-132/212 has been found in drug resistant breast tumors and cell lines. Moreover, the miR-132/212 has been shown responsible for regulation of drug accumulation through blocking the PTEN expression levels in vitro. Also, downregulation of PTEN has been found inversely associated with miR-132/212 expression in drug resistance breast cancers. Upregulation of miR-132/212 cluster was partially connected with transactivation via the NF-ƙB transcription factor and this mechanism may

have importance in acquisition of drug resistance [136]. Additionally, current statistical analysis showed that another aforementioned miRNAs, miR-410-3p and miR-503 was found to be in association with Gleason score value > 6, which demonstrates their potential prognostic role at an early stage of prostate cancer development.

MiR-573 and miR-203 was found to be associated only with Gleason score > 7, therefore we consider that these molecules exhibit a lower prognostic value for predicting EMT. However, miR-573 and miR-203 can be used to predict Gleason score ranges from 7 to above. Wang et al. have investigated that upregulation of miR-573 may be stabilized by miR-573 mimic, leading to inhibition of proliferation and progression in melanoma cells [137]. On the other hand, Wang and other authors have revealed that downregulation of miR-573 in PCa is precisely associated with the Gleason score and cancer-related mortality ($p = 0.41$). The latest study showed that miR-573 could regulate the activity and function of Fibroblast growth factor receptor 1 (FGFR1) in response to fibroblast growth factor 2 (FGF2). Additionally, in the same study, transcription factor GATA3 was indicated as an enhancer of miR-573 expression, causing downregulation of FGFR1, EMT and invasion of PCa cells [22].

MiR-203 has been found to be a tumor suppressing miRNA in some cancers: hepatocellular cancer [138], breast cancer [139, 140], esophageal cancer [141], leukemia [142], glioma [143], bladder cancer [144], leading to inhibition EMT by targeting various genes and downregulation of signal transduction pathways, thus is involved in cancer progression (tumor proliferation and invasion). However, its role in some cancers has been found different. MiR-203 can function as an oncogene in ovarian cancer [145]. In SKOV3 and OVAR3 ovarian cancer cell lines, high expression of miR-203 leads to restrain of EMT process by linking to BIRC5 and causes its downregulation and attenuates the TGF-β signal transduction pathway [92, 146]. Chen and Ding et al. have investigated the effect of elevated miRNA expression on PC-3M prostate cancer tumor cells behavior and they found that downregulation of miR-203 was connected with adriamycin (ADM) resistance, thus elevation of miR-203 repress tumor cells proliferation, enhance apoptosis and reduce ADM resistance through influencing on MAPK kinase 1 (MEK1) expression [147]. MEK1 is responsible for phosphorylation of the Tyr/Thr residue on ERK protein and leads to induction of ERK/MAPK transduction pathway. In turn, excessive stimulation of ERK/MAPK pathway intensifies progression of tumor cells [147].

Finally, we analyzed the association between miRNAs and the pT stage of PCa. The process of determining tumor stages among prostate cancer patients relies on evaluation of probability of tumor spread before including the individual treatment [148]. The increased expression of both miR-410-3p and miR-543 as the same as decreased expression of miR-503 have a significant relationship with rising probability of tumor extending and higher T stage at the time of diagnosis. Our results may be useful for prediction of prostate cancer progression at an early stage. Forasmuch as further research is needed in the prostate cancer progression area, it is important to be prudent in devising far-reaching conclusions. However, it was noticed that progression to a higher pT stage is associated with the aberration of 3 miRNA levels: miR-410-3p, miR-543 and miR-503 involved in invading surrounding tissues and metastasizing. Du et al. at their study on CRC, using gene chip technology grouped inter alia by stage, have proven that microRNA expression profiles were significantly different at various CRC stages, thereby confirming that miRNAs are engaged in a cancer development and in particularly are significant at early diagnosis or cancer prognosis [149]. Concluding, the probability of cancer progression to its metastatic stage can be assessed at an early stage of tumor development. MiR-410-3p play a different role in various cancers. Zhang et al. have noticed that increased expression of miR-410-3p repress proliferation and invasion process in the breast cancer cells [150]. These authors also displayed that transcription factor Snail is a direct target of miR-410-3p and in this way miR-410-3-p inhibits an EMT process. MiR-410-3p has been demonstrated to inhibit the invasion and migration of rhabdomyosarcoma cells through inhibiting EMT [151]. Wang and other authors suggested that miR-410 function in CRC may be connected to negative regulation of DKK-1 through Wnt/β-catenin pathway [152]. MiR-410-3p plays a significant role as an oncogene in PCa via PTEN/PI3K/AKT pathway. Downregulation of miR-410-3p leads to an activation of PTEN, which is a crucial modulator of the PI3K/AKT signaling pathway [21]. Similarly, the role of miR-543 in tumor progression and metastasis were confirmed in many earlier publications [52, 153, 154]. It has been also reported that miR-543 shows outstanding overexpression in the metastatic PCa cell lines compared to the normal cells. Additionally, Raf kinase inhibitor protein (RKIP) was found to be a direct target of miR-543. Upregulation of miR-543 leads to decrease in expression level of RKIP and promotes proliferation and metastasis in PCa cells. [30]. Previous studies have revealed that described molecules are undeniably involved in initiation EMT transition, therefore they are ideal candidates for metastasis risk prediction.

Diagnostic, prognostic and therapeutic aspect of analyzed miRNAs

The revolution in the diagnosis of prostate diseases was measuring PSA level in blood samples, however it is questionable due to the lack of association between PSA level and prostate cancer stage, which may result in overdiagnosis and overtreatment. Currently, many scholars put immense effort into identifying new biomarkers to improve the diagnosis and prognosis accuracy of PCa entities. Previous studies have indicated that specific miRNA profiles were able to distinguish cancerous and non-cancerous tissues [155], incoming the potentiality of miRNAs in early cancer detection, prognosis and monitoring of treatment response.

Tissue biopsies represent a gold standard in prostate cancer patients' diagnosis. Nevertheless, tissue sampling technique is susceptible to misdiagnosis, whereas a negative outcome cannot completely exclude presence of cancer foci. Hence, the researchers are looking for a better diagnostic tool. miRNA molecules have multiple advantages in diagnostic or treatment approach, for example, RNA has a higher specificity and its expression alterations are characteristic for individual disease. The expression of miRNA in cancer cells is a process during which can be observed changes that appear over disease development. Additionally, in comparison of tissue-based miRNAs with detection of miRs in bodily fluids, the second one mentioned can be associated with many factors, such as age, diet or circadian rhythms [156]. miRs are less subjected to degradation due to their small size. Additionally, miRs possess great stability during FFPE tissue processing since they are encapsulated in exosomes [156].

Current research state constitutes that non-coding RNA, such as miRNAs, has great potential for PCa diagnosis. An individual gene can be targeted by various miRNAs, which accelerate atypical cell growth and provoke cell death [157]. Furthermore, miRNAs can manage cancer-related processes, such as EMT or metastasis. MiRNAs can be measured both by tissue-based biopsy or in various bodily fluids [158]. Aghdam and colleagues indicated that tissue miRNAs are appropriate for diagnosis and staging of malignancies as well as minimize the necessity for numerous biopsies to determine the appropriate diagnosis [158]. Even if the miRNA detection on tissue-based biopsies shows multiple benefits for prostate cancer patients, it is not yet applied in present clinical practice.

Certain miRNA molecules described in current meta-analysis have been studied previously for diagnostic and prognostic potential in prostate cancer patients. MiR-141 in association with miR-151-3p and miR-16 was found to have a sensitivity of 84% and a specificity of 96% regarding mCRPCa detection from liquid samples [159]. Agaoglu and colleagues presented that circulating miR-141 in compilation with miR-21 and miR-221 have potential to differentiate PCa patients in metastatic stage from those with local advance stage [160]. Other authors have shown that the combination of miR-141-3p with miR-21 and miR-375 presented a sensitivity of 93% and a specificity of 63% during prediction of PCa in serum samples [161]. MiR-141 is a widely researched molecule in various cancers, useful at each stage of diagnosis and prognosis for prostate cancer patients.

Also miR-301a was found to be a valuable diagnostic and prognostic biomarker for prostate cancer disease. The study performed on 28 prostate cancer patients and 13 controls as well as 40 radical prostatectomy cases indicate the possible correlation between miR-301a detected in serum and Gleason score [162].

MicroRNAs are validated as necessary tools in designing biomarker panels for identification prostate malignancies primarily in early but also advanced stages. Regulation of aberrant miRNA expression will repress the targets responsible for progression to higher prostate stage. Presently, TNM staging along with Gleason score and pretreatment PSA levels are utilized to predict the outcome for prostate cancer patients. The microRNA analytes in current meta-analysis have potential for higher accurate prostate cancer staging and developing therapy schemes. Additional advantage for miRNA diagnostic value in prostate cancer is the fact that miRs are situated at fragile chromosomal sites corresponding with tumor hotspots [12].

The role of miRNAs in prostate malignancies is not only limited to diagnosis or prediction and prognosis. MiRNA molecules can also function as targets or therapeutic agents. MiRNA molecules can regulate multiple target genes, therefore alterated expression constitutes a potential therapeutic value for the balance of gene expression [163]. Aside from direct application of miRNA as a valuable therapeutic option, miRNAs can facilitate selection of the best individual treatment scheme and predict response to a personal therapy [158]. The individual patients' response to a treatment application, such as radical prostatectomy, radiotherapy, testosterone suppression and hormone therapy, chemotherapy and immunotherapy is different [158, 164]. Intriguingly, miRNA can be applied as adjuvants acting as an enhancer of tumor sensitivity to therapy [158].

Zedan and other authors have investigated the relationship between circulating miR-141 and miR-375 and treatment outcome in metastatic castration resistant prostate cancer (mCRCP), where 40 patients were treated with docetaxel and 44 with abiraterone [165]. The results of the mentioned study showed prognostic importance of

miR-141 and miR-375 in treatment response monitoring among mCRPC patients. Intriguingly, Gonzales and colleagues noticed a strong correlation between miR-141 levels and chemotherapy response in patients with confirmed metastatic stage of prostate cancer. Furthermore, they proved that multivariate panel of directional alterations in PSA, circulating tumor cells (CTCs) and miR-141 had higher sensitivity of 78.9% in clinical outcome prediction [166]. Saini and other authors have studied miR-203 in advanced metastatic prostate cancer and revealed that miR-203 may act as an "antimetastatic" miRNA at numerous steps in the metastatic process by repression of prometastatic targets. Hence, miR-203 has been recognized as an attractive target for patient therapy in advanced prostate disease [27]. The prognostic value of miR-141 and miR-409-3p was demonstrated by Nguyen and other authors, investigating the differential expression of 669 miRNA prostate cancer serum. They confirmed that miR-141, miR-375 and miR-378 increase-depend manner with progression to higher prostate cancer stage, while miR-409-3p was marked to be overexpressed in high-risk group compared to low-risk group, but significantly reduced in the metastatic CRPC, which probably was associated with the androgen deprivation therapy [167]. Watahiki and colleagues proposed miRNA panel, including miR-141, miR-152 and miR-423-3p significantly deregulated in metastatic CRPC compared to localized PCa emerged from 742 miRNAs studied in plasma samples, demonstrating AUC of 0.944. Additionally, the researchers confirmed that miR-141 together with miR-151-3p, miR-152 and miR-423-3p was linked with poor outcome and higher Gleason score. Furthermore, miR-141 and miR-152 was able to detect a high probability of recurrence among patients after radical prostatectomy [159]. In contrast to serum-based miRNA studies, Porkka et al. have profiled 319 miRNA molecules in nine PCa and compared to them four BPH tissues, shown inter alia miR-210 together with other 7 miRNAs up regulated and 22 miRNAs down regulated [168]. Interestingly, Srivastava et al. have shown 30 miRNAs, including miR-210 and miR-212 down regulated and three up regulated in 40 FFPE tissue specimen blocks, comparing PCa tissues to adjacent normal tissue [169].

Clinical relevance of miRNA molecules constitutes the basis to widespread research in the therapeutic context. Therefore, investigators are underway to explore systems balancing miRNA expression levels, onco-miRNA antagonizing and TS-miRNA replacement systems. Modified anti-miRNA systems (microRNA mimetics–miRNA-mimic) can imitate or inhibit cellular miRNA functions to silence complex signal transduction pathways and control multiple physiologic functions [170]. Currently, miRNAs-based therapeutic approach constitutes a high-potential weapon to treat such heterogeneous disease, in terms of molecular abnormalities, pathologic tumor growth patterns and patients outcomes [171] as prostate cancer.

Currently, miRNAs are a part of multiple clinical trials exploring genomic signatures to solve a problem with patients' selection and recognize companion biomarkers for conventional therapeutic approach. One of the prostate cancer clinical trial is interventional study for 50 patients with low-risk prostate cancer [tumor stage cT1-2a cN0 cM0 and Gleason Score of index lesion ≤ 6 $(3+3)$] to investigate the feasibility and toxicity of focal brachytherapy. A secondary outcome for this phase II clinical trial was to verify the correlation level of miR-141 and miR-375 expressions with visible efficacy of radiation intervention (ClinicalTrials.gov Identifier: NCT02391051). Previously, miR-141 together with miR-21 and miR-221 have been studied in the plasma of 51 prostate cancer patients with locally advanced and metastatic prostate cancer, where miR-21 was related to malignancy state and miR-141 was found to be capable of distinction localized and metastatic stage of prostate cancer [160]. Another one pre-clinical study of 667 samples of serum obtained from prostate cancer patients recognized miR-141 and miR-375 as molecules strongly involved in disease progression and were considered as non-invasive blood-based circulating biomarkers for prostate cancer progression [172]. More precisely, Brase and colleagues noticed that overexpression of miR-141, miR-200b and miR-375 in prostate cancer patients serum was associated with higher tumor stage and Gleason score [172]. Cheng et al. have analyzed panel of 365 miRNAs in prostate cancer serum and emerged miR-141, miR-200a, miR-200c, miR-375 and miR-210 molecules, which was significantly expressed in metastatic CRPC patients compared to age-matched healthy controls as well as in lymph metastasis samples [173]. In another study, Kelly et al. demonstrated a normalization in miR-141 expression in prostate cancer patients who underwent a radical retropubic-prostatectomy 10 days post-operation [174, 175]. Hao et al. have found miR-141 and miR-21 twofold up regulated, while miR-10, -16, -34c and -125b down regulated in FFPE tissue compared to BPH specimens [176]. Additionally, association of PSA level with miR-141 and miR-21 expressions enhanced the positive predictive value from 40 to 87.5% [177].

Improvement in survival rate for prostate cancer patients follows from the advances in first line treatment approach like surgery, radiation and antagonists to the androgen signaling axis [156]. However, the metastatic stage of prostate cancer has no treatment opportunities due to the resistance to androgen antagonists. In turn, FDA was approved taxane-based therapy to provide patients three months-longer overall survival [178, 179].

Another interventional study concern 211 prostate cancer patients at newly diagnosed metastatic stage (stage IV) (ClinicalTrials.gov Identifier: NCT01120236), were as a secondary outcome was measured correlation level between miR-141, miR-210, miR-200b and miR-375 as well as PSA level and circulating tumor cells (CTC). In this phase II randomized trial prostate cancer patients were treated with bicalutamide, goserelin, or leuprolide acetate in combination with cixutumumab or without cixutumumab. Another one observational cohort trial has recruiting status and attempts to identify the role of exosomal microRNA and define microRNA expression profiles to predict the aggressiveness of PCa in urine (ClinicalTrials.gov Identifier: NCT03911999).

Conclusion

PCa is one of the commonest malignancies in Western countries. The GLOBOCAN STATISTICS 2018 indicates at 7.1% of all new cancer incidence worldwide in 2018 year (for both sexes) [180]. MiRNA molecules offer new possibilities for diagnostic and systemic therapeutic approaches. They can attenuate or obstruct multiple networks involved in the tumor microenvironment, not only single transduction pathway [181]. Furthermore, miRNAs can be a more effective weapon against tumors as they are secreted from malignant cells and their anomalies in expression levels are associated with progression of prostate diseases.

We consider that the higher prognosis value is presented by sub-analysis associating miRNAs expression and GS > 6, showing association with miR-410-3p which was also connected with the presence of general metastasis and the pT stage, miR-503 with significance in general or lymph node metastasis and pT stage and miR-132 connected to general metastasis and distant metastasis. Mentioned miRNAs molecules could be used as a miRNA panel to determine the progression risk at early stages of the disease. Our findings present future potential therapeutic options to treat men with PCa and could provide better post-treatment care. The above mentioned experiments strongly indicated that miRNAs expression modification utilizing miRNA mimics or antagomirs can harmonize gene regulatory circuits and signal transduction pathways. Also, such activities may stop the aggressive status of tumors and reverse the cancerous status of cells.

Abbreviations

PCa: Prostate cancer; EMT: Epithelial–mesenchymal transition; miRNAs: MicroRNAs; mRNAs: Messenger RNAs; Onc: Oncomirs; FFPE: Formalin-fixed paraffin embedded tissue; TNM: TNM Classification of Malignant Tumor System; GS: Gleason score; HRs: Hazard ratios; RRs: Relative risks; CIs: Confidence intervals; I^2: Inconsistency index; NOS: Newcastle–Ottawa Scale; RT-PCR: Real time polymerase chain reaction; pT stage: Pathologic tumor stage; TRAF5: Tumor necrosis factor receptor-associated factor 5; CRC: Colorectal cancer; MAPK: MAP kinase; GC: Gastric cancer; HIF-1α: Hypoxia-inducible factor; PTEN: Phosphatase and tensin homolog deleted on chromosome ten; FGF2: Fibroblast growth factor 2; MEK1: MAPK kinase 1; RKIP: Raf kinase inhibitor protein.

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Authors' contributions

This paper was planned by MP, and she is responsible for the overall conception of the work, collecting information, data extraction process, methodology and initial drafting of the manuscript. AG were responsible for statistical elaboration. MP and AG were responsible for creating tables with important data as well as the graphic layout. MP and AG contributed critical revisions of the manuscript for the intellectual content, edited the manuscript and made the necessary language corrections. MB and DG supervised the work. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

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Competing interests

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Prognostic significance of SDF-1 chemokine and its receptors CXCR4 and CXCR7 involved in EMT of prostate cancer

Martyna Parol-Kulczyk *, Arkadiusz Gzil, Joanna Ligmanowska, Dariusz Grzanka

Department of Clinical Pathomorphology, Faculty of Medicine, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Toruń, Poland

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ABSTRACT

Tendency to conversion from state of chronic inflammation to malignancy is a tumor characteristic trait, which encourages progression to its metastatic stage.. The inflammatory cells maintaining in the tumor inaugurate a communication with cancer cells and become tumor-fostering cells. Epithelial-mesenchymal transition (EMT) is a program supporting malignant cells during switch phenotype into metastatic form, providing looseness of cell-cell adherence and strengthens migratory or invasive features. EMT-undergone tumor cells become more aggressive and resistant to apoptosis. Additionally, malignant cells can be stimulated to manufacture proinflammatory factors throughout EMT program. Chronic inflammation is responsible for EMT induction in malignancies. Developed tumors induce inflammatory response through excretion of cytokines, chemokines and growth factors, which recruit populations of infiltrating immune cells straight to the tumor microenvironment. The inflammatory reaction potentially exerts tumor control, but instead it can be intercepted by the tumor to stimulate its own development in direction to metastatic form. Our study confirmed that SDF-1 chemokine and its receptors, CXCR4 and CXCR7 may participate in initiation of metastases formation and EMT process.

1. Introduction

The process of inflammation constitutes an integral point in progression to prostate cancer (PCa). Many recent studies have confirmed involvement of inflammatory process in prostate cancer initiation and development [1–3]. Inflammation encourages carcinogenesis, promotes tumor proliferation as well as angiogenesis through incite DNA damage [4–6]. Also, in chronically inflamed cells, a cytokine release is induced, leading to active stroma, tumor growth, activation of EMT process and metastasis [7]. The chemokines and cytokines, produced by inflammatory cells, has impact at whole organ occupied by tumor, disrupting the tumor growth balance, cell differentiation and cell migration in tumor microenvironment, encompass neoplastic cells, endothelial cells and fibroblasts [8,9]. Moreover, the neoplastic cells may lead to invert the inflammatory mechanism, providing the support of prostate cancer metastasis cascade [10,11]. Inflammatory-associated mediators related to the cancer incorporate tumor necrosis factor, cytokines, chemokines, inflammasomes, transcription factors, infiltrating and circulating immune cells, sex steroid receptors and reactive oxygen species (ROS) [12].

Chronic inflammation may initiate epithelial-mesenchymal transition (EMT), an initial step of metastasis, leading to detach of tumor cells from their origin site, flow into the angiolymphatic systems and occupy the endothelial cells, where adhesion to endothelial cells is supported by chemokines and their receptors [13].

Chemokines are small molecular weight proteins involved in cell trafficking and tissue differentiation [14]. More importantly, chemokines and their receptors are considered as a molecules mediating in chronic inflammation predisposing to the tumor development and progression including in prostate cancer patients [15,16].

One member of the CXC chemokines subfamily is SDF-1, also known as a CXCL12, which could interact with the seven-transmembrane G-protein-coupled receptor CXCR4 [17]. The chemokine stromal derived factor 1-alpha (SDF-1α) and CXCR4, its primary discovered receptor are

Abbreviations: EMT, epithelial-mesenchymal transition; PCa, prostate cancer; ROS, reactive oxygen species; SDF-1α, chemokine stromal derived factor 1-alpha; BPH, benign prostate hyperplasia; FFPE, formalin-fixed paraffin embedded tissue; H&E, hematoxylin and eosin staining; AJCC, American Joint Committee on Cancer; 25p, 25th percentile; 75p, 75th percentile; M, median; TME, tumor microenvironment; MSC, mesenchymal stem cells; NSCLC, non-small cell lung cancer.

* Corresponding author at: Department of Clinical Pathomorphology, Faculty of Medicine, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Skłodowskiej Curie 9, 85-094 Bydgoszcz, Toruń 87-100, Poland.

E-mail addresses: mparol@cm.umk.pl (M. Parol-Kulczyk), arkadiuszgzilresearch@gmail.com (A. Gzil), joanna.ligmanowska@cm.umk.pl (J. Ligmanowska), d_grzanka@cm.umk.pl (D. Grzanka).

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highly participate in regulation of metastatic process [18]. Alternated expression of CXCR4 receptor has been observed in 23 epithelial, mesenchymal and hematopoietic cancers, confirming ligand/receptor complex critical role in aggressiveness acquiring and metastasis forming [19]. The role of SDF1α/CXCR4 pathway in prostate cancer is multifaceted and it was found to be implicated in dissemination of malignant cells to specific organs, tumor cells aggregation and stimulation of growth at new sites, so functions, which are probably conduct through input of CXCR4 on adhesion, invasion and proliferation of tumor cells [20]. However, the CXC chemokine receptor 7 (CXCR7), the newly identified substitute receptor for SDF-1α can also manages cell survival and cell invasion in PCa [21]. Similar to CXCR4, CXCR7 belongs to chemokine-specific seven transmembrane G protein-coupled receptor and functions as an atypical chemokine receptor which does not participate in standard chemokine responses such as adenylyl cyclase activity or mobilization of intracellular calcium [22]. Previous studies have confirmed that affinity of CXCL12 binding to its CXCR7 receptor is ten folds greater comparing to the CXCR4 [23]. New advances in area of cancer progression disclosed SDF-1/CXCR4/CXCR7 axis as an important modulator of endothelial progenitor cells recruiting and angiogenesis or dissemination of tumor cells [24–28] The role of CXCL12-CXCR4/CXCR7 chemokine signaling pathway has been confirmed in human malignancies. Some authors have indicated that the CXCL12-CXCR4/CXCR7 axis has potential as a therapeutic target for repression of tumor growth and metastasis [29,30]. Nevertheless, the CXCL12-CXCR4/CXCR7 axis reffered to EMT in PCa still remains controversial and poorly understood.

2. Material and methods

2.1. Clinicopathological characteristics of the study population

The fifty patients with confirmed primary PCa were all managed at the Department of General and Oncological Urology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus in Torun (Poland) from January 2017 to December 2019 after the introduction of routine radical prostatectomy treating. The clinical databases of classified patients indicated classical prognostic factors such as age, histological type, grade, lymph node status, distant metastasis status or treatment information. The each patient was qualified when met the pre-defined entry criteria. Patients incorporated in the study were pT2c-pT3b, N0-1, M0-1. The age of patient cohort ranged from 52 to 82 with the mean equal to 66. The majority of included patients had lymph node metastases (66%) and 92% had tumor cells confirmed in prostate gland capsule. Gleason score was assessed for all of 50 prostate samples with 6 score in 6%, 7 score in 36%, 8 score in 26% and 9 score in 30% patients. Baseline features of study group were presented at Table 1.

Table 1
Clinicopathological characteristics of the study group.

Variables		% of cases (N = 50)
Age	≤60 years	20
	>60 years	80
Stage pT according to TNM	pT2c	18
	pT3	2
	pT3a	18
	pT3b	52
	pT4	10
Stage N according to TNM	N0	34
	N1	66
Stage M according to TNM	M0	92
	M1	8
Gleason Score	6	8
	7	36
	8	26
	9	30
Gleason pattern	3 + 3	8
	3 + 4	16
	3 + 5	8
	4 + 3	20
	4 + 4	18
	4 + 5	16
	5 + 4	14
Angioinvasion	Pos	12
	Neg	88
Nerve invasion	Pos	10
	Neg	90
Plugs in the vessels	Pos	26
	Neg	74
Capsule invasion	Pos	92
	Neg	8

2.2. Tissue specimens

The current study was approved by the Bioethical Commission of Collegium Medicum in Bydgoszcz of the Nicolaus Copernicus University in Torun, Poland with the issue number KB 248/2019. Histological assessment and classification according to the 8th Edition American Joint Committee on Cancer (AJCC) Cancer Staging system [31] of resected tumors were done with hematoxylin-eosin-stained (H&E-stained) slices by two independent pathologists. The specimens with equal to or more than 70% of cancer cell were recognized as a representative tumor areas. The control group was consisted of 20 patients with confirmed benign prostate hyperplasia (BPH).

2.3. Tissue microarray (TMA) construction

TMAs were precisely constructed from conventional paraffin blocks representing donor blocks. 5 mm diameter cores obtained from a series of paraffin-embedded donor blocks were located into an encoded tissue microarray in prepared acceptor paraffin blocks. Each of them was composed of five tissue fragments. Prepared paraffin blocks were cut into 4 μm thick slices by using manual rotary microtome (Accu-Cut, Sakura, Torrance, USA). Ready for use slices were placed on extra adhesive slides (Superfrost Plus; Mensel-Glazer, Braunschweig, Germany) and dried on a heating plate set at 60 °C for 1 h.

2.4. Immunohistochemistry

Prepared tissue sections were deparaffinized and rehydrated, followed by incubation with Antigen Unmasking Solution pH-9 to retrieve the antigen in PT-Link (Dako, USA). Subsequently, the immunostaining protocol included 10' treatment in 3% H_2O_2 in room temperature to block the activity of endogenous peroxidase and 15' incubation in 3% solution of bovine serum albumin (BSA) in room temperature to block non-specific binding sites. The next step was 30' incubation with primary anti-SDF-1 and CXCR4 antibody, diluted 1:100 and 1:500 with antibody diluent (Dako, USA) and 20' incubation in EnVision FLEX + HRP (Dako, USA) in the same temperature conditions as a previous step. Antigen-antibody complex was visualizated regarding to the appearance of brown reaction product, using DAB (3'3 diaminobenzidine) as a chromogen. Additionally, the slides were counterstained with Mayer's hematoxyline for 3' to reveal the nuclei of cells. Finally, the tissue sections were dehydrated in increasing concentrations of ethanol (80, 90, 96, 99.8%), cleared in series of xylens (I, II, III, IV) and mounted with Shandon Consul Mount (Thermo Scientific, Waltham, USA).

All steps as above with minor modifications were used to stain CXCR7 protein in tissue slices. The incubation time with 3% H_2O_2 was extended from 10 to 12 min and BSA concentration was increased from 3 to 5 percent. The dilution of anti-CXCR7 antibody was 1:100. The staining procedure of aforementioned proteins (encompassing staining of both tumor and BPH areas) was done manually and simultaneously to ensure the highest performance and support the quality of microscopic examination process.

The standarization of IHC methods was done before performing of principal IHC staining. Optimization and validation of antibodies

immunostaining was performed manually with modifications of standard protocols using the properly diluted antibody clones and tissue controls presented in Table 2. The tissue controls was recommended by the Human Protein Atlas with a link https://www.proteinatlas.org [32] and the manufacturer sheet. Positive control reaction was performed on human gallbladder, human brain carcinoma and human placenta for SDF-1, CXCR4 and CXCR7, respectively. Subcellular localization of signals was nuclear-cytoplasmic positivity in glandular cells for SDF-1, the presence of the immunising phosphopeptide for CXCR4 and cytoplasmic positivity in trophoblasts for CXCR7. The results of IHC staining was compared with external protein/gene sources, such as the UniProtKB/Swiss-Prot and the Human Protein Atlas. Our staining patterns was consistent with the results listed in aforementioned databases.

2.5. Imaging and scoring

The stained slides were assessed in a blinded manner using the light ECLISPE E400 microscope (Nikon Instruments Europe, Amsterdam, Netherlands) by two independent pathologists. Images of IHC-stained TMA slides were obtained at 10x magnification using a Ventana DPD200 slide scanner (Roche Diagnostics, USA).

The H-scores were calculated by a semi-quantitative evaluation of the intensity of staining and the percentage of tumor cells in each TMA cores. Three fields for each individual core were chosen randomly to evaluate the intensity of nuclei staining in tumor cells, scoring to 0 corresponding to negative diaminobenzidine staining, 1 + corresponding to weak, 2 + corresponding to intermediate or 3 + to strong. Next, the total number of tumor cells and the number of each staining intensity category cells were counted. The H-score results were obtained for each prostate sample fields by following formula (3x percentage of strongly stained tumor cells + 2x percentage of moderately stained tumor cells + 1x percentage of weakly stained tumor cells).

2.6. Statistical analysis

All statistical tests were performed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA, USA). The expression levels of studied proteins were demonstrated as 25th percentile (25p), the median (M) and the 75th percentile (75p). The comparative studies were analyzed statistically using the U Mann-Whitney and Kruskal-Wallis test. Spearman test was used to explore the association between protein levels and clinicopathological features: age, pathological tumor stage, Gleason pattern, total Gleason score, presence of lymph node, presence of angioinvasion or plugs in the vessels and nerve invasion. *P*-values < 0.05 were considered statistically significant.

Table 2
Primary antibody characteristics.

Antibody	Primary antibody dilution	Positive control according to antibody data sheet and Human Protein Atlas	Cellular localization/ expression in prostate cancer tissue	Catalog number
SDF-1	1:100	human gallbladder	Nuclear-cytoplasmic and stromal	ab9797
CXCR4	1:500	human brain carcinoma	Nuclear-cytoplasmic and stromal	ab74012
CXCR7	1:100	human placenta	Cytoplasmic	ab38089

3. Results

3.1. Immunohistochemical analysis of protein localization and expression of SDF-1, CXCR4 and CXCR7 in PCa

The SDF-1, CXCR4 and CXCR7 proteins exhibited following protein patterns: 1) SDF-1 was detected at nuclear-cytoplasmic and stromal level; 2) CXCR4 revealed nuclear-cytoplasmic and membrane pattern; 3) cytoplasmic localization was observed for CXCR7. Immunohistochemical representative microphotographs representing the SDF-1, CXCR4 and CXCR7 expression in PCa was shown in Fig. 1.

The range of possible scores was from 0 with negligible percentage of stained tumor cells to 300 with maximum percentage of strongly stained tumor cells. In DAB-stained TMA slides with PCa cores we noted 11 fields with 0 score for nuclear-cytoplasmic SDF-1 expression and 3 with 300 score, 20 fields with 0 score and 4 with 300 score for stromal SDF-1 expression, 35 fields with 0 score and 0 with 300 score for cytoplasmic CXCR7 expression, 40 fields with 0 score and 3 with 300 score for nuclear-cytoplasmic CXCR4 and 35 field with 0 score and 0 with 300 score for membrane CXCR4 expression. Frequency of particular IHC results in each protein subsets was presented in Table 3.

3.2. Correlation of SDF-1 staining intensity with clinicopathological features in PCa

Mann-Whitney *U* test demonstrated the higher nucleo-cytoplasmic expression of SDF-1 in cancer cells than in normal endothelial cells ($p = 0.0034$; Fig. 2A). Similarly, SDF-1 localized in stroma manifests a higher expression level in areas of cancer than in normal tissue ($p = 0.0009$; Fig. 2B). Kruskal-Wallis test showed significantly higher expression level of SDF-1 found in GS7 area of prostate stroma than in GS9 area of stroma ($p = 0.015$; Fig. 2D). Analogous observation was noted for SDF-1 with nuclear-cytoplasmic pattern in tumor cells ($p = 0.010$; Fig. 2C). The both expression patterns of SDF-1, nucleo-cytoplasmic and stromal were not associated with Gleason pattern. According to the TNM Classification System of Prostate Cancer, we found a notable higher stromal expression of SDF-1 chemokine in pT2c prostate cancer pathologic stage than in pT4 pathologic tumor stage of prostate ($p = 0.024$; Fig. 2E). Furthermore, our results indicate a remarkable higher stromal expression of SDF-1 protein in prostate cancer patients without presence of metastasis to the lymph node, than in patients with the occurrence of lymph node metastasis ($p = 0.038$; Fig. 2F). Also, we demonstrated a higher stromal expression of SDF-1 in tumor tissue without presence of tumor embolism than in cases with confirmed tumor plugs in the vessels ($p = 0.042$; Fig. 2G) by Mann-Whitney *U* test. Principal values obtained due to the analysis of correlation between proteins level and clinicopathological features are listed in Table 4.

3.3. Correlation of CXCR4 and CXCR7 staining intensity with clinicopathological features in PCa

Kruskal-Wallis test showed no difference between CXCR4 or CXCR7 expression and Gleason score or Gleason pattern manifested by tumor cells in prostate. However, our study suggest that chemokine receptors may participate in metastasis development. Mann-Whitney test revealed significantly higher the both membrane ($p = 0.0045$; Fig. 3A) and nuclear-cytoplasmic ($p = 0.0002$; Fig. 3B) CXCR4 expression in samples without presence of lymphovascular metastases than in samples with presence of metastases to the lymph nodes. Similarly, the cytoplasmic expression of CXCR7 protein was noted to be significantly higher in patients with lack of lymph node metastases than with nodal metastasis positive ($p = 0.0047$; Fig. 4B).

The results of Mann-Whitney test proposed that CXCR4 protein may be involved in nerve infiltration in PCa. Significantly higher membrane CXCR4 expression was observed in cases without infiltration of nerve than in cases with presence of nerve infiltration ($p = 0.0058$; Fig. 3C).

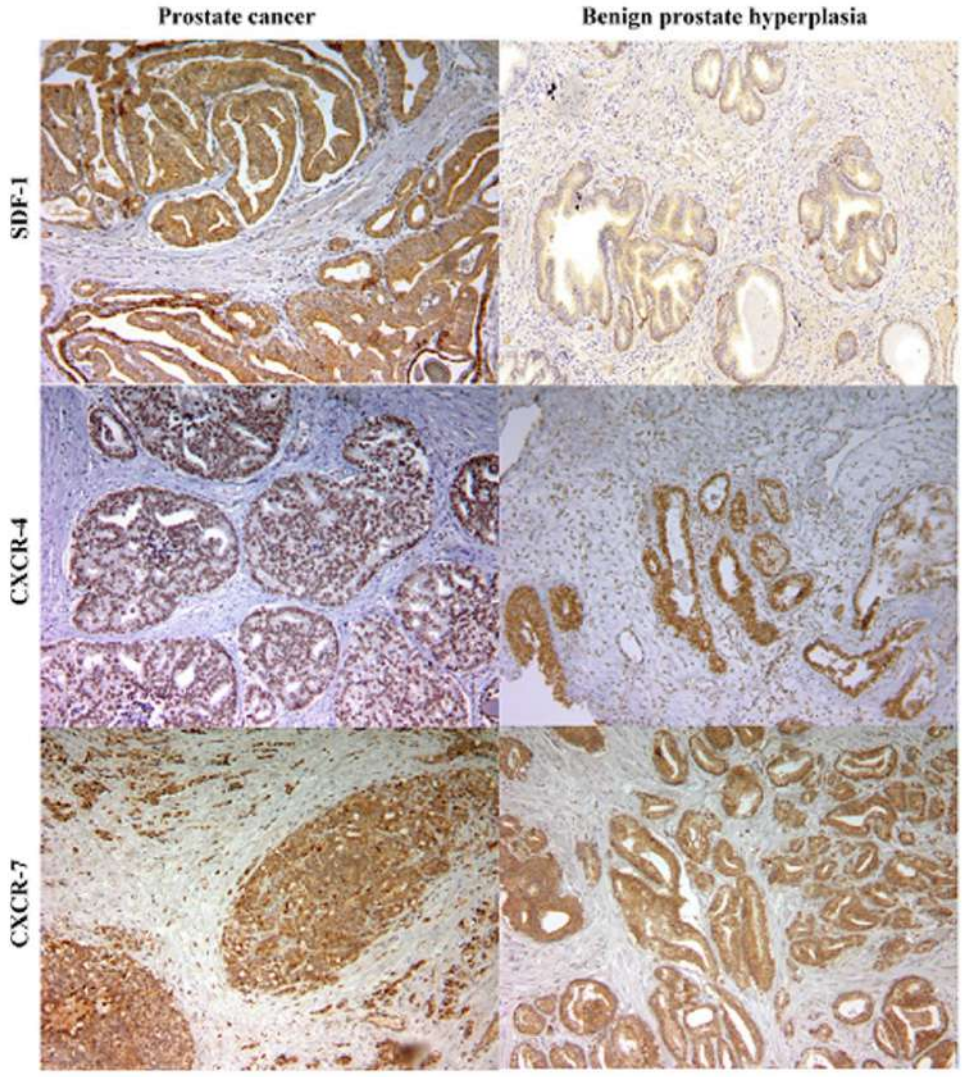

Fig. 1. Immunohistochemical representative microphotographic representing the SDF-1, CXCR4 and CXCRT expression in prostate cancer. Primary objective magnification, x10.

Table 3
Frequency of particular IHC H-score results in each protein subsets.

H-score	Number of cases, including each Fields (n = 150)				
	SDF-1 expression		CXCR4 expression		CXCR7 expression
	nuc-cyt	stroma	nuc-cyt	mem	cyt
0–50	40	39	94	70	58
51–100	33	48	28	53	40
101–150	31	15	8	12	24
151–200	30	13	5	10	15
201–250	11	29	10	4	9
251–300	5	6	5	1	4

nuc-cyt-nuclear-cytoplasmic expression; stroma-stromal expression; mem-membranous expression; cyt-cytoplasmic expression.

According to the TNM classification, the assessed the pT stage in each sample was found to be associated with CXCR7 protein. We remarked the notable higher cytoplasmic expression of CXCR7 receptor in pT2 stage, than in pT3a and pT3b stage in prostate cancer ($p = 0.0008$; Fig. 4A). The loss of CXCR7 was found to be associated with presence of angioinvasion ($p = 0.0002$, Fig. 4C)

3.4. Correlations between SDF-1, CXCR4 and CXCR7 staining intensity in PCa

Correspondence analysis based on Spearman's correlation coefficient revealed significantly positive association between chemokine receptors, CXCR4 localized at membrane and cytoplasm-localized CXCR7 at poor level ($r = 0.23$; $p = 0.003$). Interestingly, a remarkable negative correlation between SDF-1 chemokine stained in stroma and one of its receptor, CXCR7 located at cytoplasm of tumor cells was found at poor level ($r = -0.15$; $p = 0.032$). Analyzes for CXCR4 and SDF-1 revealed no significant protein–protein concordance regardless of their localization in tumor cells. However, we found a notable association between differently localized SDF-1 proteins. Our results indicate a correlation between nucleo-cytoplasmic expression of SDF-1 and SDF-1 protein localized in stroma at positive middle-high level ($r = 0.66$; $p = 0.000$). The summary of correlations between EMT-related proteins,

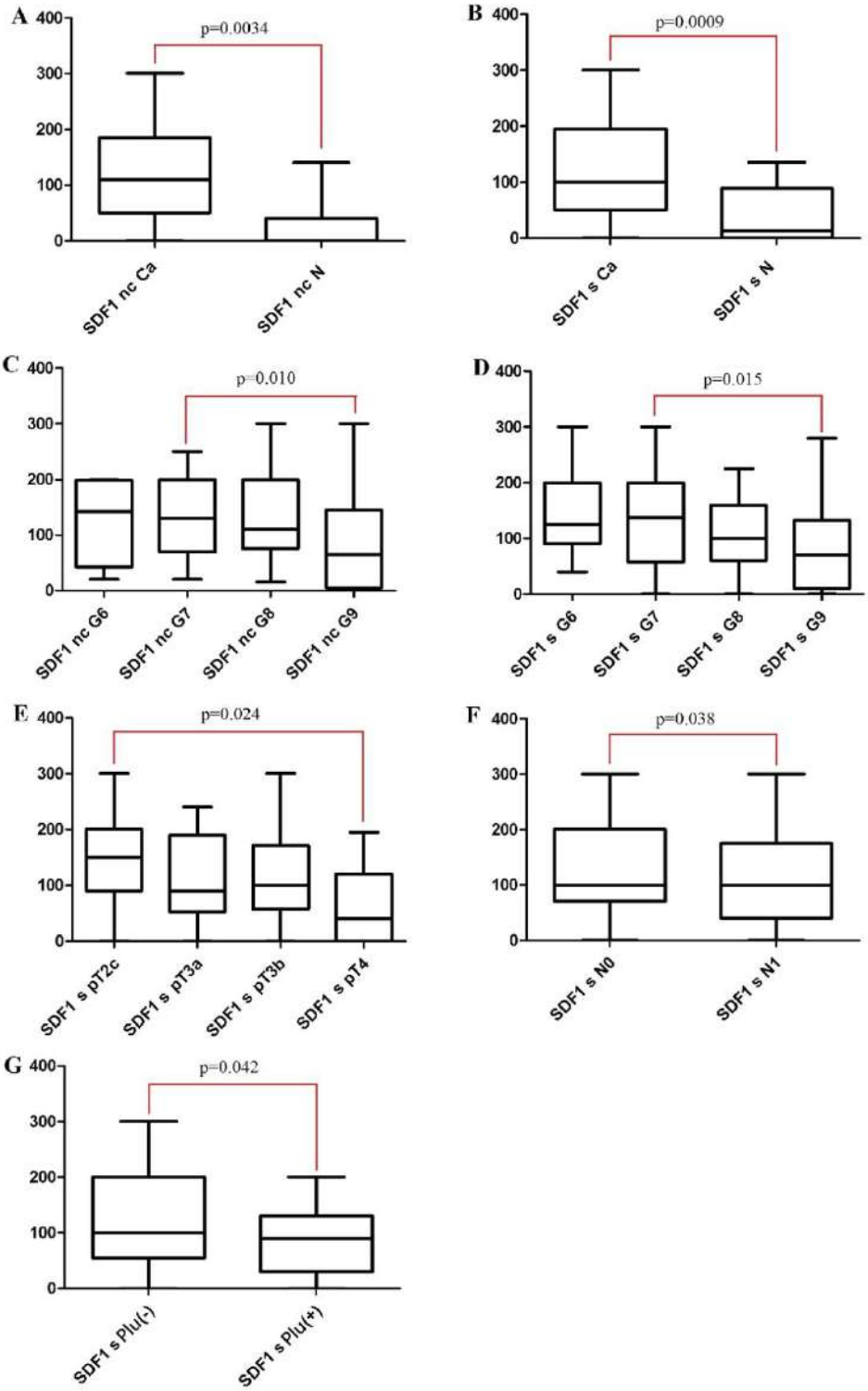

Fig. 2. The graphical demonstration of statistical analysis for SDF-I protein examined by IHC technique. The x-axis presents particular clinicopathological features for prostate cancer patients and the y-axis notices H-scores values for IHC staining. A-The correlation between SDF-1 and particular values of Gleason score. D-The correlation between stromal SDF-1 and particular values of Gleason score. E-The correlation between strornal localized SDF-1 and pathological tumor stage. F-The correlation between stromal-localized SDF-1 and present of lymph node metastases. G-the correlation between stromal expression of SDF-1 and presence of tumor phase in the vessel. The horizontal line of box plots denotes the median value of each score.

Table 4
Correlations between EMT-related proteins and clinicopathological features.

C.F.		SDF-1						CXCR4						CXCR7		
		nuc-cyt			stroma			nuc-cyt			mem			cyt		
		Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3
G.p.s	3	40.00	110.00	200.00	57.50	100.00	200.00	0.00	30.00	100.00	0.00	70.00	100.00	12.50	80.00	130.00
	4	61.25	110.00	177.50	45.00	100.00	160.00	0.00	25.00	100.00	0.00	60.00	100.00	20.00	100.00	150.00
	5	7.50	90.00	165.00	0.00	20.00	177.50	11.25	50.00	125.00	0.00	20.00	37.50	0.00	70.00	100.00
	p*		ns			ns			ns			ns			ns	
G.s.	6	42.50	142.50	198.80	90.00	125.00	200.00	0.00	10.00	38.75	60.00	87.50	107.50	77.50	140.00	195.00
	7	70.00	130.00	200.00	57.50	137.50	200.00	0.00	27.50	100.00	0.00	67.50	100.00	0.00	100.00	145.00
	8	75.00	110.00	200.00	60.00	100.00	160.00	10.00	30.00	100.00	0.00	70.00	120.00	15.00	80.00	110.00
	9	5.00	65.00	145.00	10.00	70.00	132.50	0.00	65.00	192.50	0.00	45.00	100.00	0.00	70.00	130.00
	p*		0.0104			0.0153			ns			ns			ns	
T	2c	42.50	152.50	200.00	90.00	150.00	200.00	0.00	15.00	100.00	40.00	65.00	88.75	102.50	145.00	195.00
	3a	60.00	100.00	145.00	52.50	90.00	190.00	20.00	50.00	100.00	5.00	80.00	150.00	25.00	100.00	112.50
	3b	50.00	100.00	162.50	57.50	100.00	171.30	0.00	22.50	85.00	0.00	62.50	100.00	0.00	70.00	107.50
	4	0.00	150.00	230.00	0.00	40.00	120.00	3.75	75.00	100.00	0.00	0.00	140.00	0.00	100.00	170.00
	p*		ns			0.0241			ns			ns			0.0008	
N	0	50.00	110.00	195.00	70.00	100.00	200.00	15.00	75.00	190.00	40.00	85.00	120.00	50.00	100.00	180.00
	1	50.00	100.00	185.00	40.00	100.00	175.00	0.00	20.00	60.00	0.00	47.50	100.00	0.00	80.00	112.50
	p**		ns			0.0384			0.0002			0.0045			0.0047	
Ang	(-)	50.00	117.50	198.80	60.00	100.00	200.00	0.00	30.00	100.00	0.00	65.00	100.00	30.00	100.00	140.00
	(+)	50.00	75.00	103.80	40.00	65.00	108.80	7.50	17.50	42.50	0.00	40.00	83.75	0.00	15.00	47.50
	p**		ns			ns			ns			ns			0.0002	
Plu	(-)	50.00	100.00	180.00	55.00	100.00	200.00	0.00	30.00	100.00	0.00	60.00	100.00	25.00	95.00	132.50
	(+)	40.00	120.00	200.00	30.00	90.00	130.00	0.00	37.50	100.00	0.00	55.00	100.00	0.00	100.00	140.00
	p**		ns			0.0424			ns			ns			ns	
Neu	(-)	50.00	110.00	195.00	50.00	100.00	195.00	0.00	27.50	100.00	0.00	70.00	100.00	12.50	95.00	140.00
	(+)	30.00	80.00	140.00	40.00	100.00	150.00	0.00	50.00	100.00	0.00	0.00	65.00	40.00	100.00	100.00
	p**		ns			ns			ns			0.0058			ns	
Cap	(-)	32.50	75.00	172.50	21.25	95.00	200.00	10.00	15.00	100.00	18.75	70.00	97.50	50.00	115.00	180.00
	(+)	50.00	110.00	186.30	50.00	100.00	183.80	0.00	30.00	100.00	0.00	60.00	100.00	15.00	95.00	130.00
	p**		ns			ns			ns			ns			ns	
C/N	n	0.00	0.00	40.00	0.00	12.50	88.75	0.00	60.00	140.00	0.00	82.50	133.80	35.00	97.50	155.00
	c	50.00	110.00	185.00	50.00	100.00	195.00	0.00	30.00	100.00	0.00	60.00	100.00	15.00	100.00	133.80
	p**		<0.0001			<0.0001			ns			ns			ns	

C.F.-Clinicopathological features; G.p.s-Gleason pattern separately; G.s.-Gleason score; T-tumor stage;N-nodal status; Ang- angioinvasion; Plu-Plugs in the vessels (cancer emboli); Neu-Neural infiltration; Cap-capsule invasion; C/N-cancer vs. normal tissue; nuc-cyt-nuclear-cytoplasmic expression; stroma-stromal expression; mem-membranous expression; cyt-cytoplasmic expression; p-P-value; ns-non significant; * Kruskal Wallis test; ** U Mann Whitney test.

showing its P-value and R-value are demonstrated in Table 5 and Table 6, respectively.

4. Discussion

The spread of prostate cancer cells can be subdivided into few steps, following by first critical event, where tumor cells leave the origin cancer focus and potentially undergoing EMT process. Next, migrating cancer cells invade surrounding tissues or cross endothelial barrier to penetrate into blood and lymphatic vessels, from where they spread to distal body parts and form distant metastases [33]. The recent studies have shown cross-communication between chronic inflammation, tumor outgrowth and progression to metastasis [34–38]. Especially, the chronic inflammation can expose normal cells to carcinogenetic factors through damaging their physiological barriers and stimulate oncogenes and/or disable tumor suppressors [39,40]. This process seems to be driven through growth factors and inflammatory cytokines such as chemokines [41].

The CXCR4 is one of such chemokines, that might to participate in adhesion and migration of tumor cells, cancer invasion and progression to spread disease [42,43]. In current study we found that CXCR4 protein expression could be observed in PCa as two immunostaining patterns: membranous and nucleo-cytoplasmic. Previous studies described cyto-membrane and nuclear CXCR4 expression pattern in hepatocellular carcinoma [44], breast cancer [45], lung cancer [46], nasopharyngeal carcinoma [42,47]. The most of cancers exhibit CXCR4 expression, which level increases with disease aggressiveness. Some authors have proved that CXCR4 pattern is particularly expressed in metastatic form of cancers [48,49]. Furthermore, the *meta*-analysis conducted by Lee and other authors indicated that elevated expression of CXCR4 protein in PCa patients could be notably associated with the occurrence of metastases, but not related to the Gleason score or the T stage [50]. Quite the opposite, we evaluated the higher level of CXCR4 was associated with lack of lymphatic metastases and nerve infiltration in human

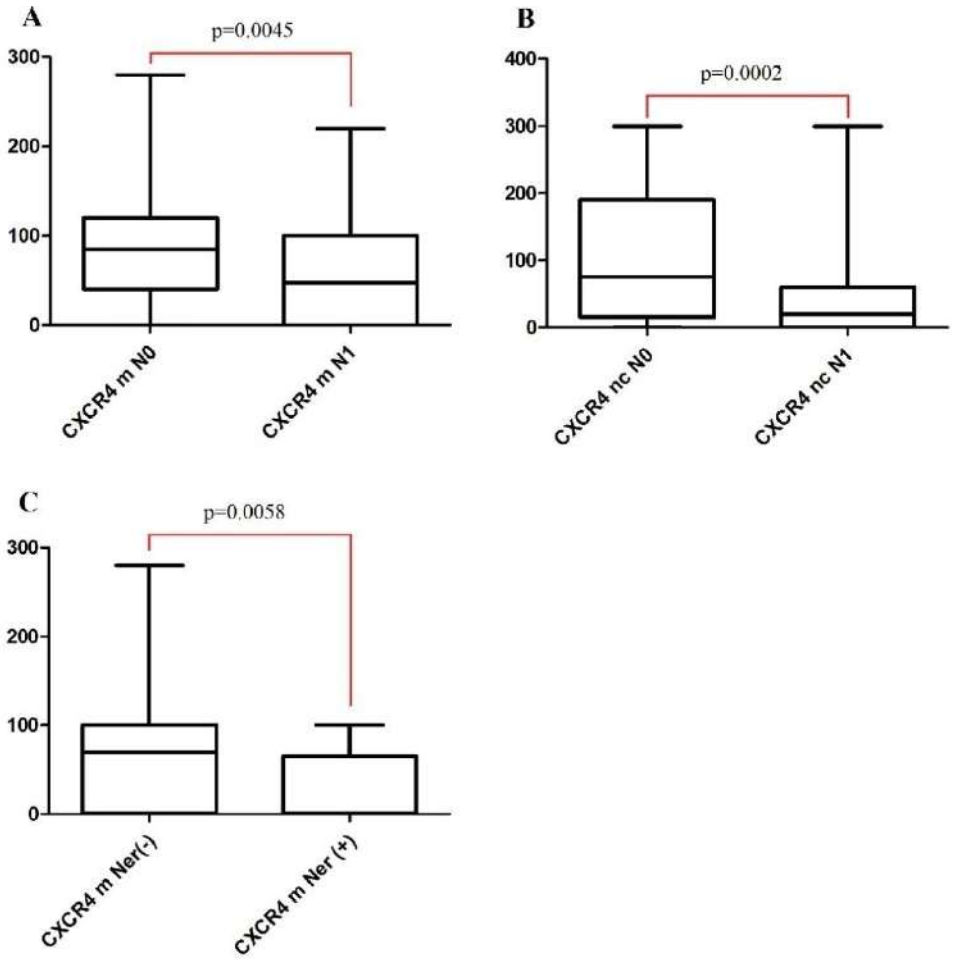

Fig. 3. The graphical demonstration of statistical analysis for CXCR4 protein examined by IHC technique. The x-axis presents particular clinicopathological features for prostate cancer patients and the y-axis notices H-score values for IHC staining. A-The correlation between membranous CXCR4 level and presence of lymph node metastases. B-The correlation between nucleo-cytoplasmic CXCR4 level and presence of lymph node metastases. C-The correlation between membranous expression of CXCR4 protein and presence of nerve invasion. The horizontal line of box plots denotes the median value of each score.

prostate cancer, so probably CXCR4 pattern was predominantly expressed in patients with lack of predisposition to metastatic form of prostate cancer and it is inconsistent with aforementioned studies.

Our data suggest that both nucleo-cytoplasmic and membranous CXCR4 fractions may get involved in the early stage of metastases formation. Yoshitake et al. have indicated that CXCR4 protein localized in nucleus of CRC tumor cells manifested more frequent lymphovascular metastasis, poor differentiation and patient outcome compared to cytomembrane CXCR4 pattern. According to Yoshitake study CXCR4 expressed in nucleus of CRC tumor cells may play crucial role in CRC progression, however our results showed that both membrane and nucleo-cytoplasmic pattern of CXCR4 had role in PCa progression [42]. It was proved in vivo that the expression of CXCR7 in PCa biopsies escalate along with higher invasive grade, similarly to CXCR4 [21,49]. Also, in vitro studies has been shown that enhanced CXCR7 expression correlated with cell adherence, angiogenesis and invasion [51]. Additionally, it was proved as the expression of CXCR7 in non-malignant diseases is highly circumscribed, while it is extensively expressed in malignancies, such as breast, lung or prostate cancer [21,25,52].

Current results proposed that the both loss of CXCR7 as well as loss of CXCR4 in PCa tumor cells could be significant in progression to the lymph node metastases. More interestingly, we marked that described two receptors for SDF-1 revealed the correlation between their expressions; hence they both may dually attend in initiation of metastases. Yang et al. in their study at CXCR7 pattern in prostate cancer have confirmed that the upregulation of CXCR7 was significantly corresponded with poor differentiation and presence of lymphovascular metastasis [53]. Additionally, the oncogenic role of CXCR7 was also confirmed by Singh et al. showing that overexpression of CXCR7 in normal PCa cells can influence on enhance of proliferative ability [54]. Hence, CXCR7 as well as aforedescribed CXCR4 may play dualistic role in progression to prostate cancer metastatic form. Our unsuspected result probably could be also associated with samples variability within our prostate cancer population. IHC methodology was scrupulously validated; therefore we exclude the influence of IHC procedure or applied antibodies on achieved result.

The next investigated protein SDF-1 is commonly described as a secreted by stromal fibroblasts ligand of aforementioned membrane receptor CXCR4 [55–58]. Our statistical analysis demonstrated higher expression of stromal SDF-1 in PCa in comparison to normal prostate tissue. Similar results have been earlier shown in a number of studies [59–61]. The high stromal SDF-1 expression in PCa could be explained due to observed in many studies relationship between a PCa and mesenchymal stem cells (MSC) [62,63]. It has been suggested that PCa-

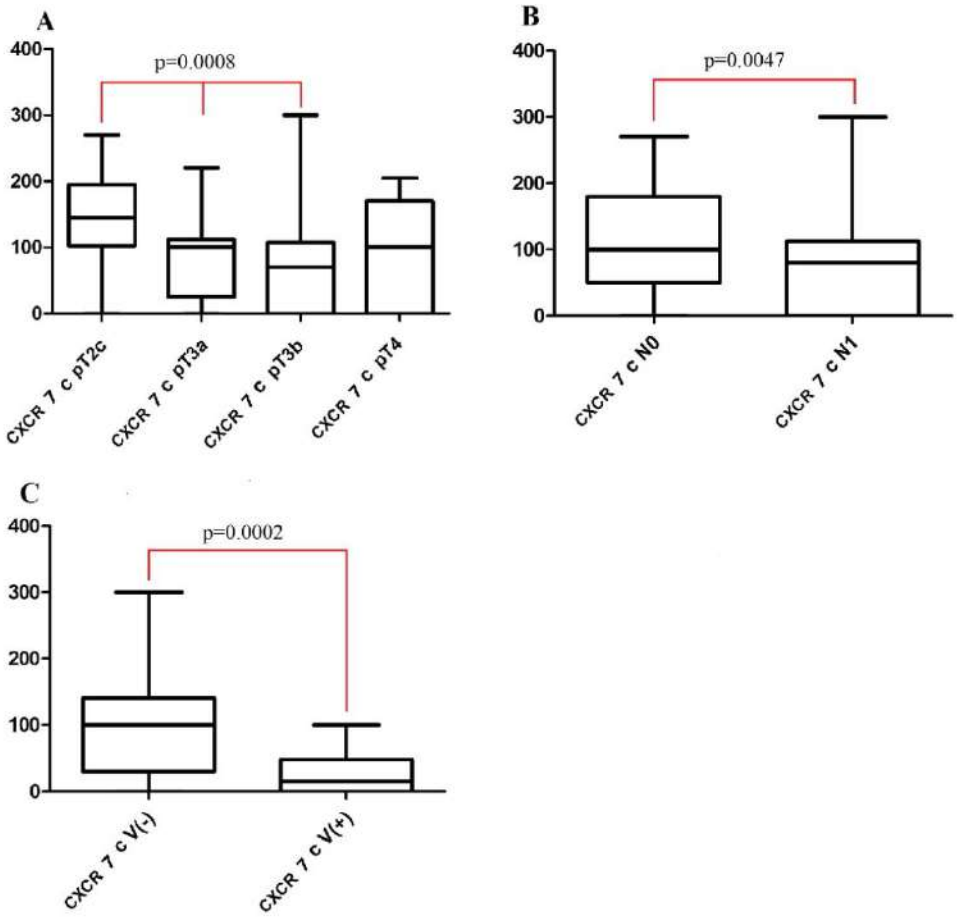

Fig. 4. The graphical demonstration of statistical analysis for CXCR7 protein examined by IHC techniques. The x-axis presents particular clinicopatholigical features for prostate cancer patients and the y-axis notices H-score values for IHCstaining. A-The correlation between cytoplasmic CXCR7 level and pathological tumor stage. B-The correlation between cytoplasmic CXCR7 expression and presence of lymph node metastases. C-The correlation between cytoplasmic CXCR7 protein and vascular invasion. The horizontal line of box plots denotes the median value of each score.

Table 5
Correlations between EMT-related proteins, presenting statistically significant P-value.

P-value	CXCR4 nuc-cyt	CXCR4 mem	SDF-1 nuc-cyt	SDF-1 stroma	CXCR7 cyt
CXCR4 nuc-cyt		0.0899	0.3891	0.1366	0.4162
CXCR4 mem	0.0899		0.4106	0.1055	0.0028
SDF-1 nuc-cyt	0.3891	0.4106		0.0000	0.2171
SDF-1 stroma	0.1366	0.1055	0.0000		0.0321
CXCR7 cyt	0.4162	0.0028	0.2171	0.0321	

associated MSC secret SDF1, what might be essential for activation other MSC and regulation of their migration, proliferation and survival and increase their population in cancer adjacent tissue [57,64–66]. As a consequence, increased MSC-population could generate increased level of excreted SDF-1. Furthermore, stromal SDF-1 may stimulate not only PCa-MSC but also influence the prostate cells to support the transformation of normal prostate cells into malignant version through the downstream signaling pathway such as MEK/ERK and PI3K/AKT [21,67]. The final effect of described pathway is proangiogenic phenotype of PCa-cells what promote survival of the tumor [68]. However, the increased stromal expression of SDF-1 has been observed in our study among PCa, without metastasis, with low T-stage measured by TNM

Table 6
Correlations between EMT-related proteins, presenting statistically significant R-value.

R-value	CXCR4 nuc-cyt	CXCR4 mem	SDF-1 nuc-cyt	SDF-1 stroma	CXCR7 cyt
CXCR4 nuc-cyt		0.1112	0.0234	0.0911	0.0177
CXCR4 mem	0.1112		0.0188	0.1038	0.2293
SDF-1 nuc-cyt	0.0234	0.0188		0.6552	0.0649
SDF-1 stroma	0.0911	0.1038	0.6552		-0.1526
CXCR7 cyt	0.0177	0.2293	0.0649	-0.1526	

classification and low Gleason grade. The microenvironment of more advanced, metastatic or undifferentiated cancers contained statistically lower secreted SDF-1 in adjacent tissue. Our result seem to be incompatible with many earlier studies, which have shown a substantial influence of SDF-1 on disease progression, migration of PCa cells and formation of metastasis [57,59–61,69–74]. Moreover, *SDF1* gene polymorphism have been associated in case of PCa with the advanced disease development with presence of bone metastasis [75]. However, SDF-1 secreting MSC could be double-edged sword in case of neoplastic disease and not only promote but also inhibit cancer progression [76]. Their appearance in tumor microenvironments may be also related to secretion of different from SDF-1 factors, such as inhibitors of matrix metalloproteinases or of the STAT3 signaling pathway and to suppression of cancer progress consequently [77–79]. In case of our investigated it is possible that detected high SDF-1 might be related to the presence of high MSC population, which secretes another, anit-tumoric cytokines leading to disease stabilization, absent in cases with lower stromal level of SDF-1. Furthermore, the prostate cancer is very heterogeneous disease, what could implicate results different from earlier studies.

In our study has been also detected higher intracellular SDF-1 expression in PCa in regard to normal prostate cells and in PCa with lower Gleason grade. Recently, similar observation have been performed by another authors [69]. Moreover, Delongchamps et al. showed recently that SDF-1 level increase from the tumor center to the distant area of tumor and is associated with high CXCR4 expression at the tumor front [80]. PCa cells may start to secrete SDF-1 too to maintain and to increase the proangiogenic stimulation at the autocrine way in the course of a disease, primarily in high differentiated cells. However, the our research was associated with a number of limitations such as precision of used methods or sample numbers, the future studies are needed to better understanding role of SDF-1 in PCa pathogenesis .

Additionally, the mechanism of SDF-1 ligand binding to its receptor, CXCR4 initiates multiple signaling pathways, leading to angiogenesis regulation, cell invasion and growth, apoptosis inhibition [50]. Cheng and colleagues have suggested that CXCL12/CXCR4 complex induces the EMT process in gastric carcinoma cells and is supported by the activation of c-MET pathway [41]. Increasing number of scholars accentuated the role of SDF-1/CXCR4/CXCR7 axis in initiation of metastasis and prognosis [28]. In current study higher ability to promote prostate disease progression revealed SDF-1/CXCR7 pathway. The both SDF-1 and CXCR7 proteins participate in lymph node metastases formation and they posse confirmed association between their stromal and cytoplasmic expression, respectively.

5. Conclusion

To the best of our knowledge, this is the first published paper investigating expression of SDF-1 chemokine and its receptors CXCR4 and CXCR7 in relation to EMT in both benign and malignant FFPE prostate tissue measured by IHC. Our results highlighted potential role of SDF-1, CXCR4 and CXCR7 in acquiring the malignant phenotype of tumor cells in PCa and may constitute potential therapeutic target in aggressive form of disease.

Authors' Contributions

This paper was planned by MPK, and she is responsible for the overall conception of the work, collecting information, data extraction process, methodology, creating tables with important data as well as the graphic layout and initial drafting of the manuscript. AG was responsible for statistical elaboration. MPK and AG contributed critical revisions of the manuscript for the intellectual content, edited the manuscript and made the necessary language corrections. JL was responsible for technical assistance. DG supervised the work. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Research Ethics

All of applied procedures in this study were in agreement with the ethical principles and standards of the institutional and national bioethical commission and with the Helsinki Declaration of 1964 and its later revisions.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2021.155778.

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RESEARCH ARTICLE

Clinicopathological significance of the EMT-related proteins and their interrelationships in prostate cancer. An immunohistochemical study

Martyna Parol-Kulczyk*, Arkadiusz Gzil, Joanna Maciejewska, Magdalena Bodnar, Dariusz Grzanka

Department of Clinical Pathomorphology, Faculty of Medicine, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, Toruń, Poland

* mparol@cm.umk.pl

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Abbreviations: PCa, prostate cancer; SEER, Surveillance, Epidemiology, and End Results Program; ECM, degrading enzymes: extracellular

Abstract

The chronic inflammation influences a microenvironment, where as a result of losing control over tissue homeostatic mechanisms, the carcinogenesis process may be induced. Inflammatory response cells can secrete a number of factors that support both initiation and progression of cancer and also they may consequently induct an epithelial-mesenchymal transition (EMT), the process responsible for development of distant metastasis. Macrophage migration inhibitory factor (MIF) acts as a pro-inflammatory cytokine that is considered as a link between chronic inflammation and tumor development. MIF can function as a modulator of important cancer-related genes expression, as well as an activator of signaling pathways that promotes the development of prostate cancer. The study was performed on FFPE tissues resected from patients who underwent radical prostatectomy. To investigate the relationship of studied proteins with involvement in tumor progression and initiation of epithelial-to-mesenchymal transition (EMT) process, we selected clinicopathological parameters related to tumor progression. Immunohistochemical analyses of MIF, SOX-4, β-catenin and E-cadherin were performed on TMA slides. We found a statistically significant correlation of overall β-catenin expression with the both lymph node metastasis ($p<0.001$) and presence of angioinvasion ($p = 0.012$). Membrane β-catenin expression was associated with distant metastasis ($p = 0.021$). In turn, nuclear MIF was correlated with lymph node metastasis ($p = 0.003$). The positive protein-protein correlations have been shown between the total β-catenin protein expression level with level of nuclear SOX-4 protein expression ($r = 0.27$; $p<0.05$) as well as negative correlation of β-catenin expression with level of nuclear MIF protein expression ($r = -0.23$; $p<0.05$). Our results seem promising and strongly highlight the potential role of MIF in development of nodal metastases as well as may confirm an involvement of β-catenin in disease spread in case of prostate cancer.

matrix degradation enzymes; EMT, epithelial-mesenchymal transition; AJ, adherens junctions; GSK3β, glycogen synthase kinase 3β; HMG, high-mobility-group; TCF, T-cell factor; LEF, lymphoid enhancer factor; SOX-4, SRY-Box Transcription Factor 4; SRY, sex-determining region Y; DBD, DNA-binding domain; MIF, Macrophage migration inhibitory factor; TPOR, thiol protein oxidoreductase; FFPE, formalin-fixed paraffin embedded; AJCC, American Joint Committee on Cancer; GS, Gleason score; TMA, tissue microarray; IHC, immunohistochemistry; IRS, immunoreactive score; 25p, 25th percentile; M, median; 75p, 75th percentile; BPH, benign prostatic hyperplasia; EMT-TFs, EMT transcription factors; TNBC, triple-negative breast cancer; NPC, nasopharyngeal carcinoma.

Introduction

Prostate cancer (PCa) is the most common malignancy as well as the fifth cause of cancer-related death among men worldwide [1]. In 2020, it was estimated approximately 191,930 of PCa new cases and 33,330 of PCa–related deaths occurred in the United States [2]. The PCa incidence rate varies depending on genetic, hormonal-dependent and environmental aspects. Besides, the PCa prevalence and mortality rate are associated with elderly age and the average age at the time of diagnosis is above 65 years [1].

PCa still remains a highly treatable neoplasm if it is diagnosed as localized disease at its an early stage and constantly monitored [3]. These patients could be usually treated by radical prostatectomy or radiotherapy, which guarantee a successful treatment outcome in most cases. However, the National Cancer Institute indicated that the lymph node metastasis are observed in 12% of PCa patients at the time of diagnosis [4]. Additionally, by the significant proportion of patients the PCa undergo progression to a highly advanced, metastatic stage of disease for which treatment options are limited and the prognosis is uncertain [5]. Based on the Surveillance, Epidemiology, and End Results Program (SEER) database, the 5-year relative survival rate for PCa cases with localized and regional stage is at 100%, whereas at the metastatic stage the level is only 29% [6].

Solid tumors, such as PCa grow in hypoxic conditions, which are characterized by inadequate blood flow and impaired tumor vessels [7]. These conditions and mutations in the *CTNNB1* gene may lead to activation, accumulation and nuclear translocation of β-catenin [8]. In the consequence there are activated or deactivated specific targets such as transcription factors, cell-surface and cytoskeletal proteins, extracellular matrix degradation enzymes (ECM-degrading enzymes) and specific microRNAs, leading to deregulation of cell proliferation management and inhibition of apoptosis [9]. Described process is called an epithelial-mesenchymal transition (EMT), during which cells lose their epithelial features responsible for cell-to-cell adhesion inter alia as a result of losing of epithelial markers and gain mesenchymal properties. Consequently, initiation of the EMT process results in the cell phenotype changes. The downregulation of epithelial markers, in particular E-cadherin, leads to destruction in cell junction proteins and in consequence to loss of the stable epithelial polarized phenotype of the involved cells. Moreover, they gain mesenchymal markers, like N-cadherin, Vimentin or Fibronectin etc. and ability to migration from their origin place, through modulating signal transduction pathways, such as Wnt/β-catenin and TGF-β pathway, as well as EMT transcription factors: zinc finger E-box binding homeobox (Zeb 1/2) and Snail [10, 11]. The cells with mesenchymal phenotype present invasive and metastatic potential as well as they acquire stemness status (ability to self-renew and differentiate) or also chemoresistance properties [12, 13].

Critical point of the EMT constitutes Wnt/β-catenin signal transduction pathway, where β-catenin acts as a central effector of multiple running separately cellular processes [11]. β-catenin forms catenin-cadherin complex due to binding to the major component of cell-cell adherens junctions (AJ), a membrane anchored E-cadherin, thus enabling modulation of localized cytoskeletal remodeling [14–16]. Mechanism of forming catenin-cadherin communication in AJ ensures β-catenin stabilization and protects it from proteasomal degradation [17, 18]. Loss of E-cadherin leads to collapse of cell-cell connections through spontaneous disruption of AJ and to β-catenin detachment from the cell adhesion complex [19]. Free membrane β-catenin released into the cytoplasm may eventually be translocated to the cell nucleus [11, 19]. β-catenin nuclear translocation is driven through Wnt/β-catenin pathway in cooperation with curly receptors and LRP5/6 co-receptors [20], resulting in the repression of glycogen synthase kinase 3β (GSK3β), which is responsible for ubiquitination of β-catenin together with proteasomal degradation through its phosphorylation and β-catenin stabilization [21]. Transport of β-catenin to the cytoplasm area or nucleus can recruit and stimulate downstream transcription

factors of TCF4, thus promoting cell proliferation and tumorigenesis [20, 22]. Stabilized β-catenin is accumulated in cell nucleus, where as a cofactor it interacts with the high-mobility-group (HMG) box family of T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors to modulate target genes of the Wnt pathway [23, 24].

Previous studies showed that transcription factor Sry-related HMG-BOX gene 4 (SOX-4) interacts with members of TCF/LEF family via its HMB domain, which regulates stability of these proteins and therefore indirectly also stability of nuclear translocated β-catenin, which is in turn modulated via TCF/LEF complex [25]. Moreover, the study with gain- and loss-of-function have demonstrated that the SOX-4 may be responsible for enhanced β-catenin/TCF activity and the tumor cells proliferation of SW480 colon carcinoma cell lines [25].

Furthermore, the latest study confirmed that SOX-4 constitutes a crucial point in metastatic progression. Under normal physiological conditions, SOX-4 belonging to sex-determining region Y (SRY) box family with special DNA-binding domain (DBD) in HMG [26] functions as a transcription factor, involved in maintenance of pluripotency in stem cells and wide range of developmental processes, such as embryogenesis, development of the central as well as peripheral nervous system, heart, osteoblastic, thymocytes and differentiation of lymphocytes [27–30]. Moreover, SOX-4 is critical in directing cell fate [31]. Recent studies have also reported that SOX-4 is associated with tumorigenesis and shows higher expression in human malignant tumors, such as prostate cancer [32], colorectal cancer [25], breast cancer [33], lung cancer [34], gastric cancer [35]. Nevertheless, in some tumors, like melanoma [36] or bladder cancer [37] SOX-4 can behave as a tumor suppressor, promoting cell cycle arrest and apoptosis [31]. Interestingly, there are some studies confirming the interactions between the canonical Wnt/β-catenin signaling pathways and the both SOX-4 as well as a Macrophage migration inhibitory factor (MIF). Currently, many researchers are focused on the inflammation-induced EMT process in various types of cancers. Previous studies have shown MIF as a potential molecular link between chronic inflammation and cancer [38]. Additionally, in pancreatic ductal adenocarcinoma, MIF was found to prompt the transition from epithelial phenotype to mesenchymal state of cancer cells [39]. However, MIF-induced activation of EMT has not been confirmed in prostate cancer patients.

MIF generally functions as a multipotent cytokine involved in regulation of immune and inflammatory responses as well as in certain pathological situations including atherosclerosis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, psoriasis and diabetes [40–42]. MIF expression was found in both extracellular and intracellular cell area. The extracellularly MIF was found to be involved in cell proliferation, adhesion, invasion and homeostasis control, while intracellularly MIF is associated with c-Jun activation domain binding protein-1 (JAB1), the tumor suppressor protein p53 and thiol protein oxidoreductase (TPOR) [43]. MIF is secreted upon inflammatory and stress stimulation by immune, parenchymal and tumor cells [40]. Furthermore, MIF affects the tumor microenvironment facilitating proliferation and growth through promotion of angiogenesis necessary for maintaining tumor growth [26, 44]. Bando et al., in their cohort study, have examined the upregulation of nuclear MIF in breast [45], whereas Verjans and colleagues have shown that the intracellular MIF is related to beneficial properties, while the extracellular MIF was involved in promoting breast cancer cell-stroma interactions [43]. Aberrant expression of MIF protein was observed also in many other types of cancers, such as colon [46, 47], melanoma [48], glioblastoma [49], lung adenocarcinomas [50], renal cancer [51], urothelial cancer [52], pancreas carcinoid [39], thyroid cancer [53] as well as prostate cancer [54].

Altered subcellular expression of MIF, SOX-4, β-catenin and E-cadherin may significantly contribute to tumor aggressiveness. The associations between aforementioned proteins as well as their clinicopathological significance in prostate cancer remains still not fully understood.

This article focuses on the study of relationships between EMT related proteins and their clinicopathological aspect.

Material and methods

The research was approved by the Bioethical Commission of Collegium Medicum in Bydgoszcz of the Nicolaus Copernicus University in Torun, Poland (decision number: *KB 248/2019*). The eighty five patients of the Department of General and Oncological Urology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun (Poland) with confirmed PCa were enrolled in the current study. The participants underwent radical prostatectomy between January 2017 and December 2019. All attendees have provided the written, signed and dated informed consent form. The patients' medical records have been completely anonymized to protect the identity of participants, before including to the research.

Tissue specimens

The present study was concerned on immunohistochemical evaluation of protein levels performed with Formalin-fixed paraffin embedded (FFPE) specimens of eighty five prostate cancers patients. The individual research stages have been conducted at the Department of Clinical Pathomorphology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, Poland.

Histological evaluation of resected tumors was performed with hematoxylin-eosin- (H&E-) stained slides to confirm diagnosis and choose representative tumor areas containing no less than 80% of tumor cells. All samples were assessed by two pathologists who independently performed histological stage classifications according to the 8th Edition American Joint Committee on Cancer (AJCC) Cancer Staging classification system [55], extending the current research by defining a precise tumor location and Gleason pattern or score of extracapsular tumor extension to show their significance in EMT. The tumor hallmarks noted in prostate cancer areas were submitted to descriptive analysis.

Patients characteristics

The age of enrolled patients ranged from 52 to 81 and the median was 66 years. According to the pathologic T stage, all patients was in advanced tumor stage following: pT3a was confirmed in 40 individuals, pT3b in 39 and pT4 in 4. At the time of diagnosis, the positive lymph node status was affirmed in 17 of cases, the positive distant metastasis occurred in approximately 9 of patients, whereas the presence of angioinvasion was found in 13 of tumor patients. Most tumors manifested Gleason pattern 3+4 and 4+3 with frequency 36.5% and 23%, respectively. Low proportion constituted specimens with Gleason pattern 5+4, 3+5, 3+3 and 4+4 at 3.5%, 5.9%, 8.2% and 9.4% level, sequentially. Samples with tumor invaded areas showing Gleason score (GS) 7 were 59%, with GS8 were 15.3% and with GS9 were 16.5%. Differently, samples with presence of cancer invasive foci that exceeded the capsule of prostate gland presented GS 8 with primary and secondary score of 4 (40%) and GS6 with primary and secondary score of 3 (28.2%). Detailed clinicopathological features of the patients with PCa that underwent the study are summarized in Table 1.

Tissue microarray (TMA) construction and immunohistochemistry (IHC) analysis

TMAs were produced by relocating of earlier marked representative tissue cores, obtained from conventional paraffin block representing particular patient and arranged on a five-cores

Table 1. Clinicopathological characteristics of identified participants.

Variables			% of cases (N = 85)
Age	≤60 years		1.6
	>60 years		82.4
Prostate weight	≤66 g		70.6
	>66 g		27
	Unk		2.4
Stage pT according to TNM	pT3a		47
	pT3b		45.9
	pT4		4.7
	unk		1.2
Stage N according to TNM	N0		80
	N1		20
Stage M according to TNM	M0		89.4
	M1		10.6
Gleason Score	6		8.2
	7		58.8
	8		15.3
	9		16.5
	unk		1.2
Gleason pattern	3+3		8.2
	3+4		36.5
	3+5		5.9
	4+3		22.4
	4+4		9.4
	4+5		12.9
	5+4		3.5
	unk		1.2
Angioinvasion	Nos		15.3
	neg		83.5
	Unk		1.2
Area of tumor extension	Top of the gland	R	100
		L	100
		R+L	100
	Right area	≤50%	76.5
		>50%	21.2
		unk	2.4
	Left area	≤50%	62.4
		>50%	35.3
		unk	2.4
	Extraprostatic extension	R	95.3
		L	87
		R+L	87
	Seminal vesicle invasion	R	44.7
		L	54.1
		R+L	44.7

(*Continued*)

Table 1. (Continued)

Variables		% of cases (N = 85)
Gleason score of extraprostatic extension	6	28.2
	7	10.6
	8	40
	9	9.4
	10	4.7
	unk	7.1
Gleason score of extraprostatic extension	3+3	28.2
	3+4	5.9
	4+3	4.7
	4+4	40
	4+5	8.2
	5+4	1.2
	5+5	4.7
	unk	7.1

unk- unknown; pos- positive; neg- negative; R- right area; L-left area.

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recipient paraffin block. Reflecting the fact that small cores from PCa with such high morphological heterogeneity might not be representative for the whole affected tumor area, we decided to use core diameters of 5 mm, including one core per patient was extracted from tumor area and one core per patient from tumor-free areas, representing tissue control. The Gleason grading and score assessment were done.

TMA blocks were cut with a rotary microtome (Accu-Cut; Sakura, Torrance, CA, USA) to 4.0 μm thick paraffin sections and mounted onto either special adhesive slides (SuperFrost-Plus, Thermo Scientific) for subsequent analyses The highest quality of microscopic evaluation process was ensure by simultaneously performing of IHC staining the both on TMA sections (encompassing tumor and adjacent healthy tissue) and on a positive control material, recommended by the producer or interactive database The Human Protein Atlas available from https://www.proteinatlas.org [56]. Deparaffinization, rehydration and heat-mediated epitope retrieval steps were done with a pH6 antigen retrieval solution in a PT-Link (Dako). The sections were subsequently incubated with the following primary antibodies;: rabbit monoclonal antibody against β-catenin (clone [E247], ab32572, Abcam, Cambridge, UK), rabbit monoclonal antibody against E-cadherin (clone [EP700Y], ab40772, Abcam, Cambridge, UK), rabbit polyclonal antibody against MIF (HPA003868, Sigma-Aldrich) and rabbit polyclonal antibody against SOX-4 (ab86809, Abcam, Cambridge, UK). The samples were incubated with monoclonal antibodies for 25 minutes in 37˚C, whereas the incubation time with polyclonal antibodies needed to be extended to 15 h at 4˚C. Characteristics of primary antibodies implicated in the current study was presented in Table 2. Immunohistochemical staining was performed

Table 2. Primary antibody characteristics.

Antibody	Primary antibody dilution	Positive control according to antibody data sheet and Human Protein Atlas	Cellular localization/expression in prostate cancer tissue	Catalog number
B-catenin	1:500	colon	Membrane/cytoplasmic/nuclear	ab32572
E-cadherin	1:500	colonic adenocarcinoma	Membrane/cytoplasmic/nuclear	ab40772
MIF	1:500	kidney	Cytoplasmic/nuclear	HPA003868
SOX-4	1:100	testis	Cytoplasmic/nuclear	ab86809

https://doi.org/10.1371/journal.pone.0253112.t002

in a Benchmark GX Platform automated slide processing system (Ventana Medical Systems, Tucson, AZ, USA) using the OptiView DAB IHC detection kit (Ventana Medical Systems) according to the manufacturer's instructions. EnVision FLEX-HRP (Dako) was used to detect the antigen-antibody complexes. Finally, the sections were counterstained with Meyer's hematoxylin, dehydrated in graded ethanols (80, 90, 96, 99.8%), cleared in series of xylenes (I–IV) and sealed with a Dako Mounting Medium.

The immunohistochemical staining of MIF and SOX-4 was performed manually, using primary antibody against MIF as well as primary antibody against SOX-4 and independently incubation overnight at 4˚C. Additionally, the ZytoChem Plus (HRP) One-Step Polymer anti-Mouse/Rabbit/Rat kit (Zytomed) was used to enhance detection signal of SOX-4/antibody complexes. Proteins localization was visualized using DAB as a chromogen.

Criterion for positive immunohistochemical staining

The protein levels (β-catenin, E-cadherin, SOX-4 and MIF) were scored by protein localization and intensity of IHC staining in malignant cellsusing light microscope ECLIPSE E800 (Nikon Instruments Europe, Amsterdam, The Netherlands). The evaluation step was provided by two independent, board-certified pathologist. Protein expressions were estimated, following the Remmele and Stegner (1987) immunoreactive score (IRS), which is based on the ratio of expression intensity and percentage of positively expressed cells. The staining intensity was evaluated in 4-point scale (expression: negative-0, weak-1, moderate-2, strong-3) and 5-point scale of percentage of positive tumor cells: 0, 1, 2, 3, 4 corresponded respectively to <10%, 10–50%, 51–80% and >80% of positive cells and giving the maximum result of 12.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software Inc.) and Microsoft Excel 2007. The expression values of analyzed proteins were presented 25^{th} percentile (25p), the median (M) and the 75^{th} percentile (75p). Our data does not fit a specific distribution and therefore we used nonparametric statistical tests. The comparative studies were analyzed statistically using the U Mann-Whitney (in case of two comparable groups) and Kruskal-Wallis test (in case of three or more comparable groups). Spearman test were used to investigate the relationship between protein levels and clinicopathological parameters: age, tumor size, pathological tumor stage, Gleason pattern, total Gleason score, presence of lymph node or distant metastases, presence of angioinvasion or plugs in the vessels as well as invasion depth. The p-values below 0.05 were considered statistically significant.

Results

Cellular redistribution of immunohistochemically stained EMT-related proteins in PCa

Immunohistochemical evaluation was provided considering protein subcellular localization and IRS score.

The nuclear expression of β-catenin was exhibited in 61 (71.8%) of specimens, while in 82 (96.4%) specimens were displayed cytoplasmic expression of β-catenin. β-catenin membrane staining was observed in 76 (89.4%) of cases. Membrane and cytoplasmic E-cadherin expression was confirmed in 98.8% (84) of specimens, in turn nuclear level of E-cadherin appeared in 97.6% (83) of cases. Strong membrane expression of β-catenin and E-cadherin have been found in normal glandular cells of adjacent healthy prostate tissues, and was treated as an internal positive control. The positive cytoplasmic expression of MIF was detected in 83

(97.6%) samples, and the positive nuclear MIF expression was assessed in 55 (64.6%) cases, whereas in healthy adjacent prostate tissue the normal glandular and immune cells appeared a strong cytoplasmic staining pattern of MIF protein and it was our internal IHC control. Immunohistochemistry studies of 85 prostate cancer cases revealed also complete absence of SOX-4 nuclear expression in 19 (22.4%) of specimens, weak SOX-4 nuclear expression in 61 (71.7%) and high nuclear expression of SOX-4 in 5 patients (5.9%). In contrast, the glandular cells of adjacent healthy prostate areas showed lack or weak SOX-4 nuclear immunostaining and they were accepted as a negative internal control (data of internal controls not shown; instead of this, all proteins data was checked with interactive database of protein expression in healthy tissue available from https://www.proteinatlas.org [56]).

The examples of all types of expression pattern of β-catenin and other EMT-related proteins are presented in Fig 1. Microscopic samples analysis revealed the following: 1) The expression pattern of β-catenin in tumor cells had mostly cytoplasmic character, what was associated with a higher stage of PCa. 2) The reduced membrane β-catenin staining was related with higher Gleason pattern and it mostly occurred with Gleason pattern 4+5 and 5+5. 4) The highest membrane β-catenin expression occurred with Gleason pattern 3+3, primarily. 5) In extracapsular extension of PCa we noticed two mostly exhibited patterns, higher β-catenin expression in relation with Gleason pattern 3+3 and lower β-catenin expression in relation with Gleason pattern 4+4. 6) The highest membrane expression of E-cadherin in tumor glandular cells was closely related to Gleason pattern 3+3 and to a lesser extent with Gleason pattern 4+5 and 5+4. 7) The decreased E-cadherin expression level in samples with the highest Gleason score. 8) The nuclear-located E-cadherin expression was at the constant level.

The first aim of the current study was to determine the connection between investigated EMT-related proteins and clinicopathological features of PCa. The clinicopathological associations of EMT-related proteins were presented in Figs 2–5.

Immunohistochemical analysis of overall β-catenin expression in PCa: Clinicopathological associations

The U Mann-Whitney analysis revealed statistically significant correlation of overall β-catenin expression with the presence of lymphovascular metastasis and angioinvasion, affirming its participation in metastasis formation. The overall expression of β-catenin level was higher in samples without infiltration of lymph nodes compared with samples with it ($p<0.001$; Fig 2A). We marked an involvement of overall β-catenin expression in vascular invasion. PCa cells showed the higher overall β-catenin expression in tumors with the presence of angioinvasion in regard to the tumors without it($p = 0.012$; Fig 2B). Similarly, we noticed elevated overall β-catenin expression in tumor samples with plugs of cancer cells in blood vessels compared to samples without it($p = 0.027$; Fig 2C).

Immunohistochemical analysis of membrane, cytoplasmic and nuclear β-catenin expression in PCa: Clinicopathological associations

Our study revealed the participation of membranous β-catenin expression of tumor cells in formation of distant metastasis. Statistical analysis showed significant correlation of membranous β-catenin expression with the presence of distant metastasis. The membranous β-catenin expression was higher in samples without metastasis($p = 0.021$; Fig 1E).

The cytoplasmic expression of β-catenin showed dependence neither with Gleason pattern and Gleason score nor with the presence of lymph node and distant metastasis and angioinvasion. The nuclear β-catenin expression remained at the constant level without any correlation with clinicopathological features. We observed that the nuclear β-catenin expression appeared

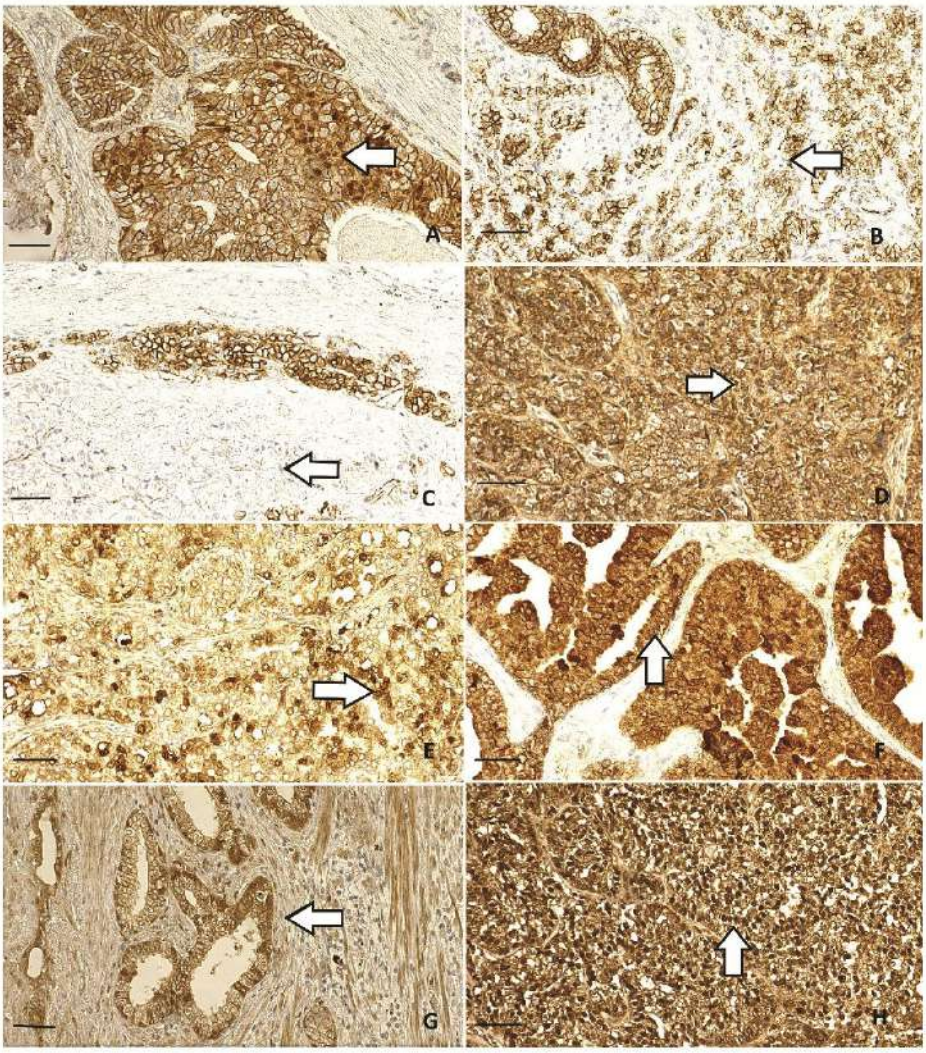

Fig 1. Microphotograph presenting the EMT-related proteins expression in prostate cancer tissue. Representative immunohistochemistry images presenting EMT-related proteins in TMA sections of prostate cancer tissue. A-white arrow demonstrates membrane and nuclear β-catenin expression; B-white arrow demonstrates partially reduced membrane β-catenin expression; C-white arrow demonstrates completely reduced membrane β-catenin expression; D-white arrow demonstrates completely reduced membrane E-cadherin expression; E-white arrow demonstrates the nuclear E-cadherin expression; F-white arrow demonstrates the cytoplasmic E-cadherin expression; G-white arrow demonstrates the nuclear SOX-4 expression; H-white arrow demonstrates the nuclear MIF expression. Original magnification was x10 for A-H figures. Nucleus counterstained with hematoxylin. Scale bar: 100 μm.

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in tumor areas related to Gleason pattern 4+4, primarily. Moreover, we found that tumor infiltration of left prostate gland area shows weak negative correlation with the membrane-located β-catenin ($r = -0.36$, $p<0.05$), the cytoplasm-located β-catenin ($r = -0.30$, $p<0.05$) as well as overall β-catenin expression ($r = -0.33$, $p<0.05$).

Data concerning correlations between β-catenin as well as other EMT-related proteins and main clinicopathological features was presented in two parts: Tables 3 and 4. Additionally, the

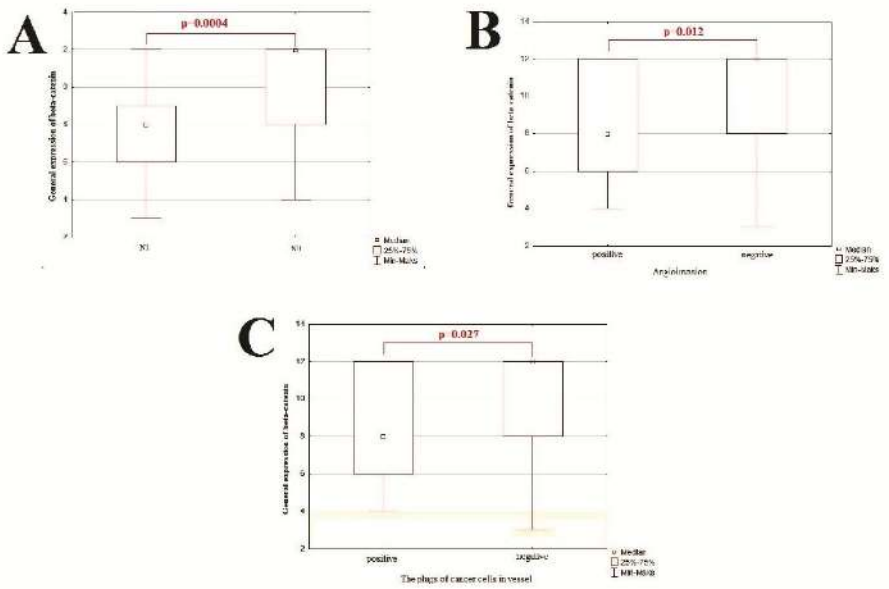

Fig 2. The graphic representation of results for general expression of β-catenin examined by IHC technique. The x-axis shows individual clinicopathological features and the y-axis shows IRS score values for IHC staining. The p-value <0.05 was considered as a statistically significant. Correlation between general β-catenin immunoexpression of PCa and presence of lymph node metastasis (A); angioinvasion (B) and presence of plugs of tumor cells in the vessel (C).

https://doi.org/10.1371/journal.pone.0253112.g002

data of correlations between investigated proteins and histological grading of tumor tissue which has grown through the prostate contain Tables 5 and 6.

Immunohistochemical analysis of membrane and cytoplasm E-cadherin expression in PCa: Clinicopathological associations

The analysis of protein expression and clinicopathological features showed that the cancer invasion of the right surface of prostate gland negatively corresponded with cytoplasm-located

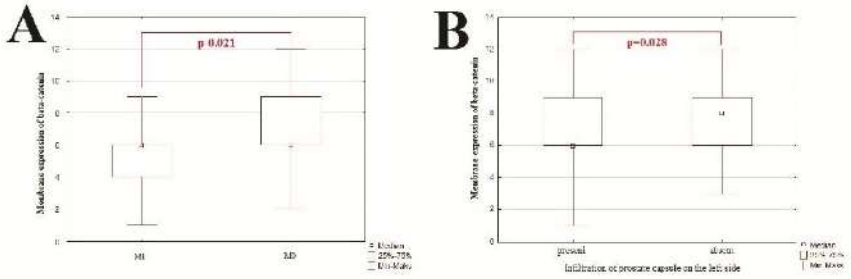

Fig 3. The graphic representation of results for membrane expression of β-catenin examined by IHC technique. The x-axis shows individual clinicopathological features and the y-axis shows IRS score values for IHC staining. The p-value <0.05 was considered as a statistically significant. Correlation between membrane expression of β-catenin and distant metastasis (A) and infiltration of prostate capsule on the left side (B).

https://doi.org/10.1371/journal.pone.0253112.g003

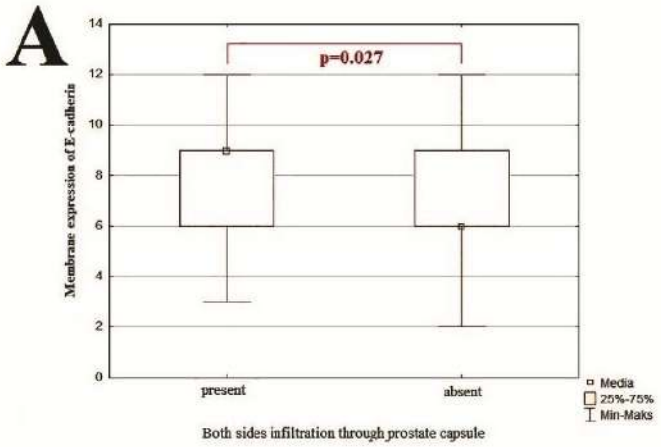

Fig 4. The graphic representation of results for membrane expression of E-cadherin examined by IHC technique. The x-axis shows individual clinicopathological features and the y-axis shows IRS score values for IHC staining. The p-value <0.05 was considered as a statistically significant. Correlation between membrane expression of E-cadherin and both sides infiltration of the prostate capsule (A).

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E-cadherin expression (r = -0.25, p<0.05) at low level, whereas tumor invasion of the left surface of the prostate gland was negatively associated with the membrane-located expression of E-cadherin (r = -0.26, p<0.05) at low level. Membrane expression of E-cadherin negatively corresponded with prostate weight (r = -0.23, p<0.05) at low level. In the specimens where tumor infiltrated the both, right and left side of the gland, we observed significant decrease of membrane E-cadherin expression level, than in specimens where tumor infiltrate only one side of gland (p = 0.019; Fig 1F). Furthermore, the cancer cells invasion of the prostate gland left area corresponded with the overall β-catenin expression at poor negative level (r = -0.33, p<0.05).

Immunohistochemical analysis of MIF and SOX-4 expression in PCa: Clinicopathological associations

We found a statistically significant increase in nuclear expression of MIF between Gleason score 7 and 8 (p = 0.039; Fig 1G). Moreover, we noted higher expression of nuclear MIF in

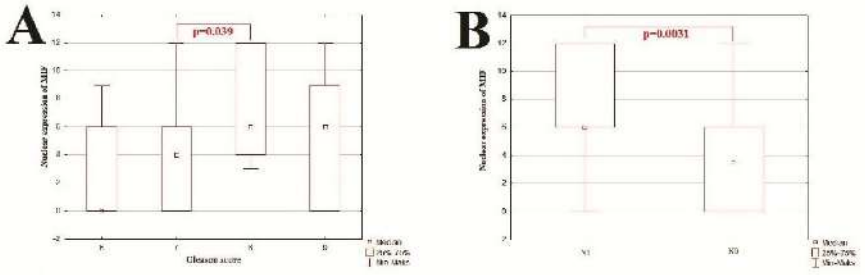

Fig 5. The graphic representation of results for nuclear expression of MIF examined by IHC technique. The x-axis shows individual clinicopathological features and the y-axis shows IRS score values for IHC staining. The p-value <0.05 was considered as a statistically significant. Correlation between nuclear expression of MIF and Gleason score (A) and presence of the lymph node metastasis (B).

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Table 3. Correlation between expression of β-catenin and SOX4 and clinicopathological features of prostate cancer.

C.F.		β-catenin												SOX4		
		gen			cyt			mem			nuc			nuc		
		Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3
G.p.	3+3	8	12	12	4	6	9	6	9	12	2	4	4	0	0	2
	3+4	12	12	12	4	6	9	6	6	9	3	4	4	2	3	4
	3+5	6	8	12	3	3	6	3	6	6	1	4	4	2	2	2
	4+3	8	8	12	3	3	6	6	6	6	2	4	4	2	2	4
	4+4	4	7	10.5	4	6	6	5	6	6	3	4	4	1	2	3
	4+5	8	12	12	4	6	6	4	6	8	1	4	4	2	2	4
	5+4	6	8	12	4	4	12	6	6	9	2	5	6	2	2	2
	p*	ns			ns			ns			ns			ns		
G.s.	6	8	12	12	4	6	9	6	9	12	2	4	4	0	0	2
	7	8	12	12	4	6	8	6	6	9	2	4	4	2	3	4
	8	6	8	12	3	6	6	4	6	6	2	4	4	2	2	3
	9	8	12	12	4	6	6	4	6	8	2	4	4	2	2	3
	p*	ns			ns			ns			ns			ns		
T	3	12	12	12	6	6	6	8	8	8	4	4	4	2	3	4
	3a	8	12	12	4	6	8	6	6	9	2	4	4	0	2	3
	3b	8	12	12	3	6	6	6	6	8	2	4	4	2	3	4
	4	7	10	12	4.5	6	9	6	7.5	9	1.5	3	5	2	2.5	3.5
	p*	ns			ns			ns			ns			ns		
N	0	8	12	12	4	6	6	6	6	9	2	4	4	2	2	4
	1	6	8	9	3	4	6	4	6	6	2	4	4	2	2	3
	p**	0.0004			ns			ns			ns			ns		
M	0	8	12	12	4	6	6	6	6	9	2	4	4	2	2	4
	1	8	9	12	4	6	6	4	6	6	1	4	4	0	2	3
	p**	ns			ns			0.021			ns			ns		
Ang	(-)	8	12	12	4	6	6	6	6	9	2	4	4	2	2	4
	(+)	6	8	12	3	4	6	6	6	9	2	4	4	2	2	2
	p**	0.012			ns			ns			ns			ns		
Plu	(-)	6	12	12	3	6	6	6	6	9	2	4	4	2	2	4
	(+)	6	8	12	3	5	8	6	6	9	2	4	4	2	2	3
	P**	0.027			ns			ns			ns			ns		

SOX4- Transcription factor SOX-4; gen- general expression; cyt- cytoplasmic expression; mem- membrane expression; nuc- nuclear expression; C.F.- Clinical Feature; G.p.- Gleason pattern; G.s.- Gleason score; T- T stage according to TNM classification; N- N stage according to TNM classification; M- M stage according to TNM classification; Ang- Presence of the angioinvasion; Plu- Presence of plugs of cancer cells in vessel, p-v- p-value; (+) -present; (-)-absent. Q1—the first quartile; M-Median; Q3—the third quartile; ns- non significant

* Kruskal-Wallis Test

** Mann–Whitney U test.

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samples with the presence of lymphovascular metastasis-N1 in comparison to samples without lymphovascular metastasis-N0 ($p = 0.003$; Fig 1H). We displayed that age has a negative association with nuclear expression of MIF ($r = -0.24$, $p<0.05$) at weak level. Also, in PCa with infiltration both right and left area of the gland, we revealed a significant increase in the cytoplasm-located MIF ($p = 0.011$) and nuclear-located MIF ($p = 0.044$), than in samples where tumor infiltrate only one side of the gland.

Additionally, we showed that SOX-4 has no correlation with any clinicopathological features and it is our unexpected result.

Table 4. Correlation between expression of MIF and E-cadherin and clinicopathological features of prostate cancer.

C.F.		MIF						E-cadherin											
		cyt			nuc			gen			mem			Cyt			nuc		
		Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3
G.p.	3+3	8	8	8	0	0	6	12	12	12	6	9	12	8	8	12	4	4	6
	3+4	8	12	12	0	4	6	12	12	12	6	9	9	8	12	12	6	6	6
	3+5	12	12	12	9	9	12	12	12	12	6	9	9	12	12	12	6	8	9
	4+3	8	12	12	0	4	6	8	12	12	6	9	9	8	12	12	4	6	6
	4+4	6	10	12	3	5	9	8	8	12	5	6	9	8.5	10.5	12	6	6	7.5
	4+5	8	12	12	0	6	9	12	12	12	6	6	9	8	8	9	4	6	8
	5+4	3	12	12	0	6	12	12	12	12	6	9	9	12	12	12	6	6	9
	p*		ns			ns			ns			ns			ns			ns	
G.s.	6	8	8	8	0	6	6	12	12	12	6	9	9	8	12	12	4	6	6
	7	8	12	12	0	4	6	12	12	12	6	9	9	8	12	12	4	6	6
	8	8	12	12	4	6	12	8	12	12	6	6	9	9	12	12	6	6	9
	9	8	12	12	0	6	9	12	12	12	6	6	9	8	9	12	6	6	8
	p*		ns			0.039			ns			ns			ns			ns	
T	3	8	8	8	0	0	0	12	12	12	9	10.5	12	6	7.5	9	6	6	6
	3a	8	10	12	0	4	6	12	12	12	6	8	9	8	12	12	4	6	8
	3b	8	12	12	0	6	9	9	12	12	6	9	9	8	12	12	4	6	6
	4	12	12	12	0	3	9	12	12	12	6	7.5	9	9	10.5	12	5	6	7
	p*		ns			ns			ns			ns			ns			ns	
N	0	8	12	12	0	3.5	6	12	12	12	6	9	9	8	12	12	4	6	6
	1	8	12	12	6	6	12	8	12	12	6	6	9	8	9	12	4	6	6
	p**		ns			0.003			ns			ns			ns			ns	
M	0	8	12	12	0	4	6	12	12	12	6	9	9	8	12	12	6	6	6
	1	6	12	12	3	6	9	8	12	12	6	6	9	8	9	9	4	4	6
	p**		ns			ns			ns			ns			ns			ns	
Ang	(-)	8	12	12	0	4	6	12	12	12	6	9	9	8	12	12	4	6	6
	(+)	6	8	12	0	6	9	8	12	12	6	9	9	8	12	12	6	6	6
	p**		ns			ns			ns			ns			ns			ns	
Plu	(-)	6	12	12	0	4	6	8	12	12	6	9	9	8	12	12	6	6	6
	(+)	6	10	12	0	6	9	8	12	12	6	9	9	8	12	12	6	6	6
	P**		ns			ns			ns			ns			ns			ns	

MIF- Macrophage migration inhibitory factor; gen- general expression; cyt- cytoplasmic expression; mem- membrane expression; nuc- nuclear expression; C.F.- Clinical Feature; G.p.- Gleason pattern; G.s.- Gleason score; T- T stage according to TNM classification; N- N stage according to TNM classification; M- M stage according to TNM classification; Ang- Presence of the angioinvasion; Plu- Presence of plugs of cancer cells in vessel, p-v- p-value; (+) -present; (-)–absent. Q1—the first quartile; M- Median; Q3—the third quartile; ns- non significant

* Kruskal-Wallis Test

** Mann–Whitney U test.

https://doi.org/10.1371/journal.pone.0253112.t004

The co-expressions between β-catenin, E-cadherin, SOX-4 and MIF proteins

The interactions between investigated proteins provide valuable information about the natures of the interacting proteins. Our protein co-expression analysis suggests novel insight into β-catenin-E-cadherin-SOX-4-MIF inter-factor dependence. Correlations between individual proteins were measured based on increasing or decreasing levels in various cell locations. The correlations between studied proteins were included in Fig 6.

Table 5. Correlations between β-catenin, SOX-4 and invasion area and histological grading of tumor tissue which has grown through the prostate.

C.F.			β-catenin												SOX4		
			gen			cyt			mem			nuc			nuc		
			Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3
oPC	R	(+)	8	12	12	4	6	6	6	6	9	2	4	4	2	2	3
		(-)	8	12	12	3	6	8	6	6	9	2	4	4	0	2	3
		p*		ns			ns			ns			ns			ns	
	L	(+)	8	12	12	3	6	6	6	6	9	2	4	4	2	2	3
		(-)	8	12	12	4	6	7	6	8	9	2	4	4	0	2	4
		p*		ns			ns			0.028			ns			ns	
	B	(+)	8	12	12	4	6	6	4	6	9	4	4	4	2	2	3
		(-)	8	12	12	3	6	8	6	6	9	2	4	4	0	2	3
		p*		ns			ns			ns			ns			ns	
PA	R	(+)	8	12	12	4	6	6	6	6	9	3	4	4	2	2	4
		(-)	8	8	12	3	4	6	6	6	6	2	4	4	0	2	3
		p*		ns			ns			ns			ns			ns	
	L	(+)	12	8	12	6	3	6	6	6	9	4	2	4	2	2	3
		(-)	12	12	12	6	4	8	8	6	9	4	4	4	2	2	4
		p*		ns			ns			ns			ss			ns	
	B	(+)	8	12	12	3	6	6	6	6	9	2	4	4	2	2	3
		(-)	8	12	12	4	6	6	6	6	9	2	4	4	0	2	4
		p*		ns			ns			ns			ns			ns	
SV	R	(+)	8	12	12	3	6	6	6	6	8	2	4	4	2	2.5	4
		(-)	8	12	12	4	6	6	6	6	9	2	4	4	0	2	3
		p*		ns			ns		ns		8		ns			ns	
	L	(+)	8	12	12	3	6	6	6	6	9	2	4	4	2	3	4
		(-)	8	12	12	4	6	8	6	6		2	4	4	0	2	3
		p*		ns			ns			ns			ns			ns	
	B	(+)	12	8	12	6	3	6	6	6	8	4	2	4	3	2	4
		(-)	12	8	12	6	4	6	6	6	9	4	3	4	2	0	3
		p*		ns			ns			ns			ns			ns	
G.p.*	3+3		8	12	12	4	6	8.5	6	8	10.5	2	4	4	2	2	4
	3+4		12	12	12	6	9	9	6	6	9	4	4	6	3	4	4
	4+3		10	12	12	4	5	7.5	6	6	7.5	3	4	4	0	1.5	5.5
	4+4		8	10.5	12	3	6	6	6	6	6	2	4	4	2	2	3
	4+5		8	12	12	3	6	6	2	4	8	0	4	4	0	2	3
	5+4		12	12	12	4	4	4	6	6	6	4	4	4	2	2	2
	5+5		8	8	10	3.5	4	6.5	4	6	6	1	3	4	1	2	3
	p*			ns			ns			ns			ns			ns	
G.s.*	6		8	12	12	4	6	8.5	6	8	10.5	2	4	4	2	2	4
	7		12	12	12	6	6	9	6	6	9	4	4	4	0	3	4
	8		8	10.5	12	3	6	6	6	6	6	2	4	4	2	2	3
	9		8	12	12	3.5	5	6	3	5	7	2	4	4	1	2	2.5
	10		8	8	10	3.5	4	6.5	4	6	6	1	3	4	1	2	3
	p*			ns			ns			ns			ns			ns	

SOX4- Transcription factor SOX-4; gen- general expression; cyt- cytoplasmic expression; mem- membrane expression; nuc- nuclear expression; Q1—the first quartile; M- Median; Q3—the third quartile, iA- invaded Area; oPC- outside the prostate capsule, PA- the apex of prostatic gland; SV- seminal vesicles; R- on the right side, L- on the left side; B- on the both sides p-v- p-value; (+) -invasion; (-)–without invasion.; ns- non significant; G.p.- Gleason pattern; G.s.- Gleason score; p-v- p-value; Q1—the first quartile; M- Median; Q3—the third quartile; ns- non significant

* Kruskal-Wallis Test.

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Table 6. Correlations between MIF and E-cadherin and invasion area and histological grading of tumor tissue which has grown through the prostate.

Features			MIF						E-cadherin											
			cyt			nuc			Gen			Mem			cyt			nuc		
			Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3	Q_1	M	Q_3
oPC	R	(+)	8	12	12	0	4	6	12	12	12	6	8.5	9	8	9	12	4	6	6
		(-)	8	8	12	0	3	9	12	12	12	6	9	9	8	12	12	4	6	6
		p*		ns			ns			ns			ns			ns			ns	
	L	(+)	8	12	12	0	4	9	12	12	12	6	9	9	8	12	12	4	6	6
		(-)	8	8	12	0	1.5	5	12	12	12	6	9	9	8	12	12	6	6	6
		p*		ns			ns			ns			ns			ns			ns	
	B	(+)	12	12	12	3	5	9	8	12	12	6	6	9	8	9	12	4	6	6
		(-)	8	8	12	0	3	6	12	12	12	6	9	9	8	12	12	6	6	6
		p*		0.022			ns			ns			0.027			ns			ns	
PA	R	(+)	8	12	12	0	4	6	12	12	12	6	9	9	8	12	12	4	6	6
		(-)	4	8	12	0	4	9	12	12	12	6	9	9	8	12	12	4	6	8
		p*		ns			ns			ns			ns			ns			ns	
	L	(+)	12	8	12	4	0	6	12	8	12	9	6	9	12	8	12	6	4	6
		(-)	8	8	12	3.5	0	9	12	12	12	9	6	12	12	8	12	6	6	6
		p*		ns			ns			ns			ns			ns			ns	
	B	(+)	8	12	12	0	4	6	8	12	12	6	9	9	8	12	12	4	6	6
		(-)	8	8	12	0	4	9	12	12	12	6	9	9	8	12	12	4	6	6
		p*		ns			ns			ns			ns			ns			ns	
SV	R	(+)	8	12	12	0	5	9	8	12	12	6	9	9	8	10,5	12	4	6	6
		(-)	8	12	12	0	4	6	12	12	12	6	8	9	8	12	12	4	6	6
		p*		ns			ns			ns			ns			ns			ns	
	L	(+)	8	12	12	0	4	6	9	12	12	6	9	9	9	9	12	6	8	9
		(-)	8	12	12	0	4	6	12	12	12	6	9	9	8	12	12	6	6	9
		p*		ns			ns			ns			ns			ns			ns	
	B	(+)	12	8	12	4	0	9	12	8	12	9	6	9	9	8	12	4	6	6
		(-)	12	8	12	4	0	6	12	12	12	9	6	9	12	8	12	4	6	6
		p*		ns			ns			ns			ns			ns			ns	
G.p.*	3+3		8	10	12	0	3.5	5	12	12	12	6	9	9	8	12	12	5	6	6
	3+4		8	12	12	0	0	4	12	12	12	8	9	12	8	12	12	4	6	6
	4+3		3	4	6	0	4.5	9	8	10	12	6	7.5	9	8	10	12	5	6	6
	4+4		8	12	12	3	5	9	8	12	12	6	9	9	8	12	12	4	6	6
	4+5		8	12	12	0	6	9	12	12	12	4	6	9	8	8	9	4	6	9
	5+4		12	12	12	6	6	6	12	12	12	6	6	6	12	12	12	9	9	9
	5+5		3.5	6	8	0	0	0	8	10	12	4.5	7	8.5	9	12	12	5	9	12
	p*			ns			ns			ns			ns			ns			ns	
G.s.*	6		8	10	12	0	3.5	5	12	12	12	6	9	9	8	12	12	5	6	6
	7		4	8	12	0	0	9	12	12	12	6	9	9	8	12	12	4	6	6
	8		8	12	12	3	5	9	8	12	12	6	9	9	8	12	12	4	6	6
	9		8	12	12	0	6	9	12	12	12	5	6	9	8	8	10.5	5	6	9
	10		3.5	6	8	0	0	0	8	10	12	4.5	7	8.5	9	12	12	5	9	12
	p*			ns			ns			ns			ns			ns			ns	

MIF- Macrophage migration inhibitory factor; gen- general expression; cyt- cytoplasmic expression; mem- membrane expression; nuc- nuclear expression; Q1—the first quartile; M- Median; Q3—the third quartile, iA- invaded Area; oPC- outside the prostate capsule, PA- the apex of prostatic gland; SV- seminal vesicles; R- on the right side, L- on the left side; B- on the both sides p-v- p-value; (+) -invasion; (-)–without invasion.; ns- non significant; G.p.- Gleason pattern; G.s.- Gleason score; p-v- p-value; Q1—the first quartile; M- Median; Q3—the third quartile; ns- non significant

* Kruskal-Wallis Test.

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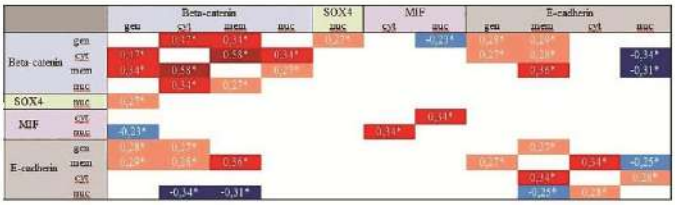

Fig 6. Correlation heat map of proteins expression data measured by immunohistochemistry. Spearman's rank correlation coefficient was calculated among 110 compilations of protein expression levels. The degrees of correlation are color-coded. The color key designates the correlation was statistically significant. As a statistically significant was considered p-value < 0.05. Red color shades indicate a positive proteins correlation, whereas blue color shades indicate negative proteins correlation. β-catenin, Transcription factor SOX-4, Macrophage migration inhibitory factor and E-cadherin were designated Beta-catenin, SOX-4, MIF, E-cadherin, respectively. Protein correlations are presented taking into account cellular localization. General, cytoplasmic, membrane and nuclear expressions were signed as gen, cyt, mem, nun, respectively.

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Regarding to the Spearman rank coefficient, the positive association was detected between the both overall β-catenin and E-cadherin expression ($r = 0.28$, $p<0.05$) and also between the both membrane expression level of β-catenin and E-cadherin ($r = 0.36$, $p<0.05$).

Next, we focused on checking the relation of SOX-4 transcription factor as well as MIF cytokine with other EMT-related proteins to show their possible impact on metastasis initiation. Consequently, we displayed a weak positive correlation between total β-catenin protein expression with level of nuclear SOX-4 protein ($r = 0.27$; $p<0.05$) and weak negative correlation of total β-catenin expression with level of nuclear MIF protein expression ($r = -0.23$), $p<0.05$). Accordingly, we supposed that MIF and SOX-4 factors may cooperate in acquiring invasive properties by tumor cells in prostate malignancies. However, we were unable to confirm immunohistochemically this relationship.

Discussion

Loss of cell-cell adhesion capacity permits the malignant cells to detach and leave the primary tumor location. In turn, changes in cell-matrix interaction allow the tumor cells to occupy the neighbouring stroma. Saha et al., have detected homogenous membrane expression of E-cadherin and β-catenin in benign prostatic hyperplasia (BPH) at similar frequency pattern, whereas in primary PCa, it was significantly reduced. Interestingly, a similar expression and frequency pattern of E-cadherin and β-catenin as in BPH was presented in invasive cells of PCa with bone metastasis [57]. Likewise, in our study we observed a particularly reduced E-cadherin and β-catenin membrane pattern in relation to Gleason pattern 4+5 or 5+4 and 4+5 or 5+5, respectively. Invasive cells that exceeded the capsule of the prostate gland exhibited higher membrane β-catenin pattern in areas with Gleason pattern 3+3 and lower expression in areas with Gleason pattern 4+4. The invasive potential of malignant cells is required to dissociate from the primary tumor cluster. Next, displaced tumor cells may invade surrounding or distant stroma and no longer require invasive phenotype. The enhanced β-catenin nuclear translocation may activate or deactivate specific targets and stimulate a transcription factors changing cell phenotype from epithelial into mesenchymal [9, 12]. In current study, the nuclear β-catenin expression remained at the constant level without any correlation with clinicopathological features. Interestingly, we observed that translocation of β-catenin to the nucleus was present primarily in tumor areas related to Gleason pattern 4+4. The increasing aggressiveness of tumors seems to be connected with the redistribution of beta-catenin in tumor cells. This event was related to the passage of membrane-located β-catenin to the

intracellular area of the tumor cells. We revealed that in our tumor samples there were only hotspots showing the transition of β-catenin from cell to cell boundaries to the cytoplasm. Probably, during the assessment of protein expression in individual sample we should focus on evaluating the several specific hotspots to standardize the results.

In most epithelial cancers the both E-cadherin and β-catenin repression is followed by transcriptional silencing provided by cooperating with EMT transcription factors (EMT-TFs). The SOX-4 may be one of the numerous EMT-TFs necessary for initiation of cytoskeletal rearrangements and supporting the multi-step metastasis process. Here, we report a weak correlation between the total expression of β-catenin and nuclear expression of SOX-4 protein ($r = 0.27$; $p<0.05$). Indeed, it appeared in evidence that β-catenin level was weak negative correlated with the nuclear MIF ($r = -0.23$, $p<0.05$). It demonstrated that MIF as a proinflammatory cytokine may actively participate in stimulating signal transduction pathways involved in EMT, controlling the acquisition of invasiveness features by tumor cells and facilitating metastasis processes. Previous studies have provided that E-cadherin, as same as β-catenin, effectively influences on enhancing tumor progression and has effect on converting to its metastatic form [58–60]. Chang et al. showed that metastasis of prostate cancer cells to lymph nodes expressed less E-cadherin level than primary PCa [61]. Multiple studies have linked loss of membrane E-cadherin in PCa associated with the acquisition of invasive properties [62]. Jaggi et al., in their semi-quantitative immunohistochemistry analysis, have shown the reduction of immunostaining for the both β-catenin and E-cadherin in PCa compared to normal glandular epithelium. The decrease of β-catenin and E-cadherin expressions were accompanied by rising tumor invasiveness [63, 64]. Moreover, Horvath et al. demonstrated that low level of nuclear β-catenin expression of the tumor cells in the early diagnosed PCa is a marker related with highly poor prognosis in patients, among who the prognosis seemed to be promising and those patients were classified into radical prostatectomy [65]. Admittedly, we did not find any correlations between E-cadherin and the presence of lymph nodes or distant metastasis, however it is indisputable that loss of E-cadherin is highly associated with metastasis capability in PCa. Otherwise, we revealed that reducing of the membranous E-cadherin in prostate epithelium may influence the enlargement of gland neoplasm. Some studies have demonstrated a direct interaction between SOX-4 and β-catenin [25, 66]. The SOX-4 transcription factor has been shown to enhance the cell proliferation in SW480 colon cancer cell lines through stabilization of β-catenin activated aberrant Wnt signaling pathways [25]. Our study suggested the expression level of β-catenin was probably activated by a transcription factor SOX-4, leading to changes in subcellular localization of β-catenin in PCa. Stimulation of β-catenin located in cell membrane via SOX-4 transcription factor may lead to translocation of β-catenin to cytoplasm or partially to the nucleus, initiating the cell phenotype transformation from epithelial to mesenchymal state. Hence, knockdown of endogenous SOX-4 notably decreases the migration and invasion ability of tumor cells in vitro, thereby leads to reverse of the EMT cascade by increasing E-cadherin [67]. Birdal et al. have demonstrated in the in vivo study that Sox4 deletion affects on decreasing of active β-catenin level [68]. It is consistent with our findings confirming that SOX-4 may be responsible for regulation of the β-catenin signaling pathway, by influencing on β-catenin in tumor cells. Makoto and colleagues have shown that SOX-4 has a positive impact on the β-catenin signal transduction through changes in TCF4 expression during the morular differentiation of endometrial carcinoma cells, thus providing the proliferation arrest [69]. There are a lot of publications showing the role of SOX-4 in promotion of EMT and confirming its strong involvement in acquisition of aggressiveness and invasiveness. Moreover, the SOX-4 transcription factor may be involved in controlling many issues of tumor expansion in different types of cancer. Many scholars have confirmed the participation of SOX-4 transcriptional factor in the PCa progression showing its

relationship with high Gleason score ($p = 0.009$) and the presence of distant metastasis ($p = 0.023$) [70]. The study on colon cancer, where the nuclear SOX-4 overexpression was closely corresponded with tumor invasion and distant metastasis [71], has been proved the crucial role of SOX-4 protein in tumor development and EMT initiation. Consequently, Liu P. et al. in their study on PCa cells also have shown that the both at the mRNA and protein level, SOX-4 was highly correlated with Gleason score [72]. Unexpectedly, our Spearman correlation analysis revealed no association between the SOX-4 protein and any clinicopathological features. We considered the discrepancies between results of current and previous studies as a consequence of dissimilarity of applied protein detection techniques, different antibody clones available to protein detection, IHC visualization kits and multifocal character of PCa. The high genomic diversity degree and morphological heterogeneity of prostate neoplasm make it difficult to obtain a repeatable results of immunohistochemical staining and microscopic analysis. A thin tissue cores may not be representative for whole cancer areas.

Our next aim was to investigate the role of inflammation in promotion of EMT in prostate adenocarcinoma. Recent studies focused on inflammation-induced epithelial cell injury have shown the fundamental role of inflammation in initiation and progression of prostate cancer [73]. MIF is one cytokine with T lymphocyte origin that presumably participates in an immune mechanism related to cancer microenvironment [74]. Activated T cells secrete a MIF factor that has impact on cell mitosis and initiate transformation of cells to malignant phenotype, thereby facilitating tumor progression [74, 75]. Meyer-Siegler and Hudson in their study have indicated that MIF excreted by metastatic cells may manage functioning of macrophages and secretion of cytokines [76]. Also, MIF molecules may participate in accumulation of macrophages related to PCa and affect on tumor maintenance [76]. MIF manifests its pro-inflammatory nature, showing high expression levels in tissues with chronic inflammation areas, such as hepatitis, gastritis and pancreatic [77]. It is worth to notice that many previous studies have shown increased MIF expression levels associated with benign prostate hyperplasia, induced by chronic conditions [78]. Nevertheless MIF has been found as a cytokine, strongly corresponded with prostate adenocarcinoma and disease progression.

MIF can activate EMT cascade through E-cadherin downregulation and N-cadherin overexpression, leading to form secondary site tumors [79]. Our study revealed that translocation of the MIF factor to the nucleus could enable β-catenin upregulation and activate the EMT process. Probably, stimulated and altered β-catenin expression, both total and membrane, inversely interacts with E-cadherin accumulated in the nucleus and consequently leads to a reduction in tumor cell-to-cell adhesion.

Funamizu et al. have used mice cells to show significant overexpressed MIF in progressive tumor growth compared to the control cells and thus highlighted the role of MIF in accelerating progression and metastasis of pancreatic ductal adenocarcinomas [39]. Our study exhibited that nuclear MIF expression is a strong predictor of lymph node metastasis, therefore MIF may act as a mediator, modulated to accelerate the tumor progression to its aggressive state. To the best of our knowledge, only present study focused the attention on examination of the both cytoplasmic and nuclear expression of MIF in PCa. Contrary to our results, suggesting that PCa patients with high nuclear MIF expression may be assigned a poor prognosis, Kamimura et al. have discovered that patients with lung cancer, without confirmed nuclear expression of MIF factor in tumor cells, had poorer prognosis than cases with confirmed MIF nuclear expression [50]. Similarly to our findings, Ren et al. have revealed that rMIF fraction contributes the invasion and migration of HCC cells by an in vitro cell migration assay [80]. Additionally, in prostate neoplasm, MIF factor has been found precisely corresponding with tumor progression and metastasis [76]. Lately, MIF has been described as a novel therapeutic target against metastatic triple-negative breast cancer (TNBC). MIF inhibitor CPSI-1306 may

silence TNBC growth and metastasis through activating apoptosis [81]. Taken as a whole, current and previous results seem to indicate that MIF molecule might be an important checkpoint inducing transformation into an aggressive and metastatic form of cancer. Interestingly, MIF is a part of several clinical trials. One of this trials was phase III interventional study with attendance of 60 PCa participants, titled "A Double-Blind, Randomised, Placebo-Controlled Study of the Effect of Transdermal Nitroglycerin (Glyceryl Trinitrate; GTN) Therapy on Biomarkers of Immune Escape in Men With Biochemical Recurrence of Prostate Cancer After Primary Therapy" (ClinicalTrials.gov Identifier: NCT01704274). For evaluating the effect of intervention with Glyceryl Trinitrate doses as a primary outcome measures was applied inter alia changes of MIF biomarker level.

Vecchio and colleagues have indicated the existence of association between level of MIF factor and cellular differentiation in untreated PCa, showing that MIF expression was stronger in low-grade adenocarcinoma (GS≤6) than in high-grade adenocarcinoma (GS≥7) [82]. In another study by Chen et al., specimens with a Gleason score of 7 was more frequently present enhanced MIF expression than the specimens with a Gleason score of 6 [83]. We showed that high expression of nuclear MIF was more likely associated in prostate tumor areas with a Gleason score of 8 than in those with a Gleason score of 7. Thus, our results support the hypothesis that nuclear MIF overexpression may predict poor prognosis for PCa patients.

Moreover, we found that upregulation of the cytoplasmic MIF, produced by the prostatic cancer epithelium, participates in the infiltrating of both the right and left zone of prostate cancer gland. Verjans et al., have shown MIF expression as significantly associated with tumor size in breast cancer ($p = 0.007$), where size of tumor above 2 cm corresponded with cancer progression and manifested in low MIF expression with IRS score less than 4 [43]. Admittedly, we did not confirm association between tumor size and MIF deregulation, however we highlighted its role in tumor growth. Meyer-Siegler et al. using more advanced molecular techniques, like Slot blot analysis of RNA in PCa, have demonstrated that elevated MIF expression is strongly corresponded with mesenchymal phenotype of prostate disease. They have shown that MIF secretes by PCa cells may play an important role in the multi-step process of metastatic cascade [76]. The enhanced immunostaining of MIF protein in prostate malignancies and its correlation with lymphovascular metastases suggested that MIF may contribute to the acceleration of PCa progression through involvement in the initiation of lymph node metastases. Bando et al., have found significant negative dependence between deregulation of MIF factor related to tumor microenvironment, detected by ELISA test and presence of nodal metastasis in breast cancer [45]. Our results are also consistent with Pei et al.' study of MIF and DJ-1 protein in Nasopharyngeal Carcinoma (NPC). They have shown that high expression of MIF protein was significantly correlated with advanced clinical stage, nodal metastasis and poor prognosis, thus had influence on initiation of cell invasion and metastasis in NPC [84].

Translocation of MIF from cytoplasm to the nucleus may play a critical role in PCa progression and seems to be important for tumor cell growth and invasion. Nuclear redistribution of MIF factors may interact with deregulation of transcription factors, such as SOX-4 leading to facilitate the process of EMT. In our study, we did not find any correlations between SOX-4 and MIF proteins, that is inconsistent with our hypothesis, in which we assumed that SOX-4 activity could be controlled by upregulation of MIF during prostate tumor progression. However, the immunohistochemical analysis in this study is insufficient to confirm this relationship. Using more advanced molecular tools, we could get satisfying results. A study on correlation between MIF and SOX-4 to check the potential role of SOX-4 as a downstream target of MIF overexpression in PCa has not been performed yet. Nevertheless, we have found the study with other SOX family members and MIF factors. In the study performed by Yuan et al., using among others the luciferase reporter assay in a 293T cell line and chromatin

immunoprecipitation (ChIP)-PCR assay, it has shown that overexpression of MIF is responsible for transcriptional activity of SOX-9 during the chondrogenesis and osteogenesis, [85]. Moreover, Shigeki et al. have shown that MIF molecules regulate the Sox6 expression in mouse NSPCs via the interaction with STAT3 molecules [86]. However, our study did not confirm that MIF factor may impact on the activity of SOX-4 protein. We did not find any significant association between the both nuclear or cytoplasmic MIF expression and nuclear expression of SOX-4 protein. We want to point out that the study at alterations in protein levels using immunohistochemistry method do not always correlate with the results reached by other techniques such as cell culture or molecular techniques.

Conclusion

Many previously carried out studies as well as the current research emphasize the significant role of β-catenin, SOX-4, MIF and E-cadherin in malignant transformation of prostate tumor cells and confirmed their attendance in one of the initial metastasis steps—epithelial–mesenchymal transition (EMT). Immunohistochemical study may not provide the final evidence that SOX-4 is not a downstream target of MIF, therefore definitive conclusions cannot be drawn due to insufficient knowledge. Advanced studies using molecular biology techniques are required to confirm the direct or indirect protein-protein interactions. At this stage, we can corroborate protein inter-relationships based on expression patterns of studied factors and outline the direction for further research. However, our results seem promising and strongly highlight the potential role of MIF as a treatment option in metastatic PCa. The MIF targeting could help to reverse disease progression at both early and advanced stages in future.

Supporting information

S1 Table. The additional values of significant correlations between protein expression and clinicopathological features.
(DOCX)

S2 Table. Comparison of significant values for Mann-Whitney U test.
(DOCX)

Author Contributions

Conceptualization: Martyna Parol-Kulczyk.

Data curation: Martyna Parol-Kulczyk.

Formal analysis: Arkadiusz Gzil, Joanna Maciejewska.

Investigation: Martyna Parol-Kulczyk.

Methodology: Martyna Parol-Kulczyk.

Project administration: Martyna Parol-Kulczyk.

Resources: Martyna Parol-Kulczyk.

Supervision: Dariusz Grzanka.

Validation: Martyna Parol-Kulczyk, Magdalena Bodnar.

Visualization: Martyna Parol-Kulczyk, Arkadiusz Gzil.

Writing – original draft: Martyna Parol-Kulczyk.

Writing – review & editing: Martyna Parol-Kulczyk, Arkadiusz Gzil.

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6. Podsumowanie

Zjawisko EMT jest wielotorowym programem z licznymi czynnikami regulacyjnymi, odpowiedzialnymi za konkretne komórkowe szlaki sygnałowe. Czynniki te współgrając ze sobą, tworzą sieć komunikacyjną, dzięki której dochodzi do progresji choroby nowotworowej. Nowoczesny model rozprzestrzeniania się komórek nowotworowych do węzłów chłonnych, czy narządów odległych pokazuje, że patologie przerzutowe są określone unikalnym zestawem zmienionych czynników specyficzno-tkankowych prowadzących do aktywacji procesu EMT i zapoczątkowania kaskady inwazyjno-przerzutowej u pacjentów z rakiem prostaty. W ogniskach wtórnych komórki nowotworowe zmieniają swój fenotyp na epitelialny w wyniku procesu odwrotnego, czyli MET. Deregulacja specyficznych czynników określających fenotyp mezenchymalny migrujących komórek jest procesem przejściowym do momentu powrotu tych komórek do stanu pierwotnego, aby utworzyć ogniska przerzutowe. Jest to dowód na to, że zmiany ekspresji czynników biorących udział w przejściu epitelialno-mezenchymalnych są zmienne i na podstawie tychże zmian można określać stopień zaawansowania choroby i oceniać rokowanie pacjentów. Dążenie do skutecznego leczenia przerzutowej postaci raka gruczołu krokowego powinno zatem koncentrować się na wyciszeniu lub zrównoważeniu rozregulowanej ekspresji czynników związanych z EMT, aby odwrócić jej funkcje do stanu wyjściowego. Dlatego też dalsze badania naukowe należy ukierunkować na wieloaspektową analizę czynników odpowiedzialnych za regulację procesu EMT, znaczenie kluczowych już w pierwszych etapach inicjacji nowotworowej. Dalszy postęp w obszarze transformacji komórek nabłonka w komórki mezenchymalne u pacjentów chorych na raka prostaty ułatwi przełożenie tego osiągnięcia na możliwość wdrożenia zaawansowanej diagnostyki onkologicznej, prognozowania choroby na wczesnym etapie i leczenia ukierunkowanego na ustabilizowanie poziomów miRNA i mRNA/białek. Zastosowanie miRNA lub mRNA/białek jako biomarkerów lub bezpośrednich targetów farmakologicznych może zmienić podejście do leczenia pacjentów z przerzutowym rakiem gruczołu krokowego i przynieść wymierne korzyści lecznicze.

7. Wnioski

1. Zmienione poziomy ekspresji określonych zestawów cząsteczek miRNA mogą stanowić potencjalne czynniki prognozujące progresję raka prostaty do stadium systemowego oraz być użyteczne w różnicowaniu rodzajów przerzutu.

2. Wysoki poziom ekspresji frakcji jądrowej białka MIF może być potencjalnym czynnikiem prognozującym zaawansowanie choroby u pacjentów z rakiem prostaty.

3. Niski poziom ekspresji frakcji podścieliskowej białka SDF-1 oraz jego receptorów wiążących: frakcji błonowej białka CXCR4 oraz frakcji jądrowo-cytoplazmatycznej białka CXCR7 mogą być niezależnymi czynnikami prognozującymi progresję choroby do stadium systemowego u pacjentów z rakiem prostaty .

4. Określanie poziomów ekspresji cząsteczek miRNA oraz białek u pacjentów chorych na raka prostaty, w zestawieniu ze standardowo stosowanymi markerami nowotworowymi, może stanowić wartościowe narzędzie prognostyczne lub posłużyć usprawnieniu procesu klasyfikacji stopnia zaawansowania tejże choroby.

8. Piśmiennictwo

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9. Oświadczenia współautorów publikacji naukowych włączonych do rozprawy doktorskiej

Bydgoszcz, 24.05.2023 r.

prof. dr hab. n. med. i n. o zdr. Dariusz Grzanka
Kierownik Katedry Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

OŚWIADCZENIE

Jako współautor pracy:

Parol, M., Gzil, A., Bodnar, M., & Grzanka, D. (2021). Systematic review and meta-analysis of the prognostic significance of microRNAs related to metastatic and EMT process among prostate cancer patients. In *Journal of Translational Medicine*. https://doi.org/10.1186/s12967-020-02644-x

Oświadczam, że mój wkład merytoryczny wniesiony do niniejszej publikacji naukowej to:

- nadzór nad prowadzonymi badaniami naukowymi i tworzeniem manuskryptu
- akceptacja ostatecznej wersji manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy naukowej przez mgr Martynę Parol-Kulczyk jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

.....
Podpis współautora

Bydgoszcz, 24.05.2023 r.

prof. dr hab. n. med. i n. o zdr. Dariusz Grzanka
Kierownik Katedry Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

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.....
Podpis współautora

Bydgoszcz, 24.05.2023 r.

prof. dr hab. n. med. i n. o zdr. Dariusz Grzanka
Kierownik Katedry Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

OŚWIADCZENIE

Jako współautor pracy:

Parol-Kulczyk, M., Gzil, A., Maciejewska, J., Bodnar, M., & Grzanka, D. (2021). Clinicopathological significance of the EMT-related proteins and their interrelationships in prostate cancer. An immunohistochemical study. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0253112

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.....
Podpis współautora

Bydgoszcz, 24.05.2023 r.

lek. Arkadiusz Gzil
lekarz
Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

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Oświadczam, że mój wkład merytoryczny wniesiony do niniejszej publikacji naukowej to:

- udział w tworzeniu manuskryptu
- opracowanie statystyczne uzyskanych wyników badań
- akceptacja ostatecznej wersji manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy naukowej przez mgr Martynę Parol-Kulczyk jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

A. Gzil

Podpis współautora

Bydgoszcz, 24.05.2023 r.

lek. Arkadiusz Gzil
lekarz
Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

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A. Gzil

Podpis współautora

Bydgoszcz, 24.05.2023 r.

lek. Arkadiusz Gzil
lekarz
Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

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A. Gzil
.....
Podpis współautora

Bydgoszcz, 24.05.2023 r.

dr n. med. Joanna Maciejewska
specjalista patomorfolog
Centrum Onkologii
ul Izabeli Romanowskiej 2
85-796 Bydgoszcz

OŚWIADCZENIE

Jako współautor pracy:

Parol-Kulczyk, M., Gzil, A., Maciejewska, J., Bodnar, M., & Grzanka, D. (2021). Clinicopathological significance of the EMTrelated proteins and their interrelationships in prostate cancer. An immunohistochemical study. *PLoS ONE.* https://doi.org/10.1371/journal.pone.0253112

Oświadczam, że mój wkład merytoryczny wniesiony do niniejszej publikacji naukowej to:

- Ocena morfometryczna preparatów mikroskopowych barwionych immunohistochemicznie

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy naukowej przez mgr Martynę Parol-Kulczyk jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Joanna Maciejewska
Podpis współautora

Bydgoszcz, 24.05.2023 r.

dr hab. n. med. Magdalena Bodnar, prof. UMK
profesor uniwersytetu
Katedra Położnictwa, Chorób Kobiecych i Ginekologii Onkologicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

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Oświadczam, że mój wkład merytoryczny wniesiony do niniejszej publikacji naukowej to:

- Korekta merytoryczna manuskryptu
- wniesienie krytycznych uwag do manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy naukowej przez mgr Martynę Parol-Kulczyk jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Podpis współautora

Bydgoszcz, 24.05.2023 r.

dr hab. n. med. Magdalena Bodnar, prof. UMK
profesor uniwersytetu
Katedra Położnictwa, Chorób Kobiecych i Ginekologii Onkologicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

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- Korekta merytoryczna manuskryptu
- wniesienie krytycznych uwag do manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy naukowej przez mgr Martynę Parol-Kulczyk jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

.....
Podpis współautora

Bydgoszcz, 24.05.2023 r.

dr n. med. Joanna Ligmanowska
adiunkt
Katedra Patofizjologii
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

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Jako współautor pracy:

Parol-Kulczyk, M., Gzil, A., Ligmanowska, J., & Grzanka, D. (2022). Prognostic significance of SDF-1 chemokine and its receptors CXCR4 and CXCR7 involved in EMT of prostate cancer. *Cytokine*. https://doi.org/10.1016/j.cyto.2021.155778

Oświadczam, że mój wkład merytoryczny wniesiony do niniejszej publikacji naukowej to:

- udział w optymalizacji i przeprowadzeniu reakcji immunohistochemicznych

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy naukowej przez mgr Martynę Parol-Kulczyk jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Ligmanowska
.....
Podpis współautora

10. Zgoda Komisji Bioetycznej na przeprowadzenie badań naukowych

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

KB 248/2019 Bydgoszcz, 26.02.2019 r.

Działając na podstawie art.29 Ustawy z dnia 5 grudnia 1996 roku o zawodzie lekarza (Dz.U. z 1997 r. Nr 28 poz. 152 (wraz z późniejszymi zmianami), zarządzenia Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych (Dz.U.Nr 47 poz.480) oraz Zarządzeniem Nr 21 Rektora UMK z dnia 4 marca 2009 r. z późn. zm. w sprawie powołania oraz zasad działania Komisji Bioetycznej Uniwersytetu Mikołaja Kopernika w Toruniu przy Collegium Medicum im Ludwika Rydygiera w Bydgoszczy oraz zgodnie z zasadami zawartymi w ICH – GCP

Komisja Bioetyczna przy UMK w Toruniu, Collegium Medicum w Bydgoszczy

(skład podano w załączeniu), na posiedzeniu w dniu **26.02.2019 r.** przeanalizowała wniosek, który złożył kierownik badania:

dr hab. n. med. Dariusz Grzanka
Katedra i Zakład Patomorfologii Klinicznej
Collegium Medicum w Bydgoszczy

z zespołem w składzie

- dr hab. n. med. Dariusz Grzanka, mgr Martyna Parol, dr n. med. Joanna Maciejewska, asystent, dr n. med. Anna Klimaszewska-Wiśniewska, Arkadiusz Gzil

w sprawie badania:

„Nowe markery w prognozowaniu przebiegu klinicznego nowotworów prostaty."

Po zapoznaniu się ze złożonym wnioskiem i w wyniku przeprowadzonej dyskusji oraz głosowania Komisja podjęła:

Uchwałę o pozytywnym zaopiniowaniu wniosku

w sprawie przeprowadzenia badań w zakresie określonym we wniosku pod warunkiem uzyskania zgody osób badanych na wykorzystanie materiału biologicznego oraz dokumentacji medycznej do celów naukowych, a w przypadku braku takiej zgody, analizowania jedynie materiału zanonimizowanego tj. pozbawionego danych personalnych (zgodnie z RODO). Zgoda obejmuje tylko materiał pobrany od pacjentów w okresie do 31.12.2017r.

Zgoda obowiązuje od daty posiedzenia (26.02.2019 r.) do końca 2021 r.

Wydana opinia dotyczy tylko rozpatrywanego wniosku z uwzględnieniem przedstawionego projektu: każda zmiana i modyfikacja wymaga uzyskania odrębnej opinii

Prof. dr hab. med. Karol Śliwka

Przewodniczący Komisji Bioetycznej

Otrzymuje:
dr hab. n. med. Dariusz Grzanka
Katedra i Zakład Patomorfologii Klinicznej
Collegium Medicum w Bydgoszczy

Lista obecności

na posiedzeniu Komisji Bioetycznej

w dniu 26.02.2019 r.

Lp.	Imię i nazwisko	Funkcja	Podpis
1.	Prof. dr hab. med. Karol Śliwka	Przewodniczący	
2.	Mgr prawa Joanna Połetek-Żygas	Z – ca przewodniczącego	
3.	Prof. dr hab. med. Mieczysława Czerwionka-Szaflarska		
4.	Prof. dr hab. med. Anna Balcar-Boroń		
5.	Prof. dr hab. med. Marek Grabiec		
6.	Prof. dr hab. med. Zbigniew Włodarczyk		
7.	Dr hab. n. med. Katarzyna Pawlak-Osińska, prof. UMK		
8.	Dr hab. n med. Maria Kłopocka		
9.	Ks. dr hab. Wojciech Szukalski, prof. UAM		
10.	Dr n. med. Radosława Staszak-Kowalska		
11.	Mgr prawa Patrycja Brzezicka		
12.	Mgr farm. Aleksandra Adamczyk		
13.	Mgr Lidia Iwińska-Tarczykowska		