UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

**Katedra Morfologii i Embriologii Człowieka**

**Zakład Histologii i Embriologii**

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# *Rola białka RBMS 3 w raku gruczołu piersiowego*

ROZPRAWA DOKTORSKA

Cykl publikacji powiązanych tematycznie

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Wrocław, 2024

Badania do publikacji, Górnicki T, Lambrinow J, Mrozowska M, Krawczyńska K, Staszko N, Kmiecik A, Piotrowska A, Gomułkiewicz A, Romanowicz H, Smolarz B, Podhorska-Okołów M, Grzegrzółka J, Rusak A, Dzięgiel P, Impact of RBMS 3 Progression on Expression of EMT Markers. Cells 2024, Sep 13, 1548, DOI: 10.3390/cells13181548, zostały sfinansowane w ramach programu MNiSW „SKN Tworzą Innowacje" nr grantu: SKN/SP/569116/2023

*Pragnę serdecznie podziękować Promotorowi rozprawy, Profesorowi Piotrowi Dzięgielowi, za nieustające wsparcie i opiekę podczas całej mojej dotychczasowej ścieżki naukowej. Za bycie mentorem oraz wzorem wybitnego naukowca.*

*Pragnę również podziękować Promotor pomocniczej rozprawy, Dr inż. Agnieszce Rusak, za nieocenioną pomoc w trakcie prowadzenia badań. Wsparcie merytoryczne oraz dobre słowo.*

*Podziękowania pragnę także złożyć na ręce Profesor Marzenny Podhorskiej-Okołów, za zawsze otwarte drzwi gabinetu i wsparcie w trudach działalności naukowej.*

*Chciałbym także serdecznie podziękować całemu Zespołowi Zakładu Histologii i Embriologii Uniwersytetu Medycznego we Wrocławiu, za przyjęcie do tak wspaniałego zespołu pełnego wybitnych specjalistów. W szczególności pragnę podziękować dr Jędrzejowi Grzegrzółce, za wprowadzenie mnie w świat nauki, mgr inż. Monice Mrozowskiej, za dzielenie trudów pracy w laboratorium oraz szczególny wkład w rozwój mojego przygotowania technicznego, mgr Klaudii Krawczyńskiej, za miliony wspólnie wyhodowanych komórek, a także dr Aleksandrze Piotrowskiej, za cenne rady.*

*Pragnę także podziękować wszystkim moim przyjaciołom, lek. Kacprowi Bułdysiowi, lek. Jakubowi Lambrinow, Panu Damianowi Haczyńskiemu, lek. Mateuszowi Mendowskiemu, lek. wet. Dawidowi Iwańskiemu, lek. wet. Rafałowi Grabce oraz inż. Michałowi Andrzejakowi, którzy wspierali mnie w trakcie tego wymagającego projektu.*

*W szczególny sposób chciałbym podziękować moim Rodzicom, Stanisławie i Henrykowi za zaufanie oraz troskę. Dzięki wam miałem możliwość spełniać marzenia oraz móc podejmować odważne życiowe decyzje. Nieocenione było także wsparcie mojej wspaniałej Narzeczonej Ewy, bez której wsparcia nie doszedłbym tak daleko. Dziękuję za cierpliwość i wyrozumiałość.*

# Spis Treści

# 1. CYKL PRAC STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ

1. Górnicki T, Lambrinow J, Mrozowska M, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J. Role of RBMS3 Novel Potential Regulator of the EMT Phenomenon in Physiological and Pathological Processes. Int J Mol Sci. 2022 Sep 17;23(18):10875

    - Cyfrowy identyfikator dokumentu elektronicznego (digital object identifier, DOI): 10.3390/ijms231810875.
    - **Współczynnik wpływu (Impact Factor, IF): 5.6**
    - **Punktacja Ministerstwa Nauki i Szkolnictwa Wyższego (MNiSW): 140**

2. Górnicki T, Lambrinow J, Mrozowska M, Romanowicz H, Smolarz B, Piotrowska A, Gomułkiewicz A, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J. Expression of RBMS3 in Breast Cancer Progression. Int J Mol Sci. 2023 Feb 2;24(3):2866.

    - DOI: 10.3390/ijms24032866.
    - **Współczynnik wpływu (Impact Factor, IF): 4.9**
    - **Punktacja MNiSW: 140**

3. Górnicki T, Lambrinow J, Mrozowska M, Krawczyńska K, Staszko N, Kmiecik A, Piotrowska A, Gomułkiewicz A, Romanowicz H, Smolarz B, Podhorska-Okołów M, Grzegrzółka J, Rusak A, Dzięgiel P, Impact of RBMS 3 Progression on Expression of EMT Markers. Cells 2024, Sep 13, 1548.

    - DOI: 10.3390/cells13181548
    - **Współczynnik wpływu (Impact Factor, IF): 5.1**
    - **Punktacja MNiSW: 140**

**Sumaryczny IF: 15.6**

**Sumaryczna Punktacja MNiSW: 420**

## 2. WYKAZ STOSOWANYCH SKRÓTÓW

| | |
|---|---|
| BC | rak gruczołu piersiowego (ang. *breast cancer)* |
| CDH1 | gen dla E-kadheryny |
| CDH2 | gen dla N-kadheryny |
| E-CAD | E-kadheryna (ang. *epithelial cadherin*) |
| EMT | przejście nabłonkowo-mezenchymalne (ang. *epithelial-mesenchymal transition*) |
| EOC | nabłonkowy rak jajnika (ang. *epithelial ovarian cancer*,) |
| HER-2 | receptor ludzkiego nabłonkowego czynnika wzrostu 2 (ang. *human epidermal growth factor receptor 2*) |
| IDC | inwazyjny rak gruczołu piersiowego (ang. *invasive ductal breast cancer*) |
| MEG3 | ang. *maternally expressed 3* |
| miRNA | mikro RNA |
| MMP2 | ang. *matrix metalloproteinase-2* |
| MMP9 | ang. *matrix metalloproteinase-9* |
| MSSPs | *c-Myc* gene single-strand binding proteins |
| N-CAD | N-kadheryna (ang. *neural cadherin*) |
| NPC | rak jamy nosowo-gardłowej (ang. *nasopharyngeal cancer*) |
| RT-qPCR | ilościowa reakcja PCR w czasie rzeczywistym (ang. *quantitative real time PCR*) |
| RBMS 3 | Białko wiążące RNA z motywem oddziałowującym z jednoniciowymi kwasami nukleinowymi 3, ang. *RNA-binding motif single-stranded-interacting protein 3* |
| SLUG | ang. *snail family transcriptional repressor 2* |
| SNAIL | ang. *snail family transcriptional repressor 1* |
| TNBC | potrójnie negatywny rak gruczołu piersiowego (ang.*triple negative breast cancer*) |
| TWIST 1 | ang. *twist-related protein 1* |
| WB | metoda Western Blot |
| ZEB 1 | ang. *Zinc finger E-box-binding homeobox 1* |

# 3. STRESZCZENIE W JĘZYKU POLSKIM

Według ostatnich statystyk, rak gruczołu piersiowego jest drugim najczęściej diagnozowany nowotworem na świecie, odpowiadając za prawie 12% wszystkich nowych przypadków nowotworów. W związku z tak wysokim wskaźnikiem zachorowalności, rak gruczołu piersiowego stanowi nie tylko poważny globalny problem zdrowotny, ale także duże wyzwanie ekonomiczne. Pomimo znacznego postępu w zakresie diagnostyki oraz leczenia raka gruczołu piersiowego w przypadku wyższych stopni zaawansowania nowotworu, w których obecne są już przerzuty odległe przeżywalność 5-letnia wynosi zaledwie 30%. Wskazuje to na ciągle istniejącą potrzebę poszukiwania nowych biomarkerów diagnostycznych oraz celów terapeutycznych mających na celu wzrost jakości procesu diagnostycznego oraz leczniczego.

Białko RBMS 3 (Białko wiążące RNA z motywem oddziałowującym z jednoniciowymi kwasami nukleinowymi 3, ang. RNA-binding motif single-stranded-interacting protein), jest bogatym w glicynę białkiem należącym do rodziny białek c-Myc (gene single-strand binding proteins MSSPs), opisanym po raz pierwszy w 2000 roku. Ekspresja RBMS 3, wiązana jest z wieloma procesami fizjologicznymi, takimi jak regulacja rozwoju embrionalnego trzustki oraz układu nerwowego, a także patologicznymi, między innymi marskości wątroby. Badania ostatnich lat wskazują dodatkowo na potencjalną rolę RBMS 3 jako markera prognostycznego oraz czynnika regulującego proces kancerogenezy w różnych typach nowotworów litych, takich jak rak jajnika, rak jamy nosowo-gardłowej czy rak żołądka. Dotychczas opublikowane, nieliczne dane literaturowe wykazały potencjalny związek pomiędzy ekspresją białka RBMS 3 a progresją raka gruczołu piersiowego.

Celem projektu stanowiącego przedmiot mojej rozprawy doktorskiej pt. „Rola białka RBMS 3 w raku gruczołu piersiowego" było określenie znaczenia poziomu ekspresji białka RBMS 3 w procesach uczestniczących w progresji raka gruczołu piersiowego, a także jego rokowniczego znaczenia.

Pierwsza publikacja wchodząca w skład cyklu (Górnicki T, Lambrinow J, Mrozowska M, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J. Role of RBMS3 Novel Potential Regulator of the EMT Phenomenon in Physiological and Pathological Processes. Int J Mol Sci. 2022 Sep 17;23(18):10875. DOI: 10.3390/ijms231810875.) jest pracą poglądową, mającą na celu usystematyzowanie aktualnego stanu wiedzy dotyczącego roli białka RBMS 3 w procesach

fizjologicznych oraz patologicznych, ze szczególnym uwzględnieniem inicjacji i progresji nowotworów litych.

Druga publikacja stanowi oryginalny artykuł badawczy (Górnicki T, Lambrinow J, Mrozowska M, Romanowicz H, Smolarz B, Piotrowska A, Gomułkiewicz A, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J. Expression of RBMS3 in Breast Cancer Progression. Int J Mol Sci. 2023 Feb 2;24(3):2866. DOI: 10.3390/ijms24032866.), w którym przedstawiono wyniki badań z wykorzystaniem reakcji immunohistochemicznych przeprowadzonych na materiale klinicznym pochodzącym od 490 pacjentek, z rozpoznaniem inwazyjnego przewodowego raka gruczołu piersiowego. W publikacji przedstawiono także analizę dostępnych baz danych w zakresie przeżycia całkowitego pacjentów z wykorzystaniem narzędzia „Kaplan-Meier Plotter". Opublikowane wyniki uwzględniały również analizy molekularne przy użyciu metod immunoblottingu (ang. western blot, WB) oraz ilościowej reakcji łańcuchowej polimerazy w czasie rzeczywistym (ang. quantitative real time PCR, RT-qPCR), przeprowadzonych na komercyjnie dostępnych liniach komórkowych raka gruczołu piersiowego, reprezentujących podstawowe podtypy molekularne tego nowotworu. Rezultaty przeprowadzonych badań pokazały istotną statystycznie niższą ekspresję RBMS 3 w przypadkach raka w porównaniu do przypadków kontrolnych mastopatii. Ekspresja RBMS 3 w komórkach nowotworowych była pozytywnie skorelowana z dodatnim statusem receptora HER-2 oraz receptora estrogenowego. Ponadto, ekspresja RBMS 3 wykryta została także, w podścielisku raka gruczołu piersiowego. Analiza statystyczna wyników wykazała, iż poziom ekspresji RBMS 3 był istotnie wyższy w podścielisku niż w komórkach raka oraz w stosunku do przypadków kontrolnych. Co więcej zaobserwowano negatywną korelację ekspresji RBMS 3 w podścielisku w przypadkach potrójnie negatywnego raka gruczołu piersiowego (ang. triple negative breast cancer, TNBC), w stosunku do pozostałych grup oraz dodatnią korelację z ekspresją receptora estrogenowego oraz progesteronowego. Analiza danych klinicznych oraz internetowych baz danych wykazała pozytywną korelację, między ekspresją RBMS 3, a całkowitym czasem przeżycia. In vitro analiza ekspresji RBMS 3, w wybranych liniach komórkowych na poziomie mRNA oraz białka wykazała istotnie wyższy poziom białka RBMS 3 w najbardziej agresywnych podtypach raka gruczołu piersiowego: HER-2 dodatnim oraz potrójnie negatywnym.

Ostatnia publikacja wchodząca w skład cyklu (Górnicki T, Lambrinow J, Mrozowska M, Krawczyńska K, Staszko N, Kmiecik A, Piotrowska A, Gomułkiewicz A, Romanowicz H, Smolarz B, Podhorska-Okołów M, Grzegrzółka J, Rusak A, Grzegrzółka J, Impact of RBMS 3

Progression on Expression of EMT Markers. Cells 2024, Sep 13, 1548. DOI: 10.3390/cells13181548) jest oryginalną pracą badawczą oraz stanowi bezpośrednie rozwinięcie badań zaprezentowanych w poprzedniej publikacji. W artykule skupiono się na roli białka RBMS 3 w dwóch najbardziej agresywnych podtypach raka gruczołu piersiowego HER-2 dodatnim oraz TNBC, a także powiązaniem jego ekspresji z jednym z kluczowych procesów odpowiedzialnych za progresję w raku gruczołu piersiowego: procesem EMT. W publikacji przedstawiono wyniki badań przeprowadzonych na materiale klinicznym, jak i na funkcjonalnych modelach komórkowych HER-2 dodatniego oraz potrójnie negatywnego raka gruczołu piersiowego z nadekspresją oraz wyciszeniem białka RBMS 3. W publikacji wykorzystano metody analizy immunohistochemicznej, immunofluorescencyjnej oraz molekularnej. Wyniki badań przeprowadzone na materiale tkankowym wykazały pozytywną korelację ekspresji RBMS 3 ze znanymi markerami EMT: TWIST 1 (ang. twist-related protein 1), N-kadheryną (ang. neural cadherin, N-CAD) oraz SNAIL (ang. snail family transcriptional repressor 1). W grupie przypadków TNBC ekspresja RBMS 3 pozytywnie korelowała z TWIST 1, SLUG (ang. snail family transcriptional repressor 2) oraz E-kadheryną (ang. epithelial cadherin, E-CAD), natomiast w przypadkach HER-2 dodatnich nie zaobserwowano istotnych korelacji. Badania funkcjonalnych modeli in vitro z wykorzystaniem metod WB oraz RT-qPCR wykazały w przypadku modelu TNBC przy nadekspresji RBMS 3, wzrost ekspresji markerów promujących EMT, a w przypadku wyciszenia, spadek ekspresji markerów promujących EMT. Odwrotna sytuacja miała miejsce w przypadku modelu HER-2 dodatniego, gdzie nadekspresja RBMS 3 wywoływała spadek ekspresji markerów promujących EMT, a wyciszenie wzrost ich ekspresji. Ponadto, wykonany test migracji wykazał w modelu TNBC większe zdolności migracyjne komórek z nadekspresją RBMS 3, w stosunku do tych z wyciszonym białkiem. Rezultaty przeprowadzonej analizy ekspresji E-kadheryny oraz N-kadheryny za pomocą mikroskopii konfokalnej pozostawały w zgodzie z rezultatami otrzymanymi za pomocą metod molekularnych.

Rezultaty projektu przedstawiają RBMS 3 jako białko o potencjalnie bardzo złożonej funkcji w raku gruczołu piersiowego. Ekspresja RBMS 3 może odgrywać rolę nie tylko w komórkach nowotworowych, ale także w komórkach podścieliska, które stanowią istotną komponentę guza. W komórkach nowotworowych gruczołu piersiowego rola białka RBMS3 może zależeć od molekularnego typu nowotworu, co znajduje swoje odzwierciedlenie w zmianach ekspresji markerów EMT. Z klinicznego punktu widzenia, rezultaty badań przeprowadzone na materiale klinicznym wskazują na RBMS 3 jako przyszły potencjalny

biomarker diagnostyczny, zaś badania in vitro mogą stanowić wstęp do dalszych doświadczeń, celem wykorzystania RBMS 3 jako punktu uchwytu w spersonalizowanej celowanej terapii onkologicznej, w raku gruczołu piersiowego. W związku ze złożonymi mechanizmami działania, niezbędne są dalsze wnikliwe badania mające na celu poznanie dokładnych molekularnych mechanizmów leżących u podstawy roli białka RBMS 3 w raku gruczołu piersiowego, w szczególności w procesie EMT.

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# 4. STRESZCZENIE W JĘZYKU ANGIELSKIM

According to recent statistics, breast cancer is the second most commonly diagnosed cancer in the world, accounting for nearly 12% of all new cancer cases. Due to such a high incidence rate, breast cancer is not only a serious global health problem but also a significant economic challenge. Despite significant advances in the diagnosis and treatment of breast cancer, the 5-year survival rate for advanced breast cancer with distant metastases is only 30%. This indicates a continuous need to search for new diagnostic biomarkers and therapeutic targets in order to improve the quality of the diagnostic process and treatment.

RBMS 3 (RNA-binding motif single-stranded-interacting protein 3) is a glycine-rich protein which belongs to the c-Myc gene single-strand binding proteins (MSSPs) family, first described in the year 2000. The expression of RBMS 3 has been linked to a number of physiological processes, including the regulation of embryonic development of the pancreas and the nervous system, as well as pathological processes such as liver cirrhosis. Recent studies have indicated the potential of RBMS 3 to serve as a prognostic marker and a factor regulating the process of carcinogenesis in various types of tumours, including ovarian cancer, nasopharyngeal cancer, and gastric cancer. The existing literature provides evidence suggesting a potential correlation between RBMS 3 expression and the progression of breast cancer.

The aim of the project which is the subject of my doctoral dissertation entitled “The Role of RBMS 3 Protein in Breast Cancer” was to determine the significance of RBMS 3 protein expression levels in processes involved in breast cancer progression, as well as its prognostic value.

The first publication (Górnicki T, Lambrinow J, Mrozowska M, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J. Role of RBMS3: Novel Potential Regulator of the EMT Phenomenon in Physiological and Pathological Processes. Int J Mol Sci. 2022 Sep 17;23(18):10875. DOI: 10.3390/ijms231810875) is a review article aimed at systematizing the current knowledge regarding the role of RBMS 3 in physiological and pathological processes, with particular emphasis on the initiation and progression of solid tumours.

The second publication is an original research article (Górnicki T, Lambrinow J, Mrozowska M, Romanowicz H, Smolarz B, Piotrowska A, Gomułkiewicz A, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J. Expression of RBMS3 in Breast Cancer Progression. Int J Mol Sci. 2023 Feb 2;24(3):2866. DOI: 10.3390/ijms24032866.) which presents the results of

studies utilizing immunohistochemical reactions performed on clinical material from 490 patients diagnosed with invasive ductal breast carcinoma. The publication also includes an analysis of available databases regarding overall patient survival using the Kaplan-Meier Plotter tool. The published results further incorporate molecular analyses using western blot (WB) and quantitative real-time PCR (qRT-PCR) methods conducted on commercially available breast cancer cell lines representing the major molecular subtypes of this cancer. The results of the studies demonstrated a statistically significant lower expression of RBMS 3 in cancer cases compared to control cases of mastopathy. RBMS 3 expression in cancer cells was positively correlated with the status of the HER-2 receptor and the estrogen receptor. Additionally, RBMS 3 expression was also detected in the breast cancer stroma. The statistical analysis revealed that the expression level of RBMS 3 was significantly higher in the stroma compared to cancer cells and control cases. Moreover, a negative correlation was observed between RBMS 3 expression in the stroma in cases of triple-negative breast cancer (TNBC) compared to other groups, and a positive correlation with estrogen and progesterone receptor expression was also noted. Clinical data analysis and online database analysis showed a positive correlation between RBMS 3 expression and overall survival. In vitro analysis of RBMS 3 expression in selected cell lines at the mRNA and protein levels revealed a significantly higher level of RBMS 3 protein in the most aggressive subtypes of breast cancer: HER-2 positive and triple-negative.

The last publication included in the series (Górnicki T, Lambrinow J, Mrozowska M, Krawczyńska K, Staszko N, Kmiecik A, Piotrowska A, Gomułkiewicz A, Romanowicz H, Smolarz B, Podhorska-Okołów M, Grzegrzółka J, Rusak A, Grzegrzółka J, Impact of RBMS3 Progression on Expression of EMT Markers. Cells 2024, Sep 13, 1548. DOI: 10.3390/cells13181548) is an original research article and represents a direct continuation of the studies presented in the previous publication. The article focuses on the role of RBMS 3 in the two most aggressive breast cancer subtypes: HER-2 positive and triple-negative breast cancer (TNBC), and its association with one of the key processes responsible for breast cancer progression, the epithelial-mesenchymal transition (EMT) process. The publication presents the results of studies conducted on clinical material as well as on functional HER-2 positive and TNBC cell models with RBMS 3 overexpression and silencing. Immunohistochemical, immunofluorescent, and molecular analyses were performed. The tissue-based studies demonstrated a positive correlation between RBMS 3 expression and selected EMT markers: TWIST1 (twist-related protein 1), N-cadherin (neural cadherin), and SNAIL (snail family transcriptional repressor 1). In the TNBC group, RBMS 3 expression was positively correlated

with TWIST1, SLUG (snail family transcriptional repressor 2), and E-cadherin (epithelial cadherin), whereas no significant correlations were observed in the HER-2 positive cases. Functional in vitro model studies using WB and qRT-PCR methods showed that in the TNBC model, RBMS 3 overexpression resulted in an increase in EMT markers, while RBMS 3 silencing led to a decrease in these markers. In contrast, the HER-2 positive model demonstrated that RBMS 3 overexpression resulted in a reduction in EMT markers, whereas their silencing led to an increase in their expression. Furthermore, a migration assay revealed that TNBC cells with RBMS 3 overexpression exhibited enhanced migratory capabilities compared to those with silenced RBMS 3. The results of E-cadherin and N-cadherin expression analysis using confocal microscopy were consistent with the results obtained through molecular methods.

The results of the project present RBMS 3 as a protein with a potentially very complex role in breast cancer. The expression of RBMS 3 may play a role not only in cancer cells but also in stromal cells, which constitute a significant component of the tumour. In breast cancer cells, the role of RBMS 3 may depend on the molecular subtype of the tumour, as reflected in changes in the expression of EMT markers. From a clinical perspective, the results of the studies conducted on clinical material present RBMS 3 as a potential future diagnostic biomarker, while the in vitro studies may serve as a foundation for further experiments aimed at utilizing RBMS 3 as a target in personalized cancer therapy for breast cancer. However, given the complex mechanisms involved, further in-depth research is necessary to understand the precise molecular mechanisms underlying the role of RBMS 3 in breast cancer, particularly in the EMT process.

# 5. WPROWADZENIE DO ROZPRAWY DOKTORSKIEJ

## 5.1 Wstęp

Według danych pochodzących z GLOBOCAN rak gruczołu piersiowego (ang. *breast cancer*, BC) stanowi drugi najczęściej rozpoznawany nowotwór na świecie odpowiedzialny za około 11.6% wszystkich nowych przypadków chorób nowotworowych, a także za 6.9% wszystkich zgonów z powodu choroby nowotworowej [1,2]. W związku z tak wysokim współczynnikiem zapadalności, pomimo znacznego postępu ostatnich lat zarówno w obszarze diagnostyki jak i leczenia, rak gruczołu piersiowego stanowi nadal istotne wyzwanie zarówno medyczne jak i ekonomiczne dla systemów ochrony zdrowia na całym świecie. W świetle powyższych danych uzasadniona jest potrzeba poszukiwania nowych markerów o znaczeniu diagnostycznym oraz prognostycznym, jak i nowych punktów uchwytu dla zaawansowanych, spersonalizowanych terapii przeciwnowotworowych.

Jednym z najważniejszych systemów klasyfikacji raków gruczołu piersiowego jest stworzony na podstawie analiz genetycznych oraz molekularnych, podział na podtypy molekularne, oparty o ekspresję receptorów estrogenowych, progesteronowych oraz receptora ludzkiego nabłonkowego czynnika wzrostu 2 (ang. *human epidermal growth factor receptor 2*, HER-2) w komórkach nowotworowych [3]. W ramach tej klasyfikacji można wyróżnić cztery podstawowe podtypy raka gruczołu piersiowego. Podtyp luminalny A oraz luminalny B są łagodniejszymi podtypami raka gruczołu piersiowego, charakteryzujące się obecnością ekspresji receptora estrogenowego oraz w przypadku luminalnego B receptora progesteronowego w ponad 20% komórek nowotworowych. Podtyp HER-2 dodatni jest bardziej agresywnym nowotworem charakteryzujących się obecnością tylko receptora HER-2. Najbardziej agresywnym podtypem raka gruczołu piersiowego jest rak potrójnie negatywny (ang. *triple negative breast cancer,* TNBC), który zgodnie ze swoją nazwą nie wykazuje w komórkach obecności żadnego z głównych receptorów ujętych w tej klasyfikacji [4,5].

Białko wiążące RNA z motywem oddziałowującym z jednoniciowymi kwasami nukleinowymi 3 (ang. *RNA-binding motif single-stranded-interacting protein 3*, RBMS 3) jest bogatym w glicynę białkiem, należącym do rodziny białek związanych z genem *c-Myc* łączących się z jednoniciowymi kwasami nukleinowymi (ang. *c-Myc gene single-strand binding proteins*, MSSPs) [6,7]. Postulowana fizjologiczna rola RBMS 3 obejmuje embrionalny rozwój trzustki, cewy nerwowej oraz twarzoczaszki, a także utrzymywanie

prawidłowej funkcji egzokrynnej trzustki. Badacze wskazują także na potencjalny udział białka RBMS 3 w procesie chondrogenezy [8,9]. Poziom ekspresji białka RBMS 3 może również być związany z szeregiem nienowotworowych procesów patologicznych. Do najważniejszych z nich należy proponowany udział we włóknieniu wątroby, zespole eksfoliacji, pierwotnym zespole Sjögrena czy twardzinie układowej [10-12]. W ostatnich latach uwagę badaczy przykuwa rola białka RBMS 3 w inicjacji oraz progresji różnych typów nowotworów, która może być odmienna w zależności od lokalizacji oraz histogenezy nowotworu. Dane literaturowe wskazują na możliwy związek ekspresji białka RBMS 3, z pogorszeniem prognozy u pacjentów z rakiem pęcherza moczowego [13,14]. Podobne hipotezy stawiane są w przypadku nabłonkowego raka jajnika (ang. *epithelial ovarian cancer*, EOC), gdzie utrata ekspresji białka RBMS 3 jest łączona z pogorszeniem prognozy oraz rozwinięciem oporności na chemioterapię [15]. W raku jamy nosowo-gardłowej (ang. *nasopharyngeal cancer*, NPC) ektopowa ekspresja białka RBMS 3 prowadzi do zatrzymania wzrostu guza oraz formowanie mikronaczyń, poprzez wzrost ekspresji białka p53 oraz MMP2 (ang. *matrix metalloproteinase-2*) i MMP9 (ang. *matrix metalloproteinase-9*) [16,17]. Potencjalna supresorowa funkcja białka RBMS 3 jest postulowana również w przypadku raka płuc, żołądka, brodawkowatego raka tarczycy oraz raka wątrobowokomórkowego [18-26]. Rola białka RBMS 3 w raku pęcherza moczowego może stanowić odstępstwo od proponowanej przeciwnowotworowej funkcji w wymienionych wyżej nowotworach. Dotychczas przeprowadzone analizy danych klinicznych wykazały korelację, pomiędzy obniżeniem ekspresji RBMS 3, a dłuższym czasem całkowitym przeżycia oraz wzrostem ekspresji RBMS 3 i wzrostem stopnia zaawansowania choroby w klasyfikacji TNM [27,28].

W przypadku raka gruczołu piersiowego, badacze wskazują na obniżenie poziomu ekspresji białka RBMS 3 w komórkach nowotworowych, w stosunku do prawidłowych komórek gruczołu piersiowego. Co więcej, przeprowadzone dotychczas badania *in vitro* na linii komórkowej MCF-7 reprezentującej podtyp luminalny A pokazały, że wyższy poziom białka RBMS 3 powodował zahamowanie wzrostu, inwazji oraz migracji komórek raka. Ponadto, wskazują iż poziom ekspresji białka RBMS 3 w kombinacji z poziomem ekspresji receptora estrogenowego może być niezależnym pozytywnym czynnikiem prognostycznym. Wśród molekularnych mechanizmów, odpowiedzialnych za funkcje białka RBMS 3 w raku gruczołu piersiowego, upatruje się udziału w regulacji szklaku Wnt/β-katenina, ekspresji mikro RNA (miRNA), a także białka MEG3 (ang. *maternally*

*expressed 3*). Dotychczas opublikowane analizy transkryptomicznych baz danych pokazały, że obniżenie ekspresja białka RBMS 3 w komórkach podścieliska jest skorelowana ze wzrastającym stopniem złośliwości guza i wiąże się z krótszym czasem przeżycia chorych na raka gruczołu piersiowego [29-32]. Niektórzy autorzy wskazują także, na możliwy związek ekspresji białka RBMS 3 w raku gruczołu piersiowego z procesem przejścia nabłonkowo-mezenchymalnego (ang. *epithelial-mesenchymal transition*, EMT), jednego z głównych mechanizmów odpowiedzialnych za powstawanie przerzutów. W literaturze znany jest potencjalny związek ekspresji białka RBMS 3, z ekspresję jednego z markerów EMT: TWIST 1 (ang. *twist-related protein 1*), a także hipoteza zakładająca istotną rolę białka RBMS 3 w zachowaniu mezenchymalnego fenotypu oraz zdolności do migracji komórek nowotworowych [33,34].

## 5.2 Cel badań

Celem badań stanowiących podstawę niniejszej rozprawy doktorskiej pt; „Rola ekspresji białka RBMS 3 w raku gruczołu piersiowego" była analiza profilu ekspresji białka RBMS 3 oraz jego wpływu na ekspresję markerów EMT w materiale klinicznym inwazyjnego przewodowego raka gruczołu piersiowego (ang. *invasive ductal breast cancer*, IDC) , a także w modelach funkcjonalnych *in vitro,* z zastosowaniem linii komórkowych raka gruczołu piersiowego. W ramach badań przeprowadzono analizę uzyskanych wyników z danymi kliniczno-patologicznymi.

Cele szczegółowe:

1. Ocena lokalizacji oraz nasilenia poziomu ekspresji białka RBMS 3 w materiale klinicznym guzów przy zastosowaniu reakcji immunohistochemicznych.

2. Określenie korelacji poziomu ekspresji białka RBMS 3 w materiale klinicznym z ekspresją markerów procesu EMT: TWIST 1, SNAIL, SLUG, ZEB 1, N-kadheryny, E-kadheryny przy zastosowaniu reakcji immunohistochemicznych

3. Ocena poziomu ekspresji RBMS 3 na poziomie mRNA oraz białka w liniach komórkowych raka gruczołu piersiowego przy zastosowaniu metody quantitative real time PCR (RT-qPCR) oraz Western Blot.

4. Korelacja uzyskanych rezultatów z wykorzystaniem metody immunohistochemicznej w materiale klinicznym oraz danymi kliniczno-patologicznymi pacjentek, ze zdiagnozowanym inwazyjnym przewodowym rakiem gruczołu piersiowego.

5. Określenie wpływu wyciszenia ekspresji białka RBMS 3 oraz nadekspresji tego białka na poziom kluczowych markerów EMT: TWIST 1, SNAIL, SLUG, ZEB 1 (ang. ang. *Zinc finger E-box-binding homeobox 1*), N-kadheryny, E-kadheryny w liniach komórkowych raka gruczołu piersiowego MDA-MB-231 oraz SKBR-3.

## 5.3 Materiały i metody

Pierwszy artykuł z cyklu pt; „*Role of RBMS 3 Novel Potential Regulator of the EMT Phenomenon in Physiological and Pathological Processes*" (Górnicki T, Lambrinow J, Mrozowska M, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J.) stanowi przegląd literatury. W celu pozyskania literatury dotyczącej ekspresji RBMS 3 w procesach fizjologicznych i patologicznych wykorzystano cztery popularne medyczne bazy danych: PubMed, Embase, Ovid oraz Scopus. W wyszukiwarki wpisano słowa kluczowe, do których należały frazy: RBMS3, RNA-binding motif single-stranded-interacting protein 3, rbms3 cancer, rbms3 EMT, EMT, epithelial-mesenchymal transition. Uzyskano 474 rekordów, które zostały przeanalizowane pod kątem obecności powtórzonych artykułów. Pełne teksty 76 pozostałych artykułów zostało ocenione w kierunku spełnienia kryterium włączenia, którym było bezpośrednie wspomnienie przez autorów RBMS 3 w tekście. Przeprowadzono ponadto, we wszystkich zakwalifikowanych artykułach przegląd ich piśmiennictwa, w celu zapewnienia włączenia wszystkich artykułów związanych z RBMS 3. Do ostatecznej wersji manuskryptu zakwalifikowane zostały 64 publikacje.

Drugi artykuł pt: „*Expression of RBMS 3 in Breast Cancer Progression*" (Górnicki T, Lambrinow J, Mrozowska M, Romanowicz H, Smolarz B, Piotrowska A, Gomułkiewicz A, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J.) stanowi pierwszą z oryginalnych prac badawczych. Wykonano w nim analizę ekspresji RBMS 3, w klinicznym materiale 490 przypadków IDC oraz 26 przypadkach mastopatii, stanowiących kontrolę w badaniach. W tym celu wykorzystano technikę mikromacierzy tkankowych oraz wykonano reakcje immunohistochemiczne. Ocenę reakcji immunohistochemicznych przeprowadzono osobno dla komórek nowotworowych oraz dla komórek podścieliska. Do analizy całkowitego czasu przeżycia pacjentów w zależności od ekspresji RBMS 3 na poziomie mRNA zastosowano internetowe narzędzie „Kaplan-Meier Plotter", które wykorzystuje bazy danych: GEO, EGA oraz TCGA [Győrffy B, et.al 2021]. Wykonano także analizy molekularne RT-qPCR oraz WB ekspresji RBMS 3 w liniach komórkowych raka gruczołu piersiowego, reprezentujących poszczególne podtypy molekularne raka gruczołu piersiowego: MCF-7 (luminalny A), BT-474 (luminalny B), SKBR-3 (HER-2 dodatni), oraz MDA-MB-231 (potrójnie negatywny), a także linię komórkową prawidłowego nabłonka gruczołu piersiowego h-TERT-HME1 (Me16C). Analiza poziomu ekspresji RBMS 3 została

przeprowadzona z zastosowaniem metody Western Blot. Ocenę normalności rozkładu danych przeprowadzono z zastosowaniem testu Kołmogorova-Smirnova. Analizę statystyczną wykonano z zastosowaniem testów Mann-Whitney'a oraz ANOVA z testem post hoc Bonferroniego dla porównań wielokrotnych oraz korelacji Spearmana, co pozwoliło na porównanie różnic i wzajemnych zależności w ekspresji między RBMS 3 we wszystkich grupach pacjentów z uwzględnieniem danych kliniczno-patologicznych. Z kolei metoda Kaplana-Meier'a oraz Gehan-Breslow-Wilcoxon'a zostały zastosowane do analizy przeżycia całkowitego pacjentów. Wszystkie analizy statystyczne zostały wykonane przy użyciu programów Prism 9.0 oraz Statistica 13.3. Wyniki zostały uznane za istotne gdy $p < 0.05$.

Trzeci artykuł *Impact of RBMS 3 Progression on Expression of EMT Markers* (Górnicki T, Lambrinow J, Mrozowska M, Krawczyńska K, Staszko N, Kmiecik A, Piotrowska A, Gomułkiewicz A, Romanowicz H, Smolarz B, Podhorska-Okołów M, Grzegrzółka J, Rusak A, Dzięgiel P) stanowi drugą z oryginalnych prac badawczych. W ramach publikacji przeprowadzono analizę poziomu ekspresji białka RBMS 3 oraz wybranych markerów procesu EMT (TWIST 1, SNAIL, SLUG, ZEB 1, N-kadheryny, E-kadheryny) w materiale klinicznym obejmującym 449 przypadków IDC. Badania przeprowadzono wykorzystując technikę mikromacierzy tkankowych oraz reakcje immunohistochemiczne. Ocenę reakcji immunohistochemicznych badanych markerów oceniono osobno dla komórek nowotworowych oraz dla komórek podścieliska. W pracy stworzono także funkcjonalne, komórkowe modele *in vitro* z zastosowaniem komercyjnie dostępnych linii komórkowych raka gruczołu piersiowego, reprezentujących zróżnicowane podtypy molekularne tego nowotworu. Zastosowano model z wyciszeniem oraz nadkepsresją białka RBMS 3 wykorzystując linie komórkowe SKBR-3, reprezentującą HER-2 dodatni podtyp raka gruczołu piersiowego, oraz MDA-MB-231 jako model potrójnie negatywny. Skuteczność wyciszenia oraz nadekspresji potwierdzona została przy użyciu metod RT-qPCR oraz Western Blot. W celu określenia lokalizacji ekspresji białek N-kadheryny oraz E-kadheryny w strukturach komórki wykonano reakcje immunofluorescencyjne. W celu określenia wpływu zmian poziomu ekspresji białka RBMS 3 na zdolności migracyjne komórek nowotworowych, wykonano test migracji komórek modyfikowanych w ramach modeli funkcjonalnych. W pracy posłużono się także metodami analizy molekularnej, w celu detekcji zmian w profilu ekspresji wybranych białek EMT, pod wpływem modyfikacji

poziomu ekspresji białka RBMS 3. W tym celu wykorzystano metody RT-qPCR oraz Western Blot.

## 5.4 Podsumowanie wyników

W publikacji przeglądowej pokazano, że RBMS 3 może pełnić istotną rolę w wielu procesach fizjologicznych oraz patologicznych. Większość opublikowanych dotychczas prac dotyczących roli RBMS 3 w procesach patologicznych dotyczy zmian ekspresji tego białka w procesach nowotworowych, z racji częstej deregulacji poziomu tego białka w wielu typach nowotworów oraz związku z ogólnym czasem przeżycia. W publikacjach dotyczących raka gruczołu piersiowego badacze postulują związek obniżonej ekspresji białka RBMS 3 z procesem inicjacji oraz progresji w tym nowotworze oraz wskazują na możliwy związek z procesem EMT. Dotychczas jednak brak jest jednoznacznie określonej roli białka RBMS 3 w biologii raków gruczołu piersiowego. Analiza danych literaturowych wskazuje zatem konieczność kontynuacji badań, nad rolą oraz molekularnymi podstawami działania białka RBMS 3 w chorobach nowotworowych.

Wykonana, w ramach badań zaprezentowanych w pierwszym oryginalnym artykule wchodzącym w skład cyklu publikacji stanowiącego podstawę niniejszej rozprawy doktorskiej, analiza preparatów immunohistochemicznych wykazała, statystycznie istotnie obniżoną ekspresję RBMS 3 w komórkach raka IDC w porównaniu do komórek nabłonka przewodowego kontrolnych przypadków mastopatii ($p < 0.001$). Ekspresja RBMS 3 była również istotnie statystycznie wyższa, w przypadkach o dodatnim statusie receptorowym HER-2 ($p < 0.05$) oraz receptora estrogenowego ($p < 0.05$). Analiza ekspresji RBMS 3 w komórkach podścieliska wykazała istotnie statystycznie wyższą ekspresję RBMS 3 w przypadkach IDC, w stosunku do kontrolnych przypadków mastopatii ($p < 0.0001$). Ekspresja RBMS 3 w podścielisku była także istotnie niższa w przypadkach TNBC w stosunku do pozostałych podtypów IDC ($p < 0.001$). Ponadto ekspresja RBMS 3 w podścielisku była istotnie wyższa w przypadkach o dodatnim statusie receptora progesteronowego ($p < 0.01$) oraz estrogenowego ($p < 0.001$). Ekspresja RBMS 3 była również istotnie wyższa w komórkach podścieliska, w stosunku do komórek raka ($p < 0.0001$). Analiza ekspresji RBMS 3 w wybranych liniach komórkowych reprezentujących podstawowe podtypy molekularne raka gruczołu piersiowego wykazała różnicę w ekspresji tego białka na poziomie mRNA we wszystkich badanych liniach w stosunku do kontrolnej linii prawidłowego nabłonka gruczołu piersiowego oraz wyższą ekspresję w bardziej agresywnych podtypach raka gruczołu piersiowego. Na poziomie białka wykazano, iż ekspresja RBMS 3 w dwóch najbardziej agresywnych podtypach raka gruczołu piersiowego, HER-2 dodatnim oraz potrójnie negatywnym jest istotnie wyższa od ekspresji RBMS 3 w podtypach luminalnym A oraz B. Analiza danych klinicznych wykazała, bliski istotności statystycznej, krótszy czas

przeżycia całkowitego pacjentów bez ekspresji RBMS 3 ($p < 0.051$). Wykonana za pomocą narzędzia „Kaplan Meier Plotter" analiza korelacji ekspresji RBMS 3 na poziomie mRNA z danymi dotyczącymi przeżyć całkowitych, wykazała, że grupa pacjentów z obniżoną ekspresją RBMS 3 miała istotnie statystycznie skrócony czas przeżycia całkowitego ($p < 0.0001$).

W ramach badań zaprezentowanych w drugim oryginalnym artykule wchodzącym w skład cyklu publikacji stanowiącego podstawę niniejszej rozprawy doktorskiej, wykonano analizę korelacji poziomu ekspresji białka RBMS 3 z wybranymi markerami EMT w materiale klinicznym przypadków inwazyjnego raka przewodowego gruczołu piersiowego. Analiza reakcji immunohistochemicznych wykazała lokalizację RBMS 3, SNAIL oraz SLUG w cytoplazmie komórek nowotworowych, oraz TWIST 1 w cytoplazmie oraz jądrze komórkowym komórek raka. N-kadheryna oraz E-kadheryny obecne były w błonie komórkowej oraz cytoplazmie komórek nowotworowych. W badanych przypadkach nie zaobserwowano obecności białka ZEB 1 w komórkach nowotworowych. Analiza korelacji wykazała dodatni, słaby związek ekspresji RBMS 3 z ekspresją TWIST 1 ($p < 0.0001$, r=0.31), N-CAD ($p < 0.0001$, r=0.19) oraz SNAIL ($p < 0.0001$, r=0.18). Analiza transkryptomicznych baz danych z wykorzystaniem narzędzia UALCAN wykazała obniżenie poziomu ekspresji białka RBMS 3 w nowotworach HER-2 dodatnich oraz TNBC w stosunku do nowotworów o podtypach luminalnych. Analiza korelacji uwzględniająca podział na molekularne podtypy raka gruczołu piersiowego wykazała, w grupie 80 przypadków podtypu luminalnego A brak korelacji z badanymi markerami procesu EMT, w grupie 170 przypadków podtypu luminalnego B zaobserwowano pozytywną, słabą korelację z ekspresją TWIST 1 ($p < 0.0001$, r=0.29), SNAIL ($p < 0.006$, r=0.20) oraz N-CAD ($p < 0.0001$, r=0.30). Analizowana grupa 20 przypadków podtypu HER-2 dodatniego wykazała bliską istotności statystycznej pozytywną, silną korelację z ekspresją E-CAD ($p < 0.053$). W grupie 37 przypadków raka potrójnie negatywnego zaobserwowano dodatnią, przeciętną korelację z TWIST 1 ($p < 0.01$, r=0.44), oraz bliską istotności statystycznej dodatnią, przeciętną korelację z SLUG ($p < 0.09$, r=0.3) oraz E-CAD ($p < 0.0001$, r=0.31). Analiza korelacji uwzględniająca stopień złośliwości guza ujawniła, w grupie podtypie HER-2 dodatnim, w stopniu złośliwości G2 dodatnią, silną korelację ekspresji RBMS 3 z N-CAD ($p < 0.003$, r=0.56), natomiast w podtypie potrójnie negatywnym, w stopniu złośliwości G2 dodatnią, silną korelację z ekspresją TWIST 1 ($p < 0.025$, r=0.58). Biorąc pod uwagę obecność przerzutów komórek nowotworowych do węzłów chłonnych, analiza korelacji wykazała dodatnią, silną korelację ekspresji RBMS 3 w przypadkach z inwazją do węzłów chłonnych w podtypie HER-2 dodatnim z ekspresją TWIST

1 ($p < 0.035$, r=0.65) oraz dodatnią, bardzo wysoką korelację, w podtypie potrójnie negatywnym z TWIST 1 ($p < 0.031$, r=0.77) oraz N-CAD ($p < 0.035$, r=0.85).

W ramach badań *in vitro* przygotowano modele nadekspresji i wyciszenia ekspresji białka RBMS 3 w dwóch najbardziej agresywnych podtypach raka gruczołu piersiowego HER-2 dodatniego (linia komórkowa SKBR-3) i potrójnie negatywnego (linia komórkowa MDA-MB-231). Analiza z wykorzystaniem metody Western Blot oraz RT-qPCR potwierdziła skuteczność wywołania nadekspresji oraz wyciszenia RBMS 3 w wybranych liniach komórkowych. Wykonana analiza molekularna ekspresji wybranych markerów EMT na poziomie mRNA oraz białka wykazała obecność szeregu zależności. W modelu HER-2 dodatniego raka gruczołu piersiowego nadekspresja białka RBMS 3 powodowała istotne statystycznie obniżenie poziomu ekspresji genów *CDH2* ( gen dla N-CAD) ($p < 0.0001$), *SLUG* ($p < 0.0004$) oraz *ZEB 1* ($p < 0.0001$) na poziomie mRNA a także obserwowalne obniżenie N-CAD i SLUG na poziomie białka. Wyciszenie RBMS 3 w tym modelu wywoływało na poziomie mRNA istotne obniżenie poziomu *TWIST 1* ($p < 0.0001$), a także podniesienie poziomu ekspresji *CDH2* ($p < 0.0001$), *SNAIL* ($p < 0.0001$), *SLUG* ($p < 0.0001$), *CDH1* (gen dla E-CAD) ($p < 0.0001$) oraz *ZEB 1* ($p < 0.0001$) bez obserwowalnych zmian na poziomie białka. W przypadku modelu potrójnie negatywnego raka gruczołu piersiowego nadekspresja RBMS 3 wywołała istotne podniesienie poziomu ekspresji na poziomie mRNA w *TWIST 1* ($p < 0.002$), *SLUG* ($p < 0.0001$), *SNAIL* ($p < 0.0001$), *CDH1* ($p < 0.0001$), *CDH2* ($p < 0.0005$) oraz *ZEB 1* ($p < 0.0001$), a także obserwowalne podniesienie poziomu białka E-CAD, N-CAD oraz ZEB 1. Wyciszenie RBMS 3 skutkowało natomiast, na poziomie mRNA, wzrostem ekspresji *CDH1* ($p < 0.0001$) oraz obniżenie poziomu *ZEB 1* ($p < 0.0001$), *SNAIL* ($p < 0.0001$), *TWIST 1* ($p < 0.0001$) oraz *SLUG* ($p < 0.0001$), a także obserwowalnym obniżeniem poziomu białka SLUG oraz N-CAD. Wyniki wykonanego testu migracji pokazały, że w modelu TNBC nadekspresja białka RBMS 3 słabiej hamuje migrację komórek, niż jego wyciszenie. W modelu HER-2 dodatnim nie zaobserwowano różnic w migracji komórek. Aby wykazać potencjał migracyjny komórek w zależności od poziomu ekspresji białka RBMS 3 wykonano reakcje immunofluorescencyjne z zastosowaniem przeciwciał skierowanych przeciwko białkom adhezyjnym połączeń międzykomórkowych E-CAD i N-CAD. Rezultaty analizy reakcji immunofluorescencyjnych wykazały w HER-2 dodatnim modelu obecność E-CAD w błonie komórkowej oraz cytoplazmie, zarówno przy nadekspresji jak i wyciszeniu ekspresji białka RBMS 3, natomiast N-CAD obecna była tylko w pojedynczych komórkach, niezależnie od ekspresji RBMS 3. W modelu TNBC wykazano obecność cytoplazmatycznej ekspresji E-CAD

i N-CAD w cytoplazmie komórek z nadekspresją RBMS 3, podczas gdy w komórkach z wyciszoną ekspresją białka RBMS 3, gdzie zaobserwowano jedynie śladowe ilości kadheryn w cytoplazmie komórek. Analiza ilościowa komórek, za pomocą metod uczenia maszynowego, wykazała w obu modelach większą ilość pozytywnych komórek w kierunku E-CAD przy nadekspresji białka RBMS 3, w porównaniu do wyciszenia. W przypadku N-CAD w modelu TNBC zaobserwowano podobną zależność, jak w przypadku E-CAD, natomiast w modelu HER-2 dodatnim tylko około 20% komórek można było uznać za dodatnie.

## 5.5 Wnioski

1. Wyższy poziom ekspresji białka RBMS 3 w komórkach inwazyjnego przewodowego raka gruczołu piersiowego, w porównaniu do nienowotworowych tkanek gruczołu piersiowego (mastopatii), może wskazywać na rolę RBMS 3 w procesie transformacji nowotworowej.

2. Wyższy poziom ekspresji białka RBMS 3 w komórkach podścieliska, w stosunku do komórek inwazyjnego przewodowego raka gruczołu piersiowego, może sugerować odmienną rolę tego białka w obydwu kompartmentach.

3. Korelacja wyższego poziomu ekspresji białka RBMS 3 w komórkach nowotworowych, z dłuższym czasem przeżycia pacjentów, może wskazywać na supresyjną rolę RBMS 3 w inwazyjnym przewodowym raku gruczołu piersiowego.

4. Korelacja poziomu ekspresji białka RBMS 3, z wybranymi markerami EMT w materiale klinicznym, może sugerować rolę RBMS 3 w procesie EMT.

5. Zależność poziomu ekspresji wybranych markerów EMT od zmian poziomu białka RBMS 3 w modelu funkcjonalnym *in vitro* potrójnie negatywnego raka gruczołu piersiowego, może wskazywać na promującą proces EMT rolę tego białka w tym podtypie nowotworu.

6. Zależność poziomu ekspresji wybranych markerów EMT od zmian poziomu białka RBMS 3 w modelu funkcjonalnym *in vitro* inwazyjnego przewodowego raka gruczołu piersiowego o podtypie HER-2 dodatnim, może wskazywać na hamującą proces EMT rolę tego białka w tym podtypie nowotworu.

## 5.6 Etyka Badań

Badania przeprowadzono po uzyskaniu pozytywnej opinii właściwej Komisji Bioetycznej przy Uniwersytecie Medycznym im. Piastów Śląskich we Wrocławiu: Nr KB 625/2022.

KOMISJA BIOETYCZNA
przy
Uniwersytecie Medycznym
we Wrocławiu

# OPINIA KOMISJI BIOETYCZNEJ Nr KB –625/2022

Komisja Bioetyczna przy Uniwersytecie Medycznym we Wrocławiu, powołana zarządzeniem Rektora Uniwersytetu Medycznego we Wrocławiu nr 278/XVI R/2020 z dnia 21 grudnia 2020 r. oraz działająca w trybie przewidzianym rozporządzeniem Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. (Dz.U. nr 47, poz. 480) na podstawie ustawy o zawodzie lekarza z dnia 5 grudnia 1996 r. (Dz.U. nr 514 z 2020 r.) w składzie:

dr Joanna Birecka (psychiatria)

dr Beata Freier (onkologia)

dr hab. Tomasz Fuchs (ginekologia, położnictwo)

prof. dr hab. Dariusz Janczak (chirurgia naczyniowa, transplantologia)

dr hab. Krzysztof Kaliszewski, prof. UMW (chirurgia endokrynologiczna)

dr prawa Andrzej Malicki (prawo)

dr hab. Marcin Mączyński, prof. UMW (farmacja)

Urszula Olechowska (pielęgniarstwo)

prof. dr hab. Leszek Szenborn (pediatria, choroby zakaźne)

prof. dr hab. Andrzej Szuba (choroby wewnętrzne, angiologia

ks. prof. Andrzej Tomko (duchowny)

prof. dr hab. Mieszko Więckiewicz (stomatologia)

dr hab. Andrzej Wojnar, prof. nadzw. (histopatologia, dermatologia) przedstawiciel

Dolnośląskiej Izby Lekarskiej)

dr hab. Jacek Zieliński (filozofia)

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

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

„Rola ekspresji białka RBMS3 w rozwoju raka gruczołu piersiowego"

zgłoszonym przez prof. dr hab. Marzenę Podhorską-Okołów, zatrudnioną w Zakładzie Badań Ultrastrukturalnych Katedry Morfologii i Embriologii Człowieka Uniwersytetu Medycznego we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła **wyrazić zgodę** na przeprowadzenie badania w Zakładzie Histologii i Embriologii Człowieka UMW przez studentów: Tomasza Górnickiego i Jakuba Lambrinowa, pod nadzorem prof. dr hab. Marzeny Podhorskiej-Okołów, **pod warunkiem zachowania anonimowości uzyskanych danych.**

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

Opinia powyższa jest ważna z polisą ubezpieczeniową i dotyczy projektu badawczego będącego działalnością Studenckiego Koła Naukowego

Przewodniczący Komisji Bioetycznej
przy Uniwersytecie Medycznym

prof. dr hab. Jerzy Rudnicki

Wrocław, dnia 25 sierpnia 2022 r.

## 5.7 Piśmiennictwo


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# 6. ARTYKUŁY Z CYKLU PUBLIKACJI W FORMIE ZAŁĄCZNIKÓW

## Artykuł pierwszy

**Tytuł:**

*Role of RBMS3 Novel Potential Regulator of the EMT Phenomenon in Physiological and Pathological Processes.*

**Autorzy:**

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**Czasopismo**

International Journal of Molecular Sciences, 2022 Sep 17;23(18):10875, doi: 10.3390/ijms231810875.

**Punktacja**

IF: 5.6, punktacja MNiSW: 140 pkt

International Journal of
*Molecular Sciences*

MDPI

*Review*

# Role of RBMS3 Novel Potential Regulator of the EMT Phenomenon in Physiological and Pathological Processes

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**Abstract:** RNA-binding protein 3 (RBMS3) plays a significant role in embryonic development and the pathogenesis of many diseases, especially cancer initiation and progression. The multiple roles of RBMS3 are conditioned by its numerous alternative expression products. It has been proven that the main form of RBMS3 influences the regulation of microRNA expression or stabilization. The absence of RBMS3 activates the Wnt/β-catenin pathway. The expression of c-Myc, another target of the Wnt/β-catenin pathway, is correlated with the RBMS3 expression. Numerous studies have focused solely on the interaction of RBMS3 with the epithelial–mesenchymal transition (EMT) protein machinery. EMT plays a vital role in cancer progression, in which RBMS3 is a new potential regulator. It is also significant that RBMS3 may act as a prognostic factor of overall survival (OS) in different types of cancer. This review presents the current state of knowledge about the role of RBMS3 in physiological and pathological processes, with particular emphasis on carcinogenesis. The molecular mechanisms underlying the role of RBMS3 are not fully understood; hence, a broader explanation and understanding is still needed.

**Keywords:** target discovery; epithelial–mesenchymal transition (EMT); RNA-binding protein 3 (RBMS3); carcinogenesis; target therapy



**Citation:** Górnicki, T.; Lambrinow, J.; Mrozowska, M.; Podhorska-Okołów, M.; Dzięgiel, P.; Grzegrzółka, J. Role of RBMS3 Novel Potential Regulator of the EMT Phenomenon in Physiological and Pathological Processes. *Int. J. Mol. Sci.* **2022**, *23*, 10875. https://doi.org/10.3390/ijms231810875

Academic Editor: Laura Paleari

Received: 5 September 2022
Accepted: 14 September 2022
Published: 17 September 2022



## 1. Introduction

RNA-binding motif single-stranded-interacting protein 3 (RBMS3) is a glycine-rich protein that was described for the first time by D. Penkov et al. in 2000 [1]. The gene encoding this protein is called *RBMS3*, and it is located in the short arm of chromosome 3, specifically in the 3p24.1 region. The discovery of RBMS3 was an effect of the screening of human fibroblast cDNA with an upstream element of the a2(I) collagen promoter Box 5A. It belongs to the family of *c-Myc* gene single-strand binding proteins (MSSPs) involved in DNA replication, transcription, apoptosis induction, and cell–cycle progression [2,3]. Published papers provide evidence of the wide range of processes in which RBMS3 takes part in, including regulation of embryogenesis, pathogenesis of liver fibrosis, and bisphosphonate-related osteonecrosis of the jaw (BRONJ) [4–6]. From 2008 onwards, RBMS3 has become a potential prognostic marker of different types of cancer and a factor regulating the process of carcinogenesis [7,8]. In addition, recent articles have provided evidence of RBMS3 taking part in the epithelial–mesenchymal transition (EMT), a key process responsible for the creation of distant metastases [9].

RBMS3's ability to suppress the growth and progression of different types of cancers makes it an interesting potential target for the development of novel anticancer therapies. There is still a need for a summary of the role of RBMS3 in physiology and pathology that would provide a synthetic evaluation of the information available about it. In this article,

we are going to review and systematize the current state of knowledge about RBMS3 and its function in physiology and pathology, with a particular focus on its role in EMT.

## 2. Methods

The authors searched for topic-related materials in four big medical databases—PubMed, Embase, Ovid, and Scopus—on 12 December 2021. The searched keywords included: RBMS3, RNA-binding motif single-stranded-interacting protein 3, rbms3 cancer, rbms3 EMT, EMT, and epithelial–mesenchymal transition. The keywords were the same for all databases. The articles were screened for relevance and analyzed based on inclusion criteria. An article was presumed relevant if RBMS3 was directly mentioned by the authors of the research. References from all relevant articles were also reviewed to ensure the inclusion of all articles directly related to the topic of RBMS3 (Figure 1).


**Figure 1.** Workflow literature review.

## 3. Role in Development and Physiology

It is stated that the expression of RBMS3 may be a part of the regulatory mechanisms of pancreas embryonic development in mouse models. Authors have shown a restricted expression of protein in the embryonic pancreas, the neural tube, and the dorsal root ganglion, with peak expression in the pancreas taking place at E13.5. RBMS3 acts on a post-transcriptional level and is able to bind to the 3′-UTR of pancreas transcription factor 1, the alpha subunit (*Ptf1α*) mRNA, stabilizing it and increasing the level of Ptf1α protein in cells. Ptf1α is responsible for exocrine cell differentiation. Scientists have also provided evidence of RBMS3 expression affecting the expression of various digestive enzymes and its role in maintaining the function of mature exocrine cells in the pancreas [4].

Experiments conducted on the zebrafish model brought to light the potential impact of RBMS3 on craniofacial development and chondrogenesis [10]. RBMS3 was discovered to be expressed transiently in the cranial neural crest, and its knockdown results in severe craniofacial defects. The authors point to the TGF-β receptor pathway as the mechanism responsible for these abnormalities. RBMS3 binds to and stabilizes the transcripts of the Smad2 pathway. Further studies discovered that RBMS3 also has the ability to interact with *Smad1*, as well as cell cycle regulators, such as the TGF-β, receptor *cyclin D1* and *Rac1* transcripts, introducing RBMS3 as a global regulator of chondrogenesis [10].

RBMS3 was also discovered to take part in preventing the degeneration of the nucleus pulposus. The mechanism underlying this process consists of decreasing the activity of the Wnt/β-catenin signaling pathway and targets, such as metalloproteinase-13 (MMP13) [11]. The study also showed that RBMS3 increases nucleus pulposus cell proliferation and decreases apoptosis, inflammation, and extracellular matrix degradation levels [11].

## 4. RBMS3 in Pathological Noncancerous Processes

From the moment of the discovery of RBMS3, scientists pointed to the role of this protein and the gene that encodes it in a wide range of pathological processes, including liver fibrosis, osteonecrosis of the jaw (ONJ), and exfoliation syndrome [12–14].

Liver fibrosis is a wound-healing type of EMT process serving as a response to the injury. It can potentially develop into cirrhosis and lead to organ failure as a consequence [12]. The focal point of this process lies in the activation of hepatic stellate cells (HSCs), which are responsible for the storage of vitamin A during their quiescent state but produce an excessive amount of the extracellular matrix after activation. One of the factors involved in inducing the activation of HSCs is the pair-related homeobox transcription factor Prx1, also involved in the production of collagen type α1(I). In their work, Fritz and Stefanovic provided evidence of the role RBMS3 has in the regulation of Prx1 expression. By binding to the 3′-UTR of the *Prx1* mRNA, RBMS3 stabilizes the structure of the mRNA, increasing the effectiveness of translation and the level of the Prx1 protein, thus leading to the stimulation of collagen type α1(I) gene transcription in HSCs. These results together with the post-transcriptional regulation of collagen type α1(I) expressions show a probable mechanism of RBMS3's role in the onset of liver fibrosis [5].

Another domain of RBMS3 influence is its impact on bone density. There is evidence of a statistically significant interaction between the *RBMS3* and *ZNF516* genes that negatively impacts the hip bone mineral density (BMD). The study was conducted using the novel approach of genome-wide association studies (GWAS), a method that successfully unveiled a number of genetic loci that impact BMD [15,16]. Table 1 presents all the discovered single-nucleotide polymorphisms (SNPs) of the *RBMS3* gene discussed in this article. Although molecular mechanisms underlying this interaction are currently unknown, RBMS3's impact on collagen expression may influence the extracellular matrix of bone tissue.

**Table 1.** Single-nucleotide polymorphisms of the *RBMS3* gene and their correlation with pathological processes.

| RBMS3-Related Processes | Identified SNP | References |
|---|---|---|
| Bone-mineral-density-related disorders | rs6549904<br>rs7640046<br>rs17024608 | [15] |
| Osteonecrosis of the jaw (ONJ) | rs17024608 | [17] |
| Exfoliation glaucoma | rs12490863 | [18] |
| Exfoliation syndrome | rs12490863 | [19] |
| Primary Sjögren's syndrome | rs13079920<br>rs13072846 | [20] |
| Systemic sclerosis | rs1449292 | [21] |
| Periodontal disease | rs17718700 | [22] |
| Lymphocyte glucocorticoid sensitivity | rs6549965 | [23] |
| Short-acting bronchodilator response | rs1266115<br>rs150703870 | [24] |

Another pathological process that may, among others, involve alteration in collagen type α1(I) expression is osteonecrosis of the jaw (ONJ). It is a serious adverse effect mainly connected to the administration of bisphosphonates (BPs), which are antiosteoclastic drugs used, among others, in oncological therapy to control bone metastasis and hypercalcemia. The frequency of ONJ ranges from 0.6% in breast cancer to even 15% in multiple myeloma [13]. Research conducted with the help of GWAS discovered a relation between the variation in the *RBMS3* gene and 5.8 times higher probability of developing bisphosphonate-related osteonecrosis of the jaw (BRONJ) [17]. Even though other researchers were not able to confirm this relation [13], taking into consideration the impact of RBMS3 on bone density postulated in [15], there is a wide area for researchers to establish the exact role of RBMS3 in ONJ [18].

Exfoliation syndrome (XFS) is an age-related systemic disease that is the most common risk factor for open-angle glaucoma, which can cause irreversible blindness. Based on familial aggregation studies, XFS is suspected to be a genetic disease. Specific loci in the *RBMS3* gene are proven to be correlated with susceptibility to XFS and exfoliation glaucoma, although the exact mechanism of this impact is yet to be discovered [14,19,25].

RBMS3 was also found to be potentially involved in autoimmune diseases. Specifically, there is evidence that certain SNPs in the *RBMS3* gene are responsible for an increased susceptibility to systemic sclerosis (SSc) and primary Sjögren's syndrome (PSS) [20,21]. A weak correlation was also found between RBMS3 and periodontal disease [22].

The versatility of RBMS3 reaches even the field of psychiatric health care and neurodegenerative diseases, since various authors have linked it to resistance to antidepressant therapy and susceptibility to schizophrenia and amyotrophic lateral sclerosis (ALS) [26–28]. Gastrointestinal dysfunction is a common symptom in the autism spectrum disorder (ASD). The exact underlying mechanism of this process is unknown, but researchers revealed that in a specific group of patients with *FOXP1* haploinsufficiency, downstream targets of the Foxp1 protein are dysregulated in the mice model. One of these targets is RBMS3, thus providing additional data about its role in this disorder [29].

RBMS3's impact seems to not be restricted only to the pathogenesis of different diseases. It also determines the response to some forms of therapy, with two effects described in the literature: (1) the regulation of lymphocyte sensitivity to glucocorticoids by decreasing cellular proliferation of peripheral blood mononuclear cells and (2) the modulation of the response to inhaled short-acting bronchodilators (BD) [23,24].

A recent study using CRISPR interference (CRISPRi) tried to assess the molecular mechanisms connected to the genes associated with chronic obstructive pulmonary disease (COPD) and low lung function. After a GWAS analysis searching for genes related to COPD, the experiments were conducted on human-induced pluripotent stem cell (iPSC)–derived lung epithelium. The results of this study show that the knockdown of *RBMS3* enhances the proliferation of cells, which is the basis for later experiments clarifying the exact role of RBMS3 in COPD [30].

## 5. Role of RBMS3 in Carcinogenesis

In 2008, RBMS3 was mentioned in the context of neoplastic processes for the first time [31]. From that time onwards, RBMS3 has significantly grown in popularity and importance as a potential marker and regulator in many different types of cancer. The increasing amount of scientific data provided by researchers has started to unveil the specific mechanisms of RBMS3's impact on carcinogenesis and metastasis (Table 2).

**Table 2.** Role of RBMS3 in carcinogenesis.

| Tumor Type | Correlation with High or Low Expression of RBMS3 | Mechanism of Action | References |
|---|---|---|---|
| Bladder cancer | High expression correlates with poorer prognosis.<br>Low expression correlates with shorter OS. | Further research is needed. | [32,33] |
| Gallbladder carcinoma | High expression inhibits growth and promotes apoptosis in vitro. | Further research is needed. | [34] |
| Prostate cancer | Upregulation of *RBMS-AS3* correlates with faster tumor growth, angiogenesis, and migration. | *RBMS-AS3*/miR-4534/VASH1 axis. | [35–37] |
| Ovarian epithelial cancer | Loss of *RBMS3* gene is correlated with poorer prognosis.<br>Deletion of *RBMS3* promotes efflux and induces chemoresistance. | RBMS3 promotes efflux.<br>Lack of RBMS3 activates the Wnt/β-catenin pathway. | [38,39] |
| Nasopharyngeal cancer | Ectopic expression inhibits tumor growth and foci formation. | RBMS3 increases the level of p53, and thus p21 and MMP2 and MMP9.<br>c-Myc/Wnt/β-catenin axis. | [40,41] |
| Gastric cancer | Low expression correlates with poorer prognosis, poor histological grade, and angiogenesis. | Wnt/β-catenin pathway.<br>Low expression of RBMS3 induces overexpression of HIF1-A. | [8,42,43] |
| Esophageal squamous cell carcinoma | Low expression correlates with poorer prognosis.<br>Ectopic expression inhibits tumor growth. | RBMS3 induces downregulation of c-Myc and CDK4. | [44,45] |
| Lung cancer | Low expression correlates with worse OS. | Downregulation of RBMS3 and upregulation of c-Myc and β-catenin. | [46] |
| Papillary thyroid cancer | High expression of *RBMS3-AS1* correlates with shorter OS. | Further research is needed. | [47] |
| Breast cancer | High expression inhibits tumor growth, invasion, and migration.<br>Low expression correlates with poorer prognosis and shorter OS.<br>Levels of expression of ER and RBMS3 are correlated. | Wnt/β-catenin axis.<br>MEG3-miR-141-3p-RBMS3 axis. | [7,48–51] |

### 5.1. Bladder Cancer

The results showed that the downregulation of RBMS3 in bladder cancer was specifically related to a better overall survival (OS), with a higher expression of RBMS3 implicating a poorer prognosis. This was confirmed a few months later by Chen et al. The expression of RBMS3 was also significantly correlated with grade and stages T and M in the TNM scale [32,33].

### 5.2. Gallbladder Carcinoma (GBC)

The relationship between the expression of RBMS3 and bladder cancer is one of the most recently discussed in the literature. While studying the role of RBMS3 in gallbladder carcinoma, scientists found its downregulation at the mRNA, and protein levels in the tested specimens had an impact on their overall survival. A low expression correlated with a worse OS and acted as an independent negative prognostic factor. Moreover, the overexpression of RBMS3 successfully inhibits growth and promotes the apoptosis of GBC cell lines in in vitro studies. A low expression of RBMS3 also leads to increased angiogenesis, highlighting another process influenced by this protein [34].

### 5.3. Prostate Cancer

Studies on prostate cancer provided evidence of another biological mechanism of the role of RBMS3 in carcinogenesis. *RBMS3-AS3*, a long noncoding RNA (lncRNA), was found to play a significant role as an antitumor factor. LncRNAs are noncoding RNA fragments longer than 200 nucleotides with the ability to bind to different microRNAs (miRNAs) functioning as competing endogenous RNA (ceRNA) [35]. *RBMS3-AS3* binds competitively to miR-4534, increasing the level of its downstream target vasohibin 1 (VASH1), creating the molecular axis *RBMS3-AS3*/miR-4534/VASH1, which may play a pivotal role in prostate cancer development and treatment. *RBMS3-AS3* is downregulated in prostate cancer, which leads to an upregulation of miR-4534, which decreases the level of VASH1. Experimental upregulation of *RBMS-AS3* led to the inhibition of tumor growth, angiogenesis, and migration by the upregulation of VASH1. VASH1 as a downstream target is also important because it can work as an individual prognostic marker of prostate cancer, and recent studies have shown that its upregulation can inhibit lymphangiogenesis [36]. Another product of the *RBMS3* gene belongs to the group of circular RNAs (circRNAs) containing noncoding RNA with various functions. has_circ_0064644 was the most downregulated circRNA in prostate cancer. Its exact role in prostate cancer progression is yet to be revealed [37].

### 5.4. Epithelial Ovarian Cancer (EOC)

Managing patients with ovarian epithelial cancer is still an exceedingly challenging task for oncologists due to the high rate of relapses caused by chemoresistance. Platinum-based therapy, combined with surgical cytoreduction, is still one of the most effective methods of treatment in EOC. The studies conducted to elucidate the role of RBMS3 in EOC provided data to support the statement that the deletion of the region of chromosome 3 containing the gene for RBMS3 is correlated with a poorer prognosis and acts as an independent prognostic factor for relapse-free survival in this type of cancer. The deletion of *RBMS3* leads to the development of chemoresistance in the patient-derived xenograft (PDX) model and in EOC cell lines. The molecular mechanism underlying these results consists of several elements. First, the loss of *RBMS3* promotes efflux in EOC cells, preventing cytotoxic platinum from getting into the cells. The downregulation of RBMS3 significantly decreases platinum-induced DNA damage and apoptosis, indicating a potential role in restricting DNA damage repair. The lack of RBMS3 activates the Wnt/β-catenin pathway by allowing the strong negative regulator miR-126-5p to downregulate strong Wnt/β-catenin repressors. RBMS3 takes part in the competitive stabilization of many identified repressors, including DKK3, AXIN1, BACH1, and NFAT5 [38]. The *RBMS3* gene was also used in the creation of the tumor-mutation-burden-related signature model. This is a model that uses the total number of replacement and insertion/deletion (indel) mutations per basic group

in the exon coding region of the assessed gene in the genome of a tumor cell to predict overall survival in a specific cancer, in this case, ovarian cancer [39].

### 5.5. Nasopharyngeal Cancer (NPC)

Studies conducted on nasopharyngeal cancer introduced RBMS3 as a potential regulator of the cell cycle. Researchers provided evidence of the significant downregulation of RBMS3 in NPC cell lines and postoperational tumor specimens. The ectopic expression of RBMS3 proved to have the ability to inhibit tumor growth and foci formation. As the reason for these abilities, scientists provided a number of molecular mechanisms related to the cell cycle, including apoptosis and microvessel formation. RBMS3 increased the level of p53, which plays a crucial role in promoting the cell cycle from the G1 phase to the S phase. The upregulation of p53 creates a cascade of effects that prevent cells from going further in the cell cycle. An increased expression of p53 increases the expression of p21, which has the ability to suppress the cell cycle by inhibiting the complex cyclin E/CDK2. This complex has an influence on retinoblastoma proteins (RBs), decreasing their phosphorylated inactive form in favor of the unphosphorylated one, which has the ability to stop cells from reaching the next stage of the cell cycle. The overexpression of p53 along with MMP2 and MMP9 may also have an impact on the inhibition of microvessel formation by RBMS3. Changes in the expression of MMP2, MMP9, MMP7, and c-Myc may be explained by the inhibited nuclear translocation of β-catenin. C-Myc is an important downstream target of the Wnt/β-catenin pathway in this case, since its expression correlates with a poorer prognosis, and there is evidence of RBMS3's abilities to bind to the promoter region of *c-Myc*. The role of RBMS3 in the increased apoptotic activity of NPC was explained with the activation of caspase 9 and PARP by RBMS3 [40,41].

### 5.6. Gastric Cancer (GC)

All studies concerning the connection between the expression of RBMS3 and gastric cancer provided information about the downregulation of RBMS3 in this type of cancer. RBMS3 was found to have an impact on the secreted frizzled-related protein 1 (SFRP1), playing a significant role in the downregulation of the Wnt/β-catenin pathway by the competitive inhibition of Wnt-frizzled membrane receptor (Fzs) complexes. The low expression of RBMS3 and SFRP1 was found to correlate with a poorer prognosis. The expression of both proteins is statistically related to a poor histological grade and prognosis. The combined expression of RBMS3 and SFP1 acts as an independent prognostic factor in GC. Another downstream target regulated by RBMS3 in GC is the basic helix-loop-helix-PAS transcription factor α (HIF1-A) subunit of the HIF-1 protein, responsible for the induction of VEGF expression in cancer cells. VEGF is a key factor responsible for angiogenesis in tumors. The expression of HIF1-A is increased in GC cells. This, combined with a decreased level of the RBMS3 expression, correlates with a poor histopathological differentiation and a stronger angiogenesis. The overexpression of RBMS3 in GC cells revealed an increased percentage of cells in the G0/1 phase and a lower number of cells in the S phase of the cell cycle, but it had no statistically significant influence on cells in the stage G2/M. Additionally, lower expressions of CDK1, CDK6, E2F1, and MYC were observed, providing evidence of RBMS3's impact on the cell cycle in GC. RBMS3 also has an impact on circular RNA (circRNA) single-stranded enclosed RNAs, which are common regulators of carcinogenesis. *CircRBMS3* is postulated to be tied with an advanced TNM stage, poor differentiation, larger tumor size, and lymph node metastasis positivity by the regulation of miR-153 and SNAIL1. The overexpression of *circRBMS3* was also shown to be connected to a lower OS. The artificial knockdown of *circRBMS3* results in the inhibition of tumor growth and invasiveness [8,42,43].

### 5.7. Esophageal Squamous Cell Carcinoma (ESCC)

The loss of the 3p fragment of chromosome 3 is one of the most common chromosomal alterations in esophageal squamous cell carcinoma. One of the frequently lost genes is

*RBMS3* [44]. The downregulation of RBMS3 significantly correlates with poorer outcomes in patients with ESCC. The ectopic expression of RBMS3 results in tumor growth impairment confirmed by foci formation and tumor xenograft formation tests. Experimental data point to the downregulation of c-Myc and CDK4 as the mechanism mediating RBMS3's tumor suppressive gene (TSG) abilities. Interestingly, other cell-cycle-related proteins, such as CDK2 or cyclin E or D1, dysregulated in other types of cancer, do not seem to be involved in RBMS3's role in ESCC. Further studies showed that Rb, the downstream target of CDK2, was also found to be altered by the expression of RBMS3. A decreased level of CDK2 increases the level of inactivated phosphorylated Rb at Ser807/811 and Ser780 [45].

### 5.8. Lung Cancer

Depending on the type of lung cancer, different approaches to the role of RBMS3 were taken, highlighting different aspects of RBMS3's effect on lung cancer progression. Lung squamous cell carcinoma (LSCC) was characterized by the downregulation of RBMS3 and the upregulation of c-Myc and β-catenin. Oddly enough, there was only a statistically significant correlation of RBMS3's expression with c-Myc. The combined positive expression of RBMS3 and negative expression of c-Myc act as an independent prognostic factor of shorter OS [46]. As for small-cell lung cancer (SCLC), Xiuwei Li et al. provided evidence of the downregulation of RBMS3 and its upstream miRNA hsa-miR-7-5p by using bioinformatic methods. Hsa-miR-7-5p was previously reported to display tumor-suppressive properties in glioma and glioblastoma by the regulation of the EGFR, PI3K/ATK, Raf/MEK/ERK, and IGF-1R pathways [52,53]. Another type of lung cancer discussed in the context of RBMS3 expression was non-small-cell lung cancer (NSCLC). By using computational methods, scientists identified RBMS3 as a core transcription factor regulating lung-adenocarcinoma-associated genes [54]. Other bioinformatic analyses provided evidence of RBMS3 belonging to the group of genes most negatively correlated with tumorigenesis and being dysregulated in precancer cells. Furthermore, this dysregulation advances through cancer progression [55].

### 5.9. Papillary Thyroid Cancer

The analysis of lncRNA in papillary thyroid cancer revealed that another product of *RBMS3*'s expression, *RBMS3-AS1*, is closely associated with a patient's shorter OS, broadening the variety of tumors in which RBMS3 has the potential to be a diagnostic marker [47].

### 5.10. Hepatocellular Carcinoma (HCC)

Studies conducted on hepatocellular carcinoma present RBMS3 in a position of effector instead of regulator. In this case, an upregulated miR-1269 is responsible for altering the expression of *RBMS3* and eight other genes: *AGAP1*, *AGK*, *BMPER*, *BPTF*, *C16orf74*, *DACT1*, *LIX1L*, and *ZNF706* [56].

### 5.11. Neuroblastoma

The potential role of RBMS3 in the carcinogenesis of the neuroblastoma was discovered through high-resolution array copy number analyses that showed the presence of homozygous deletion on 3p. However, there are no further studies on this issue [31].

### 5.12. Breast Cancer (BC)

The role of RBMS3 has been most extensively explored in breast cancer among all types of cancer. The expression profile of *RBMS3* at the protein and RNA levels is downregulated. The overexpression of *RBMS3* inhibits the growth, invasion, and migration of BC cells. In vivo experiments conducted in mice also showed an attenuation of tumor growth. As for the clinicopathological characteristics, the downregulation of *RBMS3* correlates with a poor prognosis and a shorter OS. A negative ER status corresponding with the expression of RBMS3 and the combined expression of both these parameters act as independent

prognostic factors. The molecular mechanisms underlying these effects include the impact on the Wnt/β-catenin pathway and the cell cycle, confirmed by the inhibited expression of β-catenin, c-Myc, and cyclin D1 in RBMS3 expressing cancer cells [7,48]. Another point of regulation lies in the lncRNA (long noncoding RNA) maternally expressed gene 3 (MEG3)-miR-141-3p-RBMS3 axis. LncRNA encoded by *MEG3* was found to have tumor-suppressive abilities in different types of tumors, including glioma, gastric cancer, and melanoma. MiR-141-3p is a microRNA (miRNA) belonging to the miR-200 family dysregulated in many tumors. An overexpression of miR-141-3p was found in bladder cancer and esophageal squamous cell carcinoma. A low expression of MEG3 upregulates miR-141-3p, which anterogradely downregulates RBMS3 in BC. MEG3 is a tumor-suppressive gene regulating AKT and NF-κB signaling pathways, inducing apoptosis through its impact on Bcl-2 and C casp-3 and p53 signaling. MiR-141-3p is a miRNA whose role depends on the type of tumor, with capabilities ranging from tumor-suppressive abilities to overexpression correlated with poor prognosis and chemoresistance [49–51]. Moreover, a recent study showed that the *RBMS3* gene expression in the tumor-associated stromal cells of breast tumor was gradually downregulated among grade I, II, and III of breast cancer. The downregulation of this gene was also correlated with worse clinical outcome and poorer survival prognosis [57].

## 6. Epithelial–Mesenchymal Transition and Role of RBMS3 in This Process

Epithelial–mesenchymal transition (EMT) is a biological process that allows epithelial cells to switch their phenotype to quasi-mesenchymal [58–60]. EMT causes epithelial cells to lose characteristic features, such as tight cell–cell junctions [61] and cell polarity [59], and acquire mesenchymal properties instead [62]. This is first observed during embryogenesis, in gastrulation or tissue morphogenesis [63]. Furthermore, the process plays a crucial role in wound healing, fibrosis, and tumor progression [60–66]. The reverse process is called MET, from mesenchymal–epithelial transition, and it occurs when the mesenchymal cells acquire epithelial characteristics [67].

Typically, epithelial cells appear as cells attached to basal lamina, with tight cell–cell junctions and apical–basal polarity [68]. When it comes to EMT, epithelial cells lose these properties and the ability of the expression of E-cadherin—a molecule that is essential to maintaining the epithelial phenotype [58]. The loss of E-cadherin is considered to be a hallmark of EMT along with the acquisition of the expression of vimentin of N-cadherin [68]. During EMT, the epithelial cells, which have a typical cobblestone morphology, transform into quasi-mesenchymal cells, which have a rather fibroblastic-like phenotype [69]. This transition allows cells to acquire a migratory phenotype and become more invasive [70]. These changes in phenotype require rearrangements of the cytoskeleton and the cell metabolism [71]. Due to the acquisition of these properties, EMT plays a significant role in tumor progression, metastasis, and malignancy [58,71].

Three types of EMT processes can be distinguished. Type 1 describes an EMT that occurs in the development of tissues. The EMT subtype that occurs in fibrosis and wound healing is type 2, with type 3 being observed in cancer cells [72]. Although, historically, EMT was discovered by developmental biologists [63], modern studies focus on the link between EMT and cancer [73]. Recent observations suggest that EMT is also involved in the therapeutic resistance of various tumors [67,74,75].

EMT is a process that is strictly determined by genetic mechanisms. Several transcription factors involved in this phenotype change have been discovered [76]. Some well-described EMT-TFs (epithelial–mesenchymal transition transcription factors) are SNAIL1, SNAIL2, TWIST1, and ZEB1. However, the list of EMT-TFs is way longer, and there are many more transcription factors involved in EMT, for instance, FOX- or SOX-TF [76]. The crucial signaling pathways of EMT are Wnt and TGF-β, but other pathways, such as Notch or Hedgehog, are also involved [68]. Some of the descriptions of the molecular mechanisms seem to be quite preposterous; thus, there is still a lot of speculation and uncertainty surrounding the topic.

As it has already been mentioned, EMT plays a major role in cancer progression. EMT allows cancer cells to become more mesenchymal-like. EMT is probably responsible for the creation of circulating tumor cells (CTCs), which are strictly connected to the ability to metastasize [77]. CTCs are an element of the invasion-metastatic cascade, and EMT is believed to be involved it this type of tumor progression [58]. It is worth noticing that the reverse process, mesenchymal–epithelial transition (MET), is also important for the ability of cancer cells to metastasize [67,78,79]. EMT is considered to be a relevant process in the development of cancer steam cells (CSCs). Therefore, it could be responsible for therapeutic resistance [58,78].

With a better understanding of EMT's complexity and its importance and vital role in cancer progression, invasion, and the development of metastases and CSCs comes the necessity to find and describe the key regulators of this process. RBMS3 is a novel potential regulator of EMT, with an increasing amount of data trying to unveil its molecular role in this process. Figure 2 and Table 3 present the currently proposed mechanisms of the impact of RBMS3 on the EMT process.

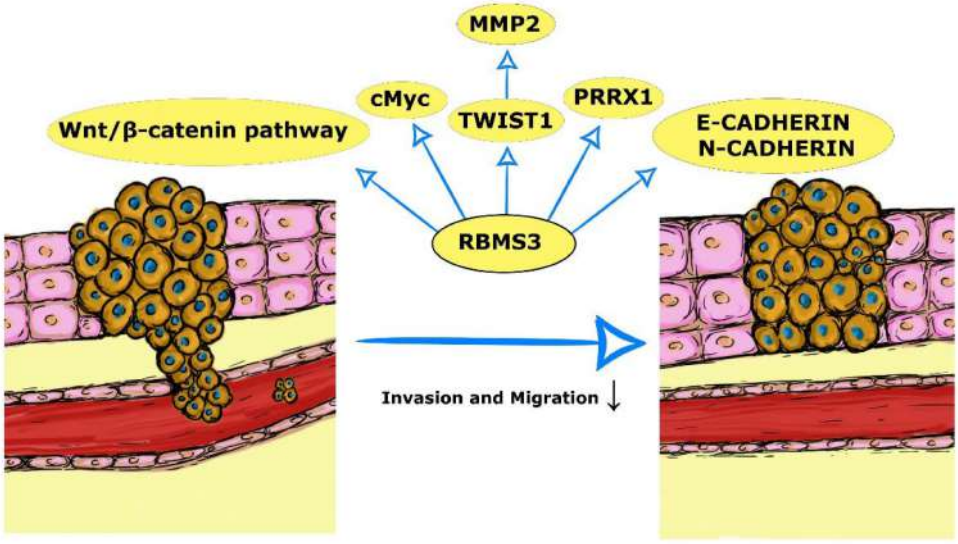


**Figure 2.** Mechanisms of the impact of RBMS3 on the EMT process.

The Wnt/β-catenin signaling pathway is a critical molecular mechanism regulating the EMT process. Downstream targets of Wnt include, among others, Twist, Snail, and MMP7 genes facilitating EMT [9]. In several of the previously discussed types of cancer, the Wnt/β-catenin pathway was inhibited by RBMS3's expression. The expression of c-Myc, another downstream target of the Wnt/β-catenin pathway, was also investigated and found statistically correlated with RBMS3's expression.

Several studies focus solely on RBMS3's interaction with the EMT machinery. While studying breast cancer in 2019, Zhu L et al. identified a regulatory axis consisting of RBMS3, TWIST1, and matrix metalloproteinase 2 (MMP2), responsible for the migration and invasion of the tumor. The expression of RBMS3 downregulated the expression of TWIST1, one of the key factors of EMT, and consecutively, its downstream target MMP2, leading to EMT impairment and invasion and migration inhibition [9]. Another study conducted on breast cancer provided interesting data stating that the expression of RBMS3 is a required factor for EMT induction in immortalized mammary epithelial cell lines. In the triple negative breast cancer (TNBC) model, RBMS3 was essential for maintaining the mesenchymal phenotype, invasiveness, and migration ability. In vivo experiments showed the loss of RBMS3 to impair the growth of the tumor and its ability to create metastasis. As the potential molecular basis of this process, the authors indicated RBMS3's ability to influence expression and stabilize *PRRX1* mRNA, a transcription factor regulating EMT [80]. Research conducted on gastric cancer by Zhao seems to be coherent with Zhu's results, showing an increased expression of E-cadherin and a decreased expression of

N-cadherin and β-catenin in RBMS3-overexpressing gastric cancer cells. Moreover, an increased expression of RBMS3 significantly decreased the invasive abilities of cells [81].

Taking into consideration all the information contained in this chapter, there is convincing evidence of RBMS3 being one of the regulators involved in the EMT process, even though the exact mechanism of this regulation requires further investigation and may differ depending on the molecular subtype of cancer.

**Table 3.** Proposed molecular mechanisms of RBMS3's impact on EMT.

| Type of Cancer | Currently Proposed Mechanisms of RBMS3' Impact on EMT |
| --- | --- |
| Breast cancer | 1. The expression of RBMS3 downregulates the expression of TWIST1 and, consecutively, its downstream target MMP2, leading to EMT impairment [9]<br>2. Loss of RBMS3 impairs the growth of the tumor and its ability to create metastasis by influencing expression and stabilizing *PRRX1* mRNA, a transcription factor regulating EMT [80] |
| Gastric cancer | 1. Increased expression of E-cadherin and decreased expression of N-cadherin and β-catenin in RBMS3-overexpressing cancer cells [81] |

## 7. Conclusions

All the information provided in this review depicts *RBMS3* as a functionally versatile gene that uses its main and multiple alternative products of expression to play a significant role in embryonic development and the pathogenesis of many different diseases, especially the induction and progression of cancers. The main ways in which RBMS3 impacts cells are the regulation of the expression or stabilization of miRNA, inhibiting Wnt/β-catenin signaling pathway and other EMT-related transcription factors. These molecular characteristics make RBMS3 a promising biomarker of OS and a prognostic factor in neoplastic processes, where statistical data support this statement for many different types of cancer. Another potential use of RBMS3 is as a target for anticancer drugs, thanks to its function as TSG and its proven ability to suppress cancer migration and invasive abilities. Artificially increased expression of RBMS3 utilizing genome editing techniques may potentially improve the outcome of standard therapies in many types of cancers. Increased expression of RBMS3 may prevent the creation of micrometastases that are too small to be picked up in diagnostic imaging and may lead to relapse of tumor.

There are some limitations to targeting RBMS3 mainly concerning the lack of a deep understanding of molecular mechanisms that are responsible for RBMS3 tumor suppressive abilities and the regulation of this properties. Additionally, currently, there are not enough data concerning the role of RBMS3 expression in different types of healthy human tissues and the consequences of RBMS3 level alteration. However, the year-on-year increasing amount of data and the incoherencies of some of the results indicate that the molecular role of RBMS3, especially in the regulation of cancer development, is a good subject for further research that may lead to the development of novel diagnostic and therapeutic strategies that will improve the outcome of patients with neoplastic diseases.

**Author Contributions:** Conceptualization, T.G. (Tomasz Górnicki), J.L. and M.M.; methodology, T.G.; software, T.G.; validation, J.G., M.P.-O. and P.D.; formal analysis, J.G. and M.M.; investigation, T.G. and J.L.; writing—original draft preparation, T.G., J.L. and M.M.; writing—review and editing, J.G., M.P.-O. and P.D.; visualization, T.G.; supervision, J.G., P.D. and M.P.-O.; project administration, J.G., P.D. and M.P.-O. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

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

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# 7. ARTYKUŁY Z CYKLU PUBLIKACJI W FORMIE ZAŁĄCZNIKÓW

## Artykuł drugi

**Tytuł:**

*Expression of RBMS3 in Breast Cancer Progression.*

**Autorzy:**

Górnicki T, Lambrinow J, Mrozowska M, Romanowicz H, Smolarz B, Piotrowska A, Gomułkiewicz A, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J.

**Czasopismo**

- International Journal of Molecular Sciences, 2023 Feb 2;24(3):2866, doi: 10.3390/ijms24032866.

**Punktacja**

IF: 4.9, punktacja MNiSW: 140 pkt

International Journal of
*Molecular Sciences*

MDPI

*Article*

# Expression of RBMS3 in Breast Cancer Progression

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**Abstract:** The aim of the study was to evaluate the localization and intensity of RNA-binding motif single-stranded-interacting protein 3 (RBMS3) expression in clinical material using immunohistochemical (IHC) reactions in cases of ductal breast cancer (in vivo), and to determine the level of RBMS3 expression at both the protein and mRNA levels in breast cancer cell lines (in vitro). Moreover, the data obtained in the in vivo and in vitro studies were correlated with the clinicopathological profiles of the patients. Material for the IHC studies comprised 490 invasive ductal carcinoma (IDC) cases and 26 mastopathy tissues. Western blot and RT-qPCR were performed on four breast cancer cell lines (MCF-7, BT-474, SK-BR-3 and MDA-MB-231) and the HME1-hTERT (Me16C) normal immortalized breast epithelial cell line (control). The Kaplan–Meier plotter tool was employed to analyze the predictive value of overall survival of *RBMS3* expression at the mRNA level. Cytoplasmatic RBMS3 IHC expression was observed in breast cancer cells and stromal cells. The statistical analysis revealed a significantly decreased RBMS3 expression in the cancer specimens when compared with the mastopathy tissues ($p < 0.001$). An increased expression of RBMS3 was corelated with HER2(+) cancer specimens ($p < 0.05$) and ER(−) cancer specimens ($p < 0.05$). In addition, a statistically significant higher expression of RBMS3 was observed in cancer stromal cells in comparison to the control and cancer cells ($p < 0.0001$). The statistical analysis demonstrated a significantly higher expression of *RBMS3* mRNA in the SK-BR-3 cell line compared with all other cell lines ($p < 0.05$). A positive correlation was revealed between the expression of RBMS3, at both the mRNA and protein levels, and longer overall survival. The differences in the expression of RBMS3 in cancer cells (both in vivo and in vitro) and the stroma of breast cancer with regard to the molecular status of the tumor may indicate that RBMS3 could be a potential novel target for the development of personalized methods of treatment. RBMS3 can be an indicator of longer overall survival for potential use in breast cancer diagnostic process.

**Keywords:** RNA-binding protein 3 (RBMS3); carcinogenesis; cancer prevention; target discovery; target therapy; epithelial–mesenchymal transition (EMT)



**Citation:** Górnicki, T.; Lambrinow, J.; Mrozowska, M.; Romanowicz, H.; Smolarz, B.; Piotrowska, A.; Gomułkiewicz, A.; Podhorska-Okołów, M.; Dzięgiel, P.; Grzegrzółka, J. Expression of RBMS3 in Breast Cancer Progression. *Int. J. Mol. Sci.* **2023**, *24*, 2866. https://doi.org/10.3390/ijms24032866

Academic Editor: Laura Paleari

Received: 15 December 2022
Revised: 25 January 2023
Accepted: 30 January 2023
Published: 2 February 2023



## 1. Introduction

According to the WHO GLOBOCAN 2020 report data, breast cancer (BC) is the most commonly diagnosed cancer, with nearly 2.3 million new cases worldwide in 2020 [1]. It is also the fifth leading cause of cancer mortality, responsible for 6.9% of all cancer-related deaths in 2020 [1]. Based on the Global Cancer Observatory forecast, by the year 2030, the number of new cases will increase to 2.7 million per year [2]. Genetic and molecular analyses have allowed researchers to identify four main intrinsic subtypes of BC: luminal A, luminal B, HER2-enriched and triple-negative breast cancer (TNBC, also called basal-like) [3]. As

indicated in Table 1, each subtype differs in its expression of biomarkers, especially estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [4,5]. The expression of these biomarkers plays a significant role, among other anatomical features, in estimating the prognosis of BC [3].

**Table 1.** Different molecular subtypes of breast cancer. Each molecular subtype is defined by the expression of three main receptors: estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 [4].

| Molecular Subtype of Breast Cancer | Receptor Status | | |
|---|---|---|---|
| | Estrogen Receptor (ER) | Progesterone Receptor (PR) | Human Epidermal Growth Factor Receptor 2 (HER2) |
| Luminal A | + | ≥20% | - |
| Luminal B | + | <20% | +/- |
| HER2-enriched | - | - | + |
| Triple-negative breast cancer (TNBC) | - | - | - |

RBMS3 is a glycine-rich protein that belongs to the family of c-Myc gene single-strand binding proteins (MSSPs). RBMS3, similarly to other MSSPs, is involved in processes that are crucial for cell life, such as cell-cycle progression and apoptosis [6,7]. RBMS3 participates in various processes, both physiological and pathological, e.g., embryogenesis or liver fibrosis [8,9]. Published papers indicate that RBMS3 can be viewed as a regulating factor of carcinogenesis in various cancers, including ovarian and nasopharyngeal cancers [10–12]. RBMS3 is postulated to regulate the progression of nasopharyngeal cancer by influencing the expression of the p53 protein, becoming a potential regulator of the cell cycle in this type of cancer [12]. In ovarian cancer, it can be involved in drug-resistance mechanisms [10]. It has been reported that RBMS3 participates in the carcinogenesis process of breast cancer, since it is often described as a suppressor protein. In recent studies, authors have provided data that point to the fact that a certain level of RBMS3 is necessary for cancer progression [13–18]. The currently postulated mechanisms explaining the role of RBMS3 in the progression of breast cancer include involvement in the epithelial–mesenchymal transition (EMT) by inhibiting the Wnt/β-catenin signaling pathway and other EMT-related transcription factors, such as TWIST1 or PRRX1 [12,17,19,20]. Another mechanism of influence of RBMS3 in breast cancer is its presence in the miR-141-3p/RBMS3 axis that inhibits proliferation and promotes apoptosis in breast cancer cells [15]. Another study reported data related to RBMS3′s suppression leading to downregulation of cell programmed death ligand-1 (PD-L1) in TNBC, resulting in increased anti-tumor immune activities [18]. There is also evidence that the expression of RBMS3 in the stroma cells of breast cancer could have an impact on the progression of BC [16]. Although RBMS3 seems to play a major role in carcinogenesis, there remains a need for extensive research because of its complex influence on breast cancer.

The aim of this study is to discuss the role of RBMS3 in breast cancer. Using immunohistochemical staining performed on paraffin-embedded blocks of breast cancer samples and molecular analysis performed with breast cancer cells from cell-line cultures, we showed the correlation between RBMS3 levels and particular intrinsic subtypes of BC. A further aim of this study is to discuss RBMS3 as a novel potential therapeutic target and biomarker of overall survival in breast cancer.

## 2. Results

### *2.1. The Immunohistochemical Intensity of RBMS3′s Expression Varies in Cancer Cells, the Stroma of the Tumor, and the Control Mastopathy Cases, Exhibiting a Dependence on the Expression of Crucial Breast Cancer Receptors*

The analysis of the immunohistochemical expression of RBMS3 in 490 cases of IDC and 26 cases of mastopathy showed a statistically significant decrease in RBMS3 expression in the cancer specimens compared to the mastopathy samples (Mann–Whitney test $p < 0.001$, Figure 1a, Figure 2a,b). Furthermore, the statistical analysis of the clinical data together with the immunohistochemical expression of RBMS3 showed a significantly increased expression of RBMS3 in the cancer cells of the HER2 positive cases (Mann–Whitney test $p < 0.05$, Figure 1b). Meanwhile, increased expression of RBMS3 correlated with the negative status of the estrogen receptor (Mann–Whitney test $p < 0.05$, Figure 1c). However, there was no statistically significant difference in the expression of RBMS3 in cancer cells between progesterone-positive and -negative cases.

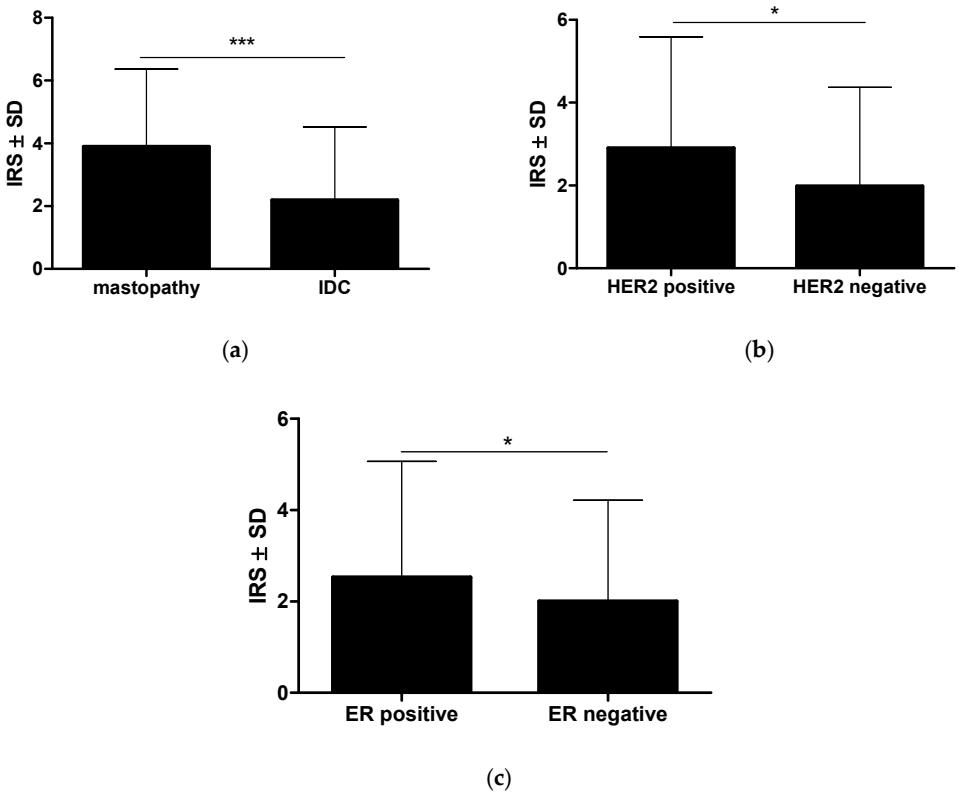
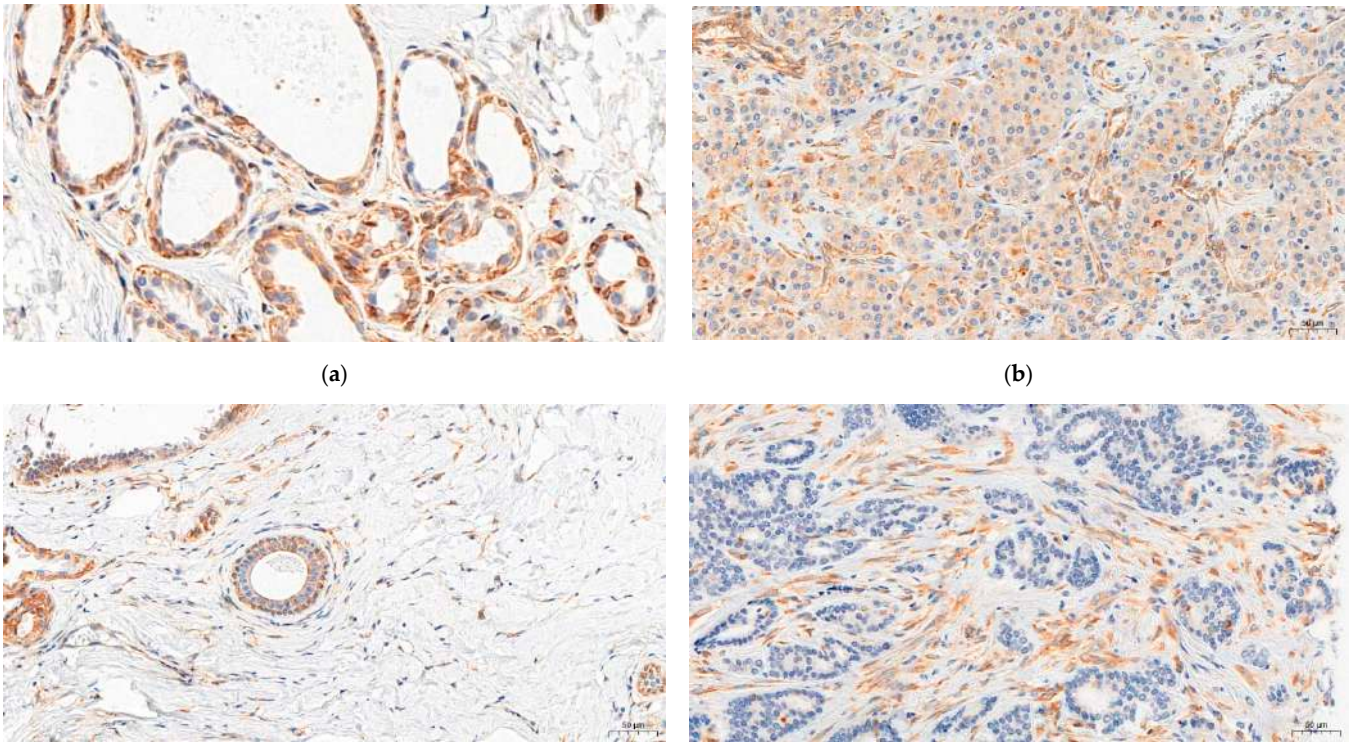



**Figure 1.** (**a**) The statistical analysis revealed a significantly higher RBMS3 expression as assessed by the immunoreactive score in the control mastopathy cases compared with the cancer cells in breast cancer. (**b**) The breast cancer cells with positive expression of HER2 and (**c**) estrogen receptors presented a higher expression of RBMS3 compared with tumors lacking expression of these receptors (Mann–Whitney test * $p < 0.05$; *** $p < 0.001$) (IDC—invasive ductal carcinoma, IRS—immunoreactive score).

Expression of RBMS3 in the stroma of the cancer cases was significantly higher than in the control specimens (Mann–Whitney test $p < 0.0001$, Figure 3a, Figure 2c,d). Moreover, RBMS3 expression in TNBC samples was significantly lower than in the other molecular types (Mann–Whitney test $p < 0.001$, Figure 3b). Further investigation in the stroma of breast cancer showed significant increases in RBMS3 expression in the specimens with positive expression of the progesterone receptor and samples with positive expression of the estrogen receptor (respectively, Mann–Whitney test $p < 0.01$ and $p < 0.001$, Figure 3c,d). On the other hand, we observed no correlation of RBMS3 expression with expression of the

HER2 receptor. RBMS3 expression in the stroma of IDC was significantly higher than in the cancer cells (Mann–Whitney test $p < 0.0001$, Figure 3e).

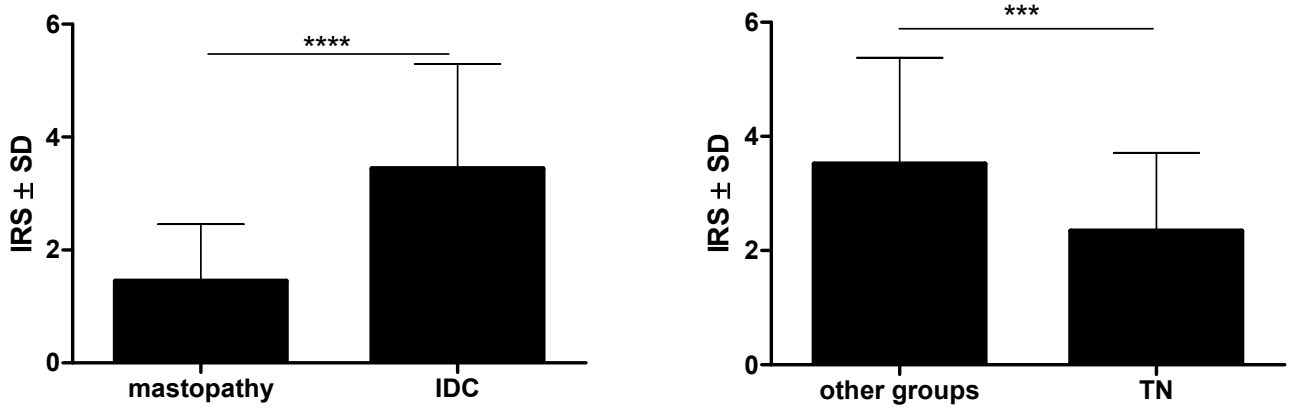


(**a**) (**b**)

(**c**) (**d**)

**Figure 2.** Immunohistochemical visualization of RBMS3 expression. (**a**) High cytoplasmic expression of RBMS3 in mastopathy cases. (**b**) Low expression of RBMS3 in breast cancer cells. (**c**) Low cytoplasmic expression of RBMS3 in the stroma of mastopathy cases and (**d**) high expression in the stroma cells of the breast cancer cases. Magnification ×200.

There were no statistically significant differences in the expression of RBMS3 with regard to the grade, TNM, and stage of the cancer. This absence of difference was observed in the cancer cells and the stroma.




(**a**) (**b**)

**Figure 3.** *Cont.*

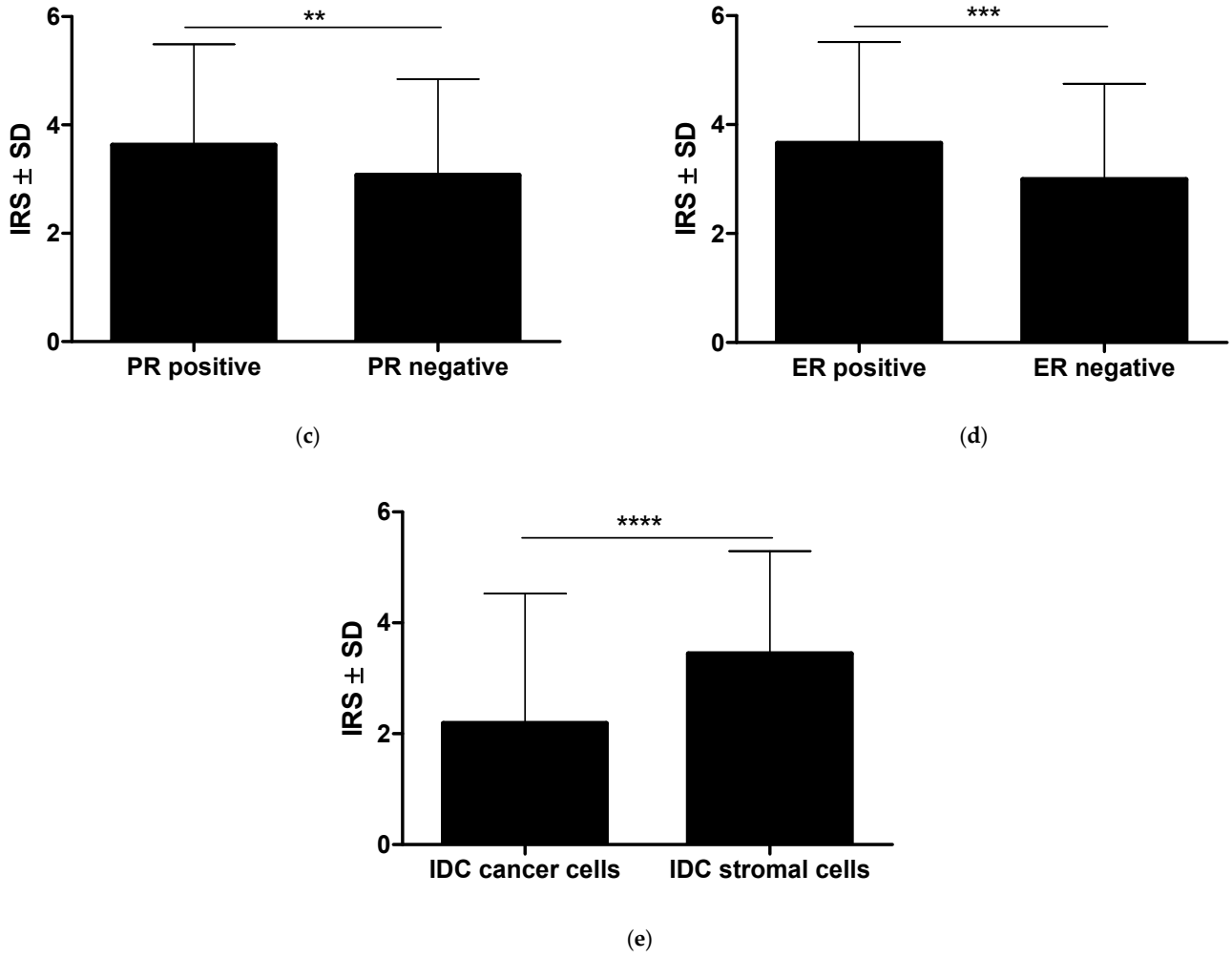
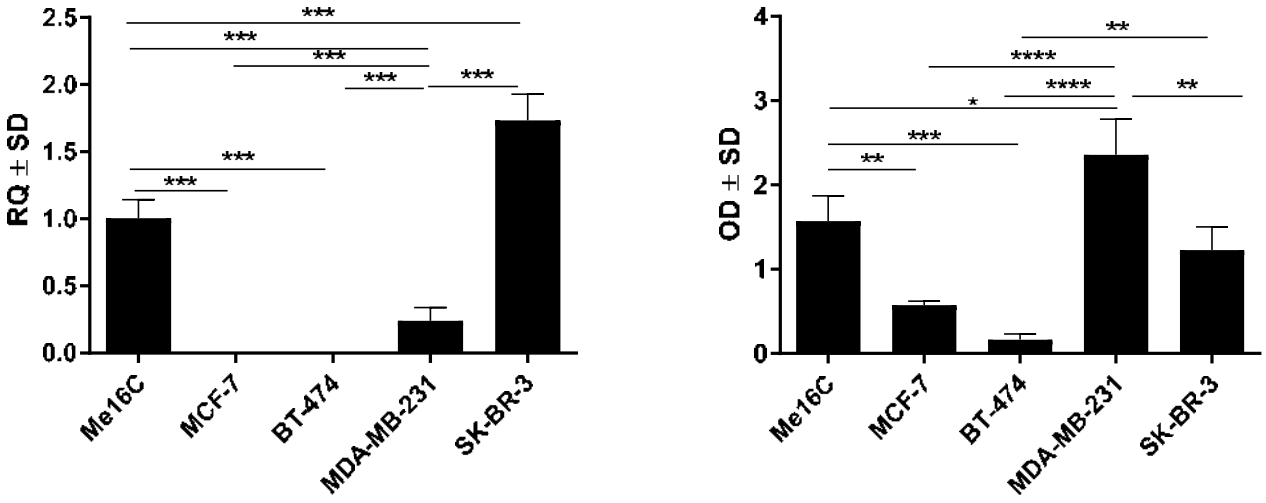



**Figure 3.** Analysis of RBMS3 expression in the stroma of breast cancer: (**a**) RBMS3 expression in the stroma of breast cancer was statistically higher than in the mastopathy cases; (**b**) Triple-negative (TN) cases of breast cancer displayed lower expression of RBMS3 in the stroma than the other molecular types of breast cancer combined. (**c**) Significant increase in RBMS3 expression in the specimens with positive expression of the progesterone receptor and (**d**) with positive expression of the estrogen receptor. The expression of RBMS3 was statistically lower in the cancer cells than in the stromal cells of the breast cancer specimens (**e**). (Mann–Whitney test ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) (IDC—invasive ductal carcinoma).

### *2.2. In Vitro Analysis of RBMS3 Expression Differs from RBMS3 Expression in Clinical Material*

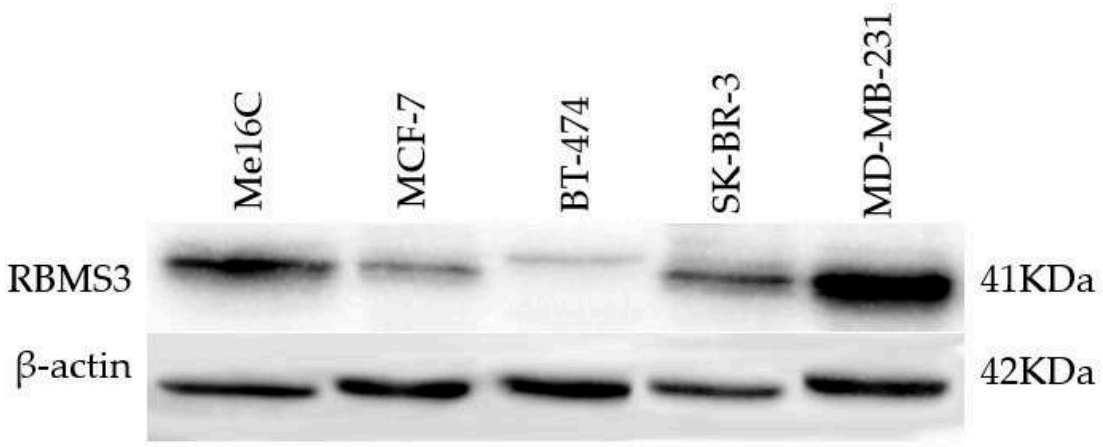
For further investigation of the difference in RBMS3 expression in the different molecular types of breast cancer, we performed an RT-qPCR analysis of *RBMS3* expression at the mRNA level in the chosen cell lines representing the various molecular types of breast cancer. When compared to the control Me16C cell line (ANOVA and Bonferroni's multiple comparison test $p < 0.05$, Figure 4a), the expression of *RBMS3* was significantly different (mostly lower) in all the examined cell lines, with the only exception being the SK-BR-3 cell line. *RBMS3* expression was highest in the SK-BR-3 cell line among all the investigated cell lines. The Western blot analysis of the protein expression showed a significantly higher expression of RBMS3 in the control cell line than in the MCF-7 and BT-474 cancer cell lines (ANOVA and Bonferroni's multiple comparison test $p < 0.05$, Figure 4b) There is a visible and statistically significant trend that the more aggressive types of breast cancer, including TNBC and HER-2-positive cancers, presented higher expression of RBMS3 than their benign counterparts.




(a)




(b)




(c)

**Figure 4.** In vitro analysis of RBMS3 expression in breast cancer cell lines representing the four main molecular types of breast cancer and a control cell line (Me16C). (**a**) The statistical analysis of *RBMS3*'s expression at the mRNA level showed a significantly different expression of *RBMS3* in all the examined cell lines in comparison to the control Me16C cell line. (**b**,**c**) Analysis at the protein level showed a significantly higher expression of RBMS3 in the MDA-MB-231 and SK-BR-3 cell lines than in the MCF-7 and BT-474 cell lines; ((**a**) Bonferroni's multiple comparison test, (**b**) Bonferroni's multiple comparison test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

### 2.3. RBMS3 Expression May Be an Indicator of Longer Overall Survival

The analysis of the clinical data regarding the survival of patients showed shorter overall survival in the group of patients without an IHC expression of RBMS3 (Gehan–Breslow–Wilcoxon test $p = 0.051$, Figure 5). The univariate and multivariate Cox analyses of the overall survival indicated that only G, pT, and pN were independent prognostic factors (Table 2).

Additionally, using the Kaplan–Meier estimator we performed an analysis of the *RBMS3* mRNA expression of 2976 cases of breast cancer. This revealed that the group of patients with lower *RBMS3* expression (cut-off point: median) had statistically significant shorter overall survival ($p < 0.0001$, Figure 6) [21].

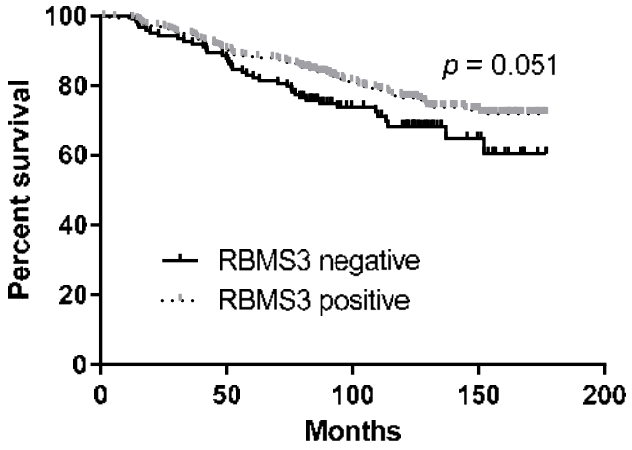



**Figure 5.** The analysis of the clinical data regarding the survival of patients showed shorter overall survival in the group of patients without IHC expression of RBMS3 (Gehan–Breslow–Wilcoxon test $p = 0.051$).

**Table 2.** Univariate and multivariate Cox analyses of overall survival in cases of invasive ductal carcinoma.

| Characteristics | Univariate Cox Analysis of Survival | | | | Multivariate Cox Analysis of Survival | | | |
|---|---|---|---|---|---|---|---|---|
| | *p*-Value | Hazard Ratio | HR 95% CI Lower | HR 95% CI Upper | *p*-Value | Hazard Ratio | HR 95% CI Lower | HR 95% CI Upper |
| G1 vs. G2-G3 | **<0.0100** | **3.0873** | **1.5179** | **6.2792** | **<0.0100** | **2.5309** | **1.2509** | **5.1208** |
| pT1 vs. pT2-pT4 | **<0.0001** | **2.4469** | **1.7123** | **3.4966** | **<0.0010** | **2.0371** | **1.4201** | **2.9221** |
| pN0 vs. pN1-pN3 | **<0.0001** | **2.6544** | **1.8541** | **3.8001** | **<0.0001** | **2.1583** | **1.4997** | **3.1062** |
| ER negative vs. ER positive | 0.2260 | 0.7987 | 0.5550 | 1.1493 | | | | |
| PR negative vs. PR positive | 0.1416 | 0.7626 | 0.5313 | 1.0946 | | | | |
| HER2 0-HER2 2 vs. HER2 3 | 0.4485 | 1.3206 | 0.64338 | 2.7105 | | | | |
| Triple-negative vs. other groups | 0.3742 | 1.3843 | 0.67566 | 2.8361 | | | | |
| RBMS3 IRS stromal: 0 vs. 1–12 | 0.3196 | 0.6548 | 0.2844 | 1.5075 | | | | |
| RBMS3 IRS cancer: 0 vs. 1–12 | **<0.0500** | **0.6470** | **0.4470** | **0.9365** | 0.1429 | 0.7576 | 0.5226 | 1.0983 |

ER—estrogen receptor, PR—progesterone receptor, RBMS3—RNA-binding motif single-stranded-interacting protein 3, IRS—immunoreactive scale, HR—hazard ratio, CI—confidential interval.

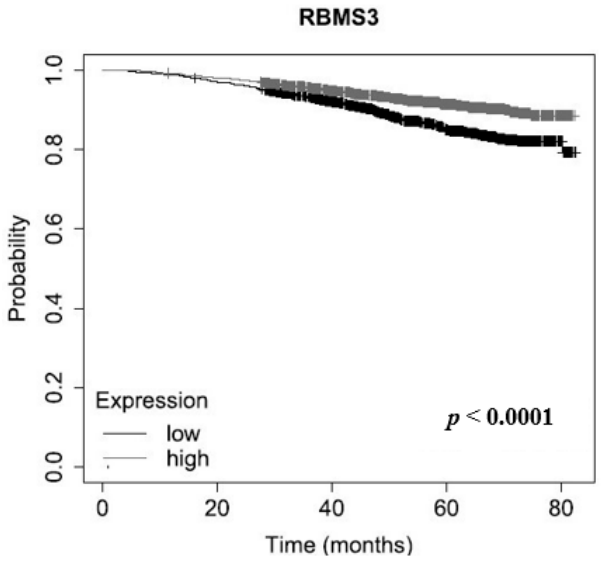



**Figure 6.** Analysis of *RBMS3* mRNA expression in 2976 cases of breast cancer, using the Kaplan–Meier estimator. The analysis revealed a significant positive correlation between the expression of *RBMS3* and overall survival ($p < 0.0001$) [21].

## 3. Discussion

RBMS3 is reported to be deregulated in many different types of neoplastic processes, for example, gastric cancer, esophageal squamous cell carcinoma, breast cancer, or gall bladder carcinoma [13,22–24]. In this study, we have discussed the role of RBMS3 in the progression of breast cancer with particular emphasis on receptor expression and the molecular type. We provided an analysis of RBMS3 expression in clinical material and cell lines, and presented experimental data supporting the statement of the potential role of RBMS3 expression in tumor stromal cells.

The results of our experiments apparently support previous studies' results that indicate the downregulation of RBMS3 expression in breast cancer cells [13,19] and its correlation with negative estrogen-receptor status [14]. In addition, we discovered another potential interaction of RBMS3 with a positive HER2-receptor status, supported by an immunostaining analysis and the high expression of *RBMS3* at the mRNA level in the SK-BR-3 line (representing the HER2-enriched subtype). At the protein level, the expression levels of RBMS3 in the SK-BR-3 and MDA-MB-231 cell lines were the highest among all the examined breast cancer cell lines and were significantly higher than in MCF-7 and BT-474 cell lines. A significantly higher expression of RBMS3 in more aggressive types of tumors characterized by the lack of estrogen-receptor expression, and in the case of the SK-BR-3 cell line the presence of the HER2 receptor, may indicate that a certain level of RBMS3 expression is necessary for specific types of cancer progression and their ability to create metastasis [20,25]. RBMS3's anticancer function could be related to the mechanisms that regulate adhesiveness and invasiveness, which are also associated with the EMT process in cancer. These findings are in partial agreement with recent reports that provide evidence of RBMS3 knockdown resulting in the impairment of in vivo tumor growth and a decreased level of angiogenesis [17,18]. It is important to mention that the research carried out by Block et.al and Zhu et.al was conducted only on the triple-negative type of breast cancer cells. The results provided in this study support the claim that RBMS3 expression in the TNBC and HER-2-enriched types is similarly high as in the control cell lines, meaning that RBMS3 could possibly act as a suppressor in Lum-A and Lum-B types of breast cancer. They may also suggest that a normal level of RBMS3 expression is necessary for the growth of TNBC and HER-2-enriched types of breast cancer; this observation requires more detailed investigation.

The role of the tumor microenvironment (TME) is a topic of rapidly increasing interest among scientists [26]. The TME is the unique environment in which the tumor develops. It consists of an extracellular matrix, blood vessels, signaling molecules, and multiple types of cells that play a pivotal role in tumor cancerogenesis by stimulating and facilitating uncontrolled cell proliferation [27,28]. Stromal cells are an integral part of the TME. Alongside other elements, they play a part in the maintenance of cancer stemness by promoting angiogenesis, invasion, metastasis, and chronic inflammation [29]. The transcriptomic analysis of the *RBMS3* gene's expression in the stromal cells of breast cancer provides evidence of its being gradually downregulated through all three grades of breast cancer [16]. The results that we present suggest a higher expression of the RBMS3 protein in the stroma cells of breast cancer compared with the mastopathy control cases or the cancer cells, with no significant differences between grades. Together, these data suggest that RBMS3's deregulation in the stroma of the tumor may influence the role of stromal cells in breast cancer through currently unknown mechanisms. Furthermore, there may exist a currently unknown post-transcriptional mechanism regulating the expression of RBMS3 in the stroma of the tumor, which could explain the grade-dependent expression of *RBMS3* and the lack of grade dependency at the protein level. A negative correlation of RBMS3 expression in the stromal cells with TNBC, and a positive one with ER- and PR-receptor status of the tumor, may indicate that there is a possibility for RBMS3 to display an antitumor effect depending on the molecular characteristics of the tumor. Specifically, a negative correlation with TNBC may indicate the tumor-suppressor role of RBMS3 in

breast cancer stroma. A higher expression of RBMS3 in the stroma of breast cancer may indicate the potentially important role of the TME in the progression of IDC.

In addition to its potential antitumor properties, the expression of RBMS3 may be an indicator of overall survival. These capabilities were reported by scientists researching lung squamous cell carcinoma and gastric cancer [23,24,30]. In this current study, we provide evidence of RBMS3′s potential use as a positive prognostic marker of overall survival in breast cancer. The results of our clinical data analysis are consistent with the findings of Wang et al. [14]. The analysis of *RBMS3* mRNA expression in samples from the GEO and EGA data repositories also supports the suggestion that RBMS3 may be a useful tool for breast cancer diagnosis. The analysis of RBMS3 expression can be included as a supplementary category in defining prognosis of patient survival based on the molecular characteristics of the tumor, increasing the accuracy of predictions. The correlation of RBMS3 expression with TNBC and the expression of progesterone receptor may also lead to the distinction of new molecular subtypes of breast cancer based on the analysis of combined biomarkers.

Taking into consideration all the results presented in this study, we provide evidence of a potential novel explanation of RBMS3′s role in breast cancer. Currently available reports have tried to explain RBMS3′s anticancer activity in all types of breast cancer through the inhibition of the Wnt/β-catenin pathway and the inhibition of the epithelial–mesenchymal transition process (EMT), mainly by impacting on TWIST, PRRX1, or MMP2 [14,17,19]. Our results suggest that further studies should be conducted to consider the differences in RBMS3 expression correlated with receptor expression in cancer cells and stromal cells. We distinguished a positive correlation with overall survival, supporting the idea of a potential tumor-suppressing role for the expression of RBMS3 in breast cancer stroma. These findings open the way for further studies to unveil the exact role and mechanisms of these correlations.

Although further studies on the exact molecular mechanisms underlying the role of RBMS3 in breast cancer are required, RBMS3 may be potentially used in the development of novel therapeutic and diagnostic approaches in breast cancer. These may target not only cancer cells but also tumor stroma cells, making these therapies more complex and potentially adaptive to the patient's type of tumor, which would translate into a more personalized approach to patient treatment.

## 4. Materials and Methods

### *4.1. Patients' Characteristics*

The clinical material consisted of 524 paraffin blocks with clinical data from patients operated on for IDC. The clinical and pathological characteristics of the patients are presented in Table 3. Additionally, 26 paraffin blocks and clinical data from cases of mastopathy were analyzed as a control for the breast cancer cases. Patients' clinical material was obtained from the Division of Pathomorphology of the Polish Mother's Memorial Hospital Research Institute. The experiment was performed in accordance with the ethical standards and following the approval of the Ethics Committee of Wroclaw Medical University (decision no. KB 625/2022 25.08.2022).

**Table 3.** Clinical and pathological characteristics of studied patients.

| Parameters | Patients | |
|---|---|---|
| | IHC *n* = 524 | % |
| **Age** | | |
| ≤60 | 165 | 31.49 |
| >60 | 359 | 68.51 |
| **Tumor grade** | | |
| G1 | 87 | 16.60 |
| G2 | 342 | 65.27 |
| G3 | 92 | 17.56 |
| No data | 3 | 0.57 |
| **Tumor size** | | |
| pT1 | 325 | 62.02 |
| pT2 | 168 | 32.06 |
| pT3 | 3 | 0.57 |
| pT4 | 9 | 1.72 |
| No data | 19 | 3.63 |
| **Lymph nodes** | | |
| pN0 | 314 | 59.92 |
| pN1-pN3 | 180 | 34.35 |
| pNx | 30 | 5.73 |
| **Stage** | | |
| I | 224 | 42.75 |
| II | 257 | 49.05 |
| III | 18 | 3.44 |
| IV | 0 | 0.00 |
| **ER** | | |
| Neg. | 177 | 33.78 |
| Pos. | 344 | 65.65 |
| No data | 3 | 0.57 |
| **PR** | | |
| Neg. | 183 | 34.92 |
| Pos. | 338 | 64.50 |
| No data | 3 | 0.57 |
| **HER2** | | |
| Neg. | 272 | 51.91 |
| Pos. | 36 | 6.87 |
| No data | 216 | 41.22 |
| **Molecular tumor types** | | |
| Triple-negative | 34 | 6.49 |
| Other types | 487 | 92.94 |
| No data | 3 | 0.57 |

### *4.2. Tissue Microarrays (TMAs)*

A total of 21 TMAs were prepared from 524 cases of IDC and 26 cases of mastopathy. Prior to performing TMA blocks, the histological slides stained with hematoxylin and eosin were obtained from whole samples of breast cancer and mastopathy cases stored in the form of paraffin blocks (donor blocks). The slides were scanned using the Pannoramic Midi II histological scanner (3DHISTECH Ltd, Budapest, Hungary). After that, using the Pannoramic Viewer program 1.15.4 (3DHISTECH Ltd.), representative areas from the entire sections where selected. In addition, to increase the representativeness of each case, 3 representative cores each with a size of 1.5 mm were selected from the donor blocks and transferred to the TMA 'recipient' block using the TMA Grand Master system 2.6.6.69657 (3DHISTECH Ltd.).

### 4.3. Immunohistochemistry

The paraffin blocks with the breast cancer and mastopathy cases were cut into 4-µm sections. The immunohistochemical reactions were performed using anti-RBMS3 rabbit polyclonal antibody (Catalog # PA5-57028, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in a 1:200 dilution. The immunohistochemical reactions were performed using a Dako Autostainer Link 48 (Dako, Glostrup, Denmark). The visualization of the reactions was carried out using EnVision™ FLEX High pH (Link, Glostrup, Denmark) reagents (Dako), according to the manufacturer's instructions. The IHC reactions for 490 cases of IDC were suitable for the further analysis. The IHC reaction for RBMS3 antigen was assessed using the immunoreactive scale (IRS) by Remmele and Stegner [31], that evaluates the percentage of positive cancer cells (A) and the intensity of color reaction (B). The final score is the product of the values A and B (see Table 4).

**Table 4.** Graphic presentation of Remmele and Stegner scale showing the available values. The final score is the multiplication of A and B values (A × B) [31].

| Points | Percentage of Positive Cancer Cells (A) | Intensity of Color Reaction (B) |
|---|---|---|
| 0 | 0% | No color reaction |
| 1 | <10% | Mild reaction |
| 2 | 10–50% | Moderate reaction |
| 3 | 51–80% | Strong reaction |
| 4 | 81–100% | |

### 4.4. Kaplan–Meier Plotter

The Kaplan–Meier plotter tool was used for correlation of *RBMS3* mRNA expression with overall survival [21]. This is a tool for Kaplan–Meier plot generation based on data from GEO, EGA, and TCGA. *RBMS3* mRNA expression data was split into two groups for analysis: "high expression" and "low expression" using the median as the cut-off value.

### 4.5. Cell Lines

Four breast cancer cell lines were used in the experiments, representing types of tumors of increasing aggressiveness (MCF-7: luminal A, BT-474: luminal B, SK-BR-3: HER2-enriched, and MDA-MB-231: triple-negative), along with a normal cell line: immortalized breast epithelial cell line (HME1-hTERT) (Me16C). All cell lines were provided by ATCC (American Type Culture Collection ATCC®, Old Town Manassas, VA, USA). Respective culture media were used to provide optimal conditions for cell growth: MEBM (Lonza, Basel, Switzerland) for the Me16C cell line, αMEM (Lonza) for the MCF-7 and BT-474 cell lines, McCoy's (ATCC) for the SK-BR-3 cell line, L-15 (Lonza) for the MDA-MB-231 cell line. All media contained 1% l-glutamine and penicillin-streptomycin solution, as well as 10% fetal bovine serum (Sigma-Aldrich®, St. Louis, MO, USA). The cells were passaged with the use of TrypLE™ (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) when they were at approximately 70% confluence.

### 4.6. Real-Time PCR

Real-time PCR was applied to determine the relative level of *RBMS3* mRNA expression in the analyzed cell lines (MDA-MB-231, SK-BR-3, BT-474, MCF-7, Me16C). Total RNA was isolated with the use of a RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription reactions were performed with the use of iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The conditions of the reactions were as follows: priming for 5 min at 25 °C, reverse transcription for 20 min at 46 °C, and final inactivation of reverse transcriptase for 1 min at 95 °C. RT-qPCR was carried out in 20-µL volumes using the TaqMan Universal PCR MasterMix (Applied Biosystems, Foster City, CA, USA). The reactions were performed using a 7500 Real-time PCR system and iTaq™ Universal Probes Supermix (Bio-Rad), according to the manufacturer's instructions.

The TaqMan probes employed were Hs01104892_m1 for RBMS3 (Applied Biosystems) and endogenous control gene Hs99999903_m1 for β-actin (Applied Biosystems), further used for normalization purposes. The experiments were run in triplicate. The reactions were carried out under the following conditions: initial denaturation for 2 min at 94 °C, followed by 40 cycles of denaturation (15 s, 94 °C) and annealing with elongation (1 min, 60 °C). The relative *RBMS3* mRNA expression levels were calculated using the ΔΔCt method.

### 4.7. Western Blotting

Whole cell lysates were obtained from the BC cell lines (MDA-MB-231, SK-BR-3, BT-474, and MCF-7) and the control cell line (Me16C) using CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich) with the addition of Halt™ Protease Inhibitor Cocktail 100x (Thermo Fisher Scientific) and 2 mM PMSF (phenylmethylsulphonyl fluoride) (Sigma-Aldrich). The protein level was determined through colorimetric analysis with the use of bicinchoninic acid (Pierce BCA Protein Assay Kit) and NanoDrop 1000 (Thermo Scientific). The lysates were mixed with 4X SDS-PAGE gel-loading buffer (200 mM Tris-HCl—pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol) for 10 min at 95 °C, loaded onto 10% acrylamide gel and separated by SDS-PAGE under reducing conditions, then transferred onto a PVDF membrane in the XCell SureLock™ Mini gel electrophoresis system (Thermo Fisher Scientific). After the protein transfer, the membrane was incubated in a blocker solution (4% BSA in TBST buffer) for 1 h at RT, followed by overnight incubation at 4 °C with anti-RBMS3 monoclonal rabbit antibody, (Catalog # PA5-57028, Invitrogen, Thermo Fisher Scientific). Subsequently, the membrane was washed with TBST with 0.1% Tween-20 and incubated for 1 h at RT with secondary antibody (Jackson ImmunoResearch, Mill Valley, CA, USA) diluted at 1:3000, then rinsed and treated with Luminata Classico (Merck KGaA, Darmstadt, Germany) chemiluminescent substrate. Rabbit anti-human β-actin monoclonal antibody (#4970; Cell Signaling Technology, Danvers, MA, USA) diluted 1:1000 was used as an internal control. The Western blotting results were analyzed using the ChemiDoc MP system (Bio-Rad). The experiments were run in triplicate.

### 4.8. Statistical Analysis

The Kolmogorov–Smirnov test was applied to evaluate the normality assumption of the groups examined. The Mann–Whitney and ANOVA with Bonferroni's multiple comparison post hoc tests were conducted to compare the differences in the expression of the examined markers in all groups of patients in vitro and in the clinicopathological data. Additionally, the Spearman's correlation test was applied to analyze the existing correlations. The Kaplan–Meier method was used to construct survival curves. The Gehan–Breslow–Wilcoxon method was applied and univariate and multivariate Cox analyses of survival were performed to evaluate the survival analysis. All statistical analyses were conducted using Prism 9.0 (GraphPad Software) and Statistica 13.3 (Tibco Software, Inc.). The results were considered statistically significant when $p < 0.05$.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24032866/s1.

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

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Wroclaw Medical University (decision no. KB 625/2022 25.08.2022) for studies involving humans.

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The data presented in this study are available in this article and supplementary materials attached to it.

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

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# 8. ARTYKUŁY Z CYKLU PUBLIKACJI W FORMIE ZAŁĄCZNIKÓW

## Artykuł trzeci

**Tytuł:**

*Impact of RBMS 3 Progression on Expression of EMT Markers.*

**Autorzy:**

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**Czasopismo**

Cells, 2024, Sep 13, 1548, doi: 10.3390/cells13181548

**Punktacja**

IF: 5.1, punktacja MNiSW: 140 pkt

**Finansowanie**

**Projekt sfinansowany w ramach programu MNiSW SKN Tworzą Innowacje SKN/SP/569116/2023**

cells

MDPI

*Article*

# Impact of RBMS 3 Progression on Expression of EMT Markers

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**Abstract:** Epithelial-to-mesenchymal transition (EMT) is a complex cellular process that allows cells to change their phenotype from epithelial to mesenchymal-like. Type 3 EMT occurs during cancer progression. The aim of this study was to investigate the role of RNA-binding motif single-stranded interacting protein 3 (RBMS 3) in the process of EMT. To investigate the impact of RBMS 3 on EMT, we performed immunohistochemical (IHC) reactions on archived paraffin blocks of invasive ductal breast carcinoma ($n$ = 449), allowing us to analyze the correlation in expression between RBMS 3 and common markers of EMT. The IHC results confirmed the association of RBMS 3 with EMT markers. Furthermore, we performed an in vitro study using cellular models of triple negative and HER-2-enriched breast cancer with the overexpression and silencing of RBMS 3. RT-qPCR and Western blot methods were used to detect changes at both the mRNA and protein levels. An invasion assay and confocal microscopy were used to study the migratory potential of cells depending on the RBMS 3 expression. The studies conducted suggest that RBMS 3 may potentially act as an EMT-promoting agent in the most aggressive subtype of breast cancer, triple negative breast cancer (TNBC), but as an EMT suppressor in the HER-2-enriched subtype. The results of this study indicate the complex role of RBMS 3 in regulating the EMT process and present it as a future potential target for personalized therapies and a diagnostic marker in breast cancer.

**Keywords:** RBMS 3; epithelial-to-mesenchymal transition (EMT); breast cancer; therapeutic strategies; biomarkers



**Citation:** Górnicki, T.; Lambrinow, J.; Mrozowska, M.; Krawczyńska, K.; Staszko, N.; Kmiecik, A.; Piotrowska, A.; Gomułkiewicz, A.; Romanowicz, H.; Smolarz, B.; et al. Impact of RBMS 3 Progression on Expression of EMT Markers. *Cells* **2024**, *13*, 1548. https://doi.org/10.3390/cells13181548

Academic Editor: Fabrizio Marcucci

Received: 17 July 2024
Revised: 6 September 2024
Accepted: 13 September 2024
Published: 14 September 2024



## 1. Introduction

Recent global cancer statistics indicate that breast cancer is the second most commonly diagnosed cancer worldwide, accounting for 11.6% of all new cases of cancer [1,2]. Given its prevalence, breast cancer represents not only a significant health concern but also an economic challenge [3]. Although considerable advances have been made in the diagnosis and treatment of breast cancer in recent decades, there is a pressing need for further research, particularly focused on the identification of new biomarkers and potential therapeutic targets [4]. From a molecular perspective, breast cancer can be classified into four main subtypes, luminal A, luminal B, HER-2-enriched, and triple negative breast cancer (TNBC), with TNBC being the only subtype lacking a dedicated targeted treatment [5,6]. Patients diagnosed with metastatic breast cancer generally have a poorer prognosis compared to those with non-metastatic disease [7].

One of the principal phenomena associated with metastasis is epithelial-to-mesenchymal transition (EMT) [8,9]. Some researchers suggest that EMT may even be necessary not only for tumor metastasis but also for the entire process of carcinogenesis [10]. Therefore, investigating EMT is particularly significant in the context of breast cancer. The term "epithelial-to-mesenchymal transition" was first used in 1982, and since then, this process has remained a key area of research globally [11,12]. EMT is a complex cellular process enabling cells to transition from an epithelial to a mesenchymal-like phenotype [13]. During EMT, epithelial cells typically lose the expression of E-Cadherin, a hallmark epithelial marker, while concurrently gaining the expression of mesenchymal markers such as N-Cadherin and vimentin [14]. Three distinct types of EMT have been identified: type one, occurring in embryonic development; type two, in tissue regeneration; and type three, which is involved in cancer progression [15]. The EMT process involved in cancer progression is mediated by EMT-inducing transcription factors (EMT-TFs), including the Snail family transcriptional repressor (SNAIL), Twist Family BHLH Transcription Factor (TWIST), and Zinc finger E-box-binding homeobox (ZEB) families [16,17]. The evidence suggests that EMT-TFs are associated with processes such as drug resistance or the acquisition of stemness [18].

In their studies on EMT, researchers focused on RNA-binding motif single-stranded interacting protein 3 (RBMS 3), a protein belonging to the c-myc gene single-strand binding proteins (MSSPs) [19,20]. RBMS 3 plays a significant role not only in cancer progression but also in other pathological processes [21–23]. Recent studies have indicated that RBMS 3 is involved in the development of various cancers, including ovarian, colorectal, lung and breast cancer [24–28]. A number of papers have suggested that RBMS 3 plays a significant role in the regulation of EMT. However the exact mechanism of action of this protein remains poorly understood.

The aim of this study was to evaluate the role of RBMS 3 in the EMT process in invasive ductal carcinoma (IDC) patient-derived tumors and also in functional in vitro models of HER-2-enriched and TNBC cancer cell lines.

## 2. Materials and Methods

### 2.1. Patients Cohort

The clinical material comprised 524 paraffin blocks with clinical data from patients operated on for IDC. The clinical and pathological characteristics of the patients are presented in Table 1. Additionally, 26 paraffin blocks and clinical data from cases of mastopathy were analyzed as a control for the breast cancer cases. Patients' clinical material was obtained from the Division of Pathomorphology of the Polish Mother's Memorial Hospital Research Institute. The experiment was performed in accordance with the ethical standards and following the approval of the Ethics Committee of Wroclaw Medical University (decision no. KB 625/2022 25 August 2022). Table 1 presents the clinical characteristics of the studied cohort of patients.

### 2.2. Immunohistochemistry

Experiments were performed on archived paraffin blocks of invasive breast ductal carcinoma ($n$ = 524) and mastopathy ($n$ = 27). Whole samples were processed into tissue microarrays (TMA) and histological slides stained with hematoxylin and eosin. The samples were obtained during surgical resections performed at the Institute of Polish Mother's Memorial Health Centre in Lodz, Poland. Immunohistochemical reactions were carried out using the following antibodies: RBMS 3 (1:200, PA5-57028, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), TWIST 1 (1:50, ab50581, Abcam, Cambridge, UK), SNAIL (1:200, 13099-1-AP, ProteinTech, Rosemont, IL, USA), E-Cadherin (RTU, IR059, Dako, Agilent Technologies, Santa Clara, CA, USA), N-Cadherin (1:50, M3613, Dako, Agilent Technologies), SLUG (1:50, sc-166476, Santa Cruz, Dallas, TX, USA) and ZEB 1 (1:100, ab203829, Abcam). A Dako Autostainer Link 48 apparatus (Dako, Glostrup, Denmark) was used to perform the immunohistochemical reactions. Reaction visualization was carried out using

EnVision™ FLEX High pH reagents (Link, Glostrup, Denmark) (Dako), according to the manufacturer's instructions [29,30]. The reactions were scored using the Remmele and Stegner immunoreactivity scale (IRS) [31]. A cytoplasmatic reaction was detected in the case of RBMS 3, E-CAD, N-CAD and the nucleocytoplasmic in SNAIL, SLUG and TWIST 1. ZEB 1 was not detected in cancer cells; this protein was present in the stroma cells of IDC.

**Table 1.** Clinical and pathological characteristics of studied patients.

| Parameters | Patients | |
|---|---|---|
| | IHC | % |
| | ***n* = 524** | |
| Age | | |
| ≤60 | 165 | 31.49 |
| >60 | 359 | 68.51 |
| Tumor grade | | |
| G1 | 87 | 16.60 |
| G2 | 342 | 65.27 |
| G3 | 92 | 17.56 |
| unknown | 3 | 0.57 |
| Tumor size | | |
| pT1 | 325 | 62.02 |
| pT2 | 168 | 32.06 |
| pT3 | 3 | 0.57 |
| pT4 | 9 | 1.72 |
| unknown | 19 | 3.63 |
| Lymph nodes | | |
| pN0 | 314 | 59.92 |
| pN1–N3 | 180 | 34.35 |
| pNx | 30 | 5.73 |
| Stage | | |
| I | 224 | 42.75 |
| II | 257 | 49.05 |
| III | 18 | 3.44 |
| IV | 0 | 0 |
| ER | | |
| Negative | 177 | 33.78 |
| Positive | 344 | 65.65 |
| Unknown | 3 | 0.57 |
| PR | | |
| Negative | 183 | 34.92 |
| Positive | 338 | 64.50 |
| Unknown | 3 | 0.57 |
| HER-2 | | |
| Negative | 272 | 51.91 |
| Positive | 36 | 6.87 |
| Unknown | 216 | 51.91 |
| Molecular tumor types | | |
| Triple negative | 37 | 7.06 |
| Other types | 484 | 92.3 |
| Unknown | 3 | 0.1 |

### 2.3. Cell Lines

Two breast cancer cell lines representing HER-2-enriched and TNBC molecular types of breast cancer, respectively (SKBR-3 and MDA-MB-231), were used in our study. All cell lines were provided by ATCC (American Type Culture Collection ATCC, Old Town Manassas, VA, USA). FBS (Sigma-Aldrich, St. Louis, MO, USA) was added to all of the media at a final concentration of 10%. The cell lines were grown in 5% $CO_2$ at 37 °C. The culture media used in the study included SKBR-3-McCoy's (ATCC) and MDA-MB-231-L-15 (Lonza, Basel, Switzerland). All media contained 1% L-glutamine and penicillin-streptomycin solutions. The cells were passaged using TrypLE™ (Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

### 2.4. Lentiviral Transduction of Breast Cancer Cell Lines

#### 2.4.1. Overexpressing RBMS 3 In Vitro Model

The lentiviral particles used for transduction in order to obtain RBMS 3 overexpression were bought from OriGene (Rockville, MD, USA). A control for overexpression was conducted using cells transduced with lentiviral control particles. The cells were transfected accordingly to the producer manual. The starting cell seeding was $0.5 \times 10^5$ on a 24-well plate. The MOI used in the experiment for both cell lines was 10. For increased transduction efficacy, Polybren (10 mg/mL) was used. The cells were selected for puromycin resistance for 1 week and maintained in a medium containing 0.5 µg/mL puromycin for SKBR-3 and 2.5 µg/mL for MDA-MB-231. The effectiveness of the transduction was determined on the mRNA and protein level using RT-qPCR and WB methods.

#### 2.4.2. Silencing RBMS 3 In Vitro Model

The lentiviral particles used for transduction in order to obtain RBMS 3 silencing were bought from OriGene (Rockville, MD, USA). The control for silencing was cells transduced with scrambled shRNA. The cells were transfected accordingly to the producer manual. The starting cell seeding was $0.5 \times 10^5$ on a 24-well plate. The MOI used in the experiment for both cell lines was 10. For increased transduction efficacy, Polybren (10 mg/mL) was used. The cells were selected for puromycin resistance for 1 week and maintained in a medium containing 0.5 µg/mL puromycin for SKBR-3 and 2.5 ug/mL for MDA-MB-231. The effectiveness of the transduction was determined on the mRNA and protein level using RT-qPCR and WB methods.

### 2.5. Migration Assay

SKBR-3 and MDA-MB-231 cells with overexpression and silenced RBMS 3, as well as wild-type SKBR-3 and MDA-MB-231 cells, were seeded in a Culture-Insert 2 Well in µ-Dish 35 mm (Ibidi, Gräfelfing Germany). The cells were seeded at $3.5 \times 10^4$ per well. After 24 h, the inserts were removed, and initial images were taken using a BX41 light microscope (Olympus, Tokyo, Japan). Twenty-four hours after the insert removal, a final set of images were taken to establish changes in the migration between types of cells.

### 2.6. Immunofluorescence Reactions

The cells were seeded at $1 \times 10^4$ on the µ-Slide 8 Well high, with Glass Bottom (Ibidi, Gräfelfing Germany). The cells were fixed in cold 70% methanol. Next, the slides were washed twice in solution in PBS/0.1% Tween 20. The slides were incubated in a blocking solution of 1% BSA in solution in PBS/0.1% Tween 20 for 30 min, followed by overnight incubation with primary antibodies against E-Cadherin (1:500, sc-8226 Santa Cruz, Dallas, TX, USA) and N-Cadherin (1:500, sc-8424, Santa Cruz). The secondary antibody Goat Anti-Mouse IgG H&L (1:2000, ab150113, Abcam, Cambridge, UK) was used. The preparations were mounted in Prolong DAPI Mounting Medium (Invitrogen, Carlsbad, CA, USA). The observations were made at objective 60 × 1.40 oil, with the use of a Fluoview FV3000 confocal microscope (Olympus, RRID:SCR_017015) coupled with Cell Sense software version 2.6 (Olympus, RRID:SCR_016238). The analysis of the

reaction presence and determination of the cell count presenting a reaction were performed using Ilastik Version 1.4.0.post1 (10 November 2023). All images were analyzed using the same parameters of threshold and size. The percentage of positive cells was calculated by dividing the number of positive reactions by the number of visible cell nuclei. For each group, measurements were taken at three distinct hot spots [32].

### 2.7. Real-Time PCR

Total RNA from the cell samples was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was obtained with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocol. A 7500 Real-Time PCR System instrument and 7500 software v2.0.6 (Applied Biosystems, Waltham, MA, USA) were applied to carry out a real-time PCR. The TaqMan probes used in the experiment were as follows: Hs01104892_m1 for RBMS3, Hs04989912_s1 for TWIST1, Hs00195591_m1 for SNAIL, Hs01023895_m1 for E-Cadherin, Hs00983056_m1 for N-Cadherin, Hs00161904_m1 for SLUG and Hs01566408_m1 for ZEB 1 (Applied Biosystems). The expression level of β-actin (Hs99999903_m1, Applied Biosystems) was used as an endogenous control for further normalization purposes. The experiments were run in triplicate. Reactions were carried out under the following conditions: polymerase activation at 50 °C for 2 min and initial denaturation at 94 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 15 s and annealing and elongation at 60 °C for 1 min. The amount of cells used for the RT-qPCR was $1.0 \times 10^6$ of each type of cells. The ΔΔCt method was used to determine the relative mRNA expression.

### 2.8. Western Blotting

For protein expression analysis, $2.5 \times 10^6$ cells of each type were lysed on ice using CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich, Burlington, MA, USA) with the addition of Halt™ Protease Inhibitor Cocktail 100× (Thermo Fisher Scientific, Waltham, MA, USA) and 2 mM PMSF (phenylmethylsulphonyl fluoride) (Sigma-Aldrich). The samples were then centrifuged at 12,000× *g* for 10 min at 4 °C to collect the protein-containing supernatant. The protein concentration was determined by colorimetric analysis using bicinchoninic acid (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA) and NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). In this study, we used total protein normalization, because the studies suggest that this method may be better than analyzing the expression of housekeeping genes [33]. We used a full stain-free system from Bio-Rad. The samples were mixed with sample loading buffer (250 mM Tris pH = 6.8, 40% glycerol, 20% (*v*/*v*) β-mercaptoethanol, 0.33 mg/mL bromophenol blue, 8% SDS) and denatured at 96 °C for 10 min. Samples containing 30 µg of protein were loaded onto Mini-Protean TGX stain-free gels (Bio-Rad) and separated by SDS-PAGE under reducing conditions. A stain-free Western blot was performed according to the supplier's protocol (Bio-Rad). The gel was activated in order to capture stain-free gel images. Proteins were transferred to a low-fluorescence PVDF (LF-PVDF) membrane using the Trans-Blot Turbo Transfer System #1704150 (Bio-Rad). After transfer, a stain-free image of the LF-PVDF membrane was obtained. Next, the membrane was incubated in a blocking solution (5% milk in TBST) for 1 h at room temperature (RT), followed by overnight incubation at 4 °C with the following antibodies: RBMS 3 (1:2000 the same antibody used for IHC reactions), TWIST1 (1:200 the same antibody used for IHC reactions), SNAIL (1:500 the same antibody used for IHC reactions), E-Cadherin (1:200, sc-8226, Santa Cruz, Dallas, TX, USA), N-Cadherin (1:200, sc-8424, Santa Cruz), SLUG (1:100) and ZEB-1 (1:500). The membrane was then washed with TBST buffer (0.1% Tween 20 in PBS) and incubated for 1 h at RT with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies, diluted 1:3000 (Jackson ImmunoResearch, Mill Valley, CA, USA). The next step was to obtain a stain-free image of LF-PVDF. Detection was performed using Immobilon Forte Western HRP (Merck KGaA, Darmstadt, Germany) chemiluminescent substrate. The Western blotting results were analyzed using the ChemiDoc MP system (Bio-Rad).

### *2.9. Statistical Analysis*

All statistical analyses were conducted using Prism 10.0 (GraphPad Software). The results were considered statistically significant when $p < 0.05$. The Spearman's Correlation Test was applied to analyze the existing correlations. The T-Student test was used to analyze the differences between the expression of RBMS 3 and the common EMT markers at the mRNA level.

## 3. Results

### *3.1. The Immunohistochemical Intensity of RBMS 3 Expression in Cancer Cells Correlates with Expression of Common EMT Markers in Invasive Ductal Breast Cancer (IDC) Tissue Samples*

In order to provide a justification for studies concerning the role of RBMS 3 in EMT in breast cancer, we performed an analysis of the correlation between RBMS 3 immunohistochemical expression in cancer cells and the expression of Twist Family BHLH Transcription Factor 1 (TWIST 1), SNAIL (Snail Family Transcriptional Repressor 1), Snail Family Transcriptional Repressor 2 (SLUG), Epithelial Cadherin (E-CAD), Neural Cadherin (N-CAD) and Zinc finger E-box-binding homeobox 1 (ZEB 1) in 449 cases of breast cancer. An analysis of the IHC reaction unveiled that RBMS 3 is a protein present in cytoplasm, TWIST 1 in cytoplasm and nucleus, SNAIL in cytoplasm and SLUG in cytoplasm and nucleus. Both E-CAD and N-CAD are present in the cell membrane and cytoplasm of cancer cells. The statistical analysis of the results revealed a significant positive correlation of RBMS 3 expression with the expression of TWIST, SNAIL and N-CAD. In our sample group, we failed to identify ZEB 1 expression in breast cancer cells. An additional analysis of the transcriptomic data of breast invasive carcinoma (BRCA) from The Cancer Genome Atlas Program (TCGA) using the UALCAN tool [34,35] showed a statistically significant lower expression of RBMS 3 in both the HER-2-enriched and TNBC subtypes of breast cancer. Representative images of RBMS 3 and all the studied proteins, showing the expression pattern of proteins in the tissue, are presented in Figure 1. Representative images of all the studied proteins on a lower magnification are presented in the Supplementary Materials. A graphical presentation of significant results can be seen in Figure 2. The results of the online database analysis are presented in Figure 3; the results of the statistical analysis performed by UALCAN are presented in the Supplementary Materials.

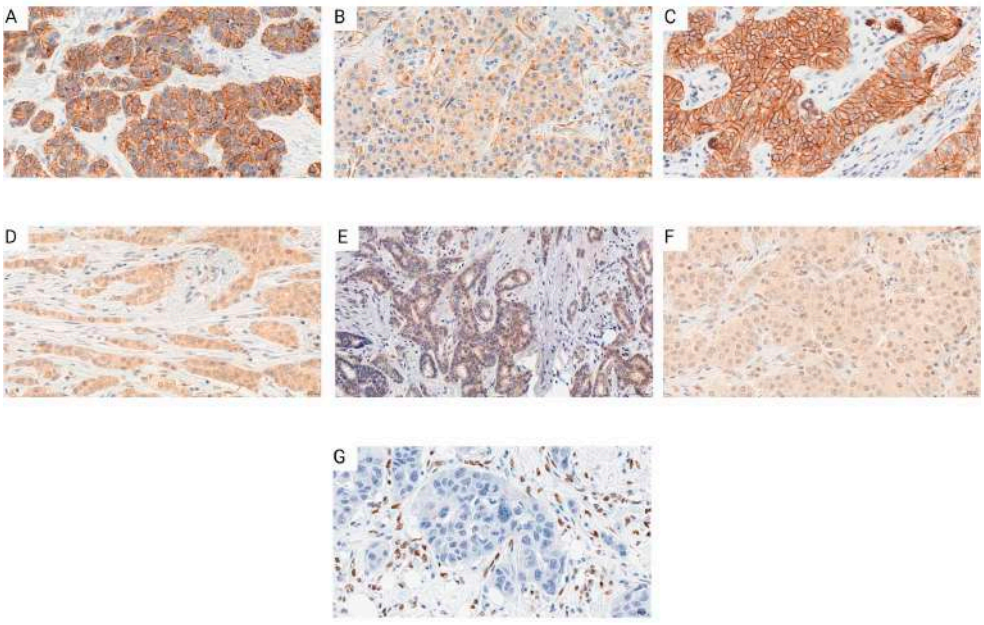


**Figure 1.** Immunohistochemical reactions performed on invasive ductal breast carcinoma tissue revealed expression of RBMS 3 and EMT markers (**A**) N-Cadherin, (**B**) RBMS 3, (**C**) E-Cadherin, (**D**) SLUG, (**E**) SNAIL, (**F**) TWIST 1, and (**G**) representation of negative ZEB 1 staining and positive in stroma of IDC. Magnification ×400. Created with BioRender.com.

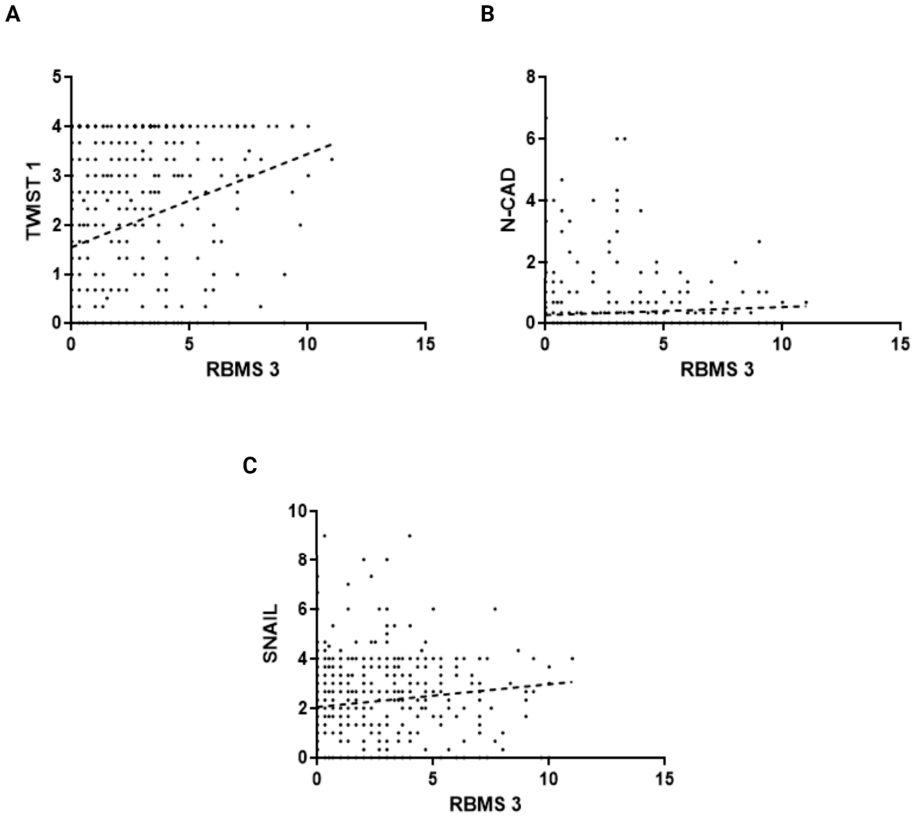


**Figure 2.** Analysis of correlation between expression of RBMS 3 in IDC with (**A**) TWIST 1 (Spearman's Correlation Test, r = 0.31, $p < 0.0001$), (**B**) N-CAD (Spearman's Correlation Test, r = 0.19, $p < 0.0001$) and (**C**) SNAIL (Spearman's Correlation Test, r = 0.18, $p < 0.0001$). Created with BioRender.com.

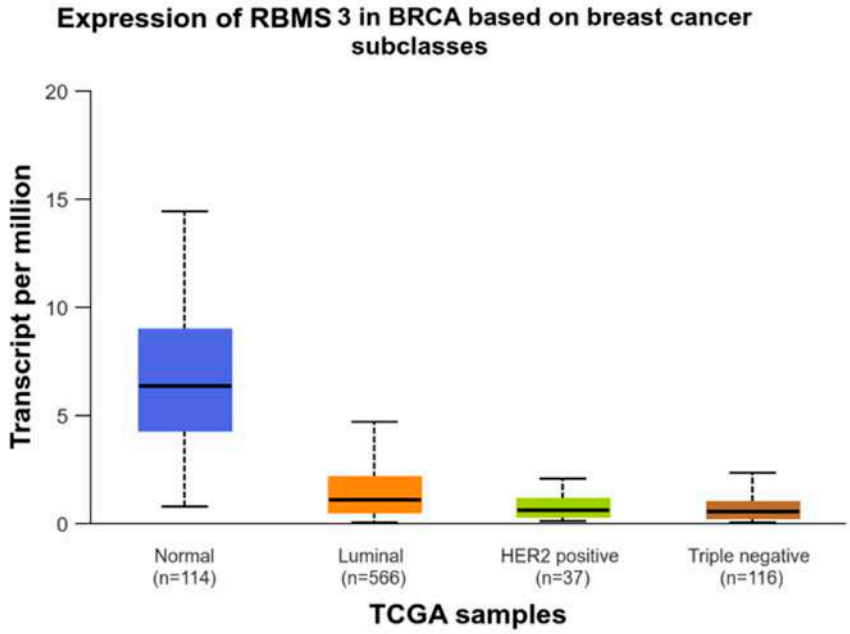


**Figure 3.** Graphical presentation of TCGA data analysis showing lower expression of RBMS 3 in both HER-2-enriched and TNBC breast cancer subtypes in comparison with healthy tissue and luminal type. Graph generated by UALCAN tool [34,35].

### *3.2. The Immunohistochemical Intensity of RBMS 3 Expression Displays Different Correlations with Common EMT Markers Depending on the Molecular Subtype of the Tumor*

For further investigation, we selected from the general pool of IDC those of luminal type A, luminal type B, triple negative (TNBC) molecular status and HER-2 enriched. The analysis of correlation in the group of 80 cases of luminal A IDC did not reveal any significant correlations between the expression of RBMS 3 and the common markers of EMT.

The analysis of 170 cases of luminal B IDC revealed statistically significant positive correlation between the expression of RBMS 3 and expression of TWIST 1, SNAIL and N-CAD. The statistically significant data are presented in Figure 4.

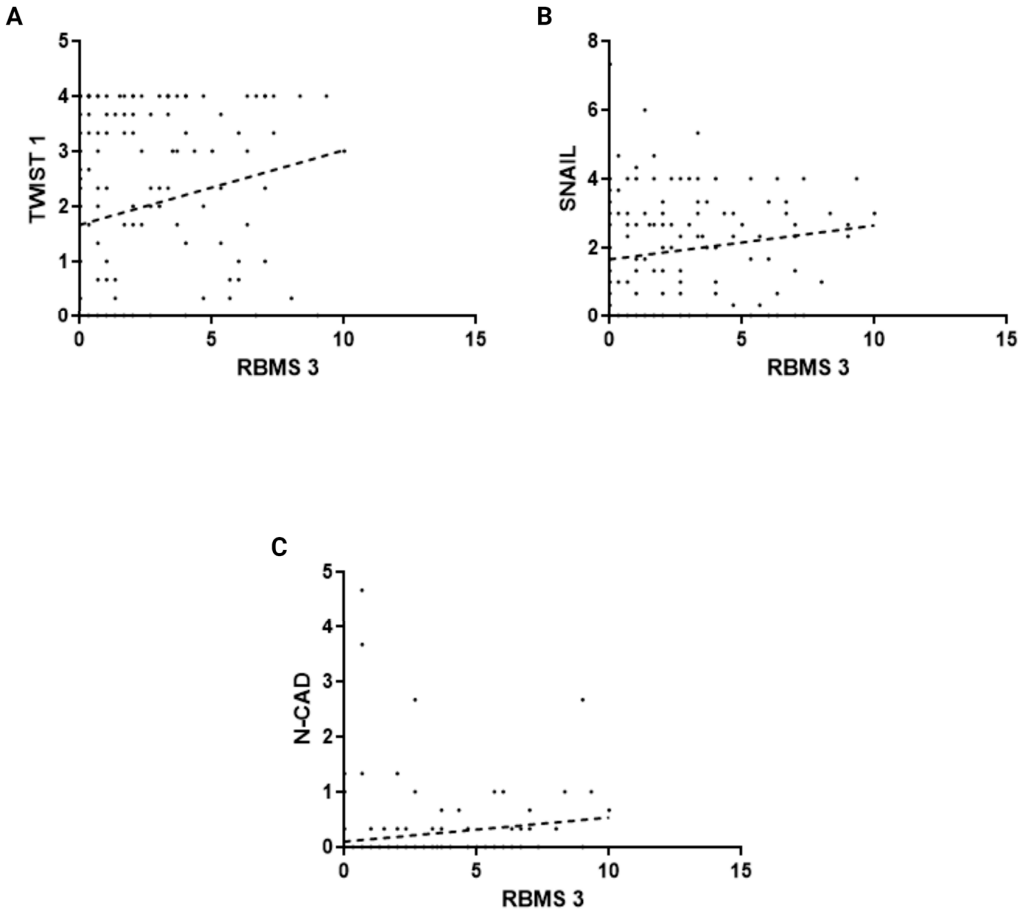


**Figure 4.** Analysis of correlation between expression of RBMS 3 in luminal B IDC with (**A**) TWIST 1 (Spearman's Correlation Test, $p < 0.0001$, r = 0.29), (**B**) SNAIL (Spearman's Correlation Test, $p < 0.006$, r = 0.20) and (**C**) N-CAD (Spearman's Correlation Test, $p < 0.0001$, r = 0.30). Created with BioRender.com.

In the group of 37 TNBC cases, a significant positive correlation was observed between RBMS 3 and TWIST 1 in cancer cells and a borderline significant correlation with SLUG expression ($p < 0.09$). With regard to other markers, there is a visible trend that the expression of markers increases with RBMS 3 expression, but the results are not statistically significant.

In 20 cases of HER-2-enriched IDC cases, we did not observe a correlation between RBMS 3 and the expression of the studied EMT markers. The positive correlation with E-CAD was close to statistical significance ($p = 0.053$). The statistical data are presented in Figure 5.

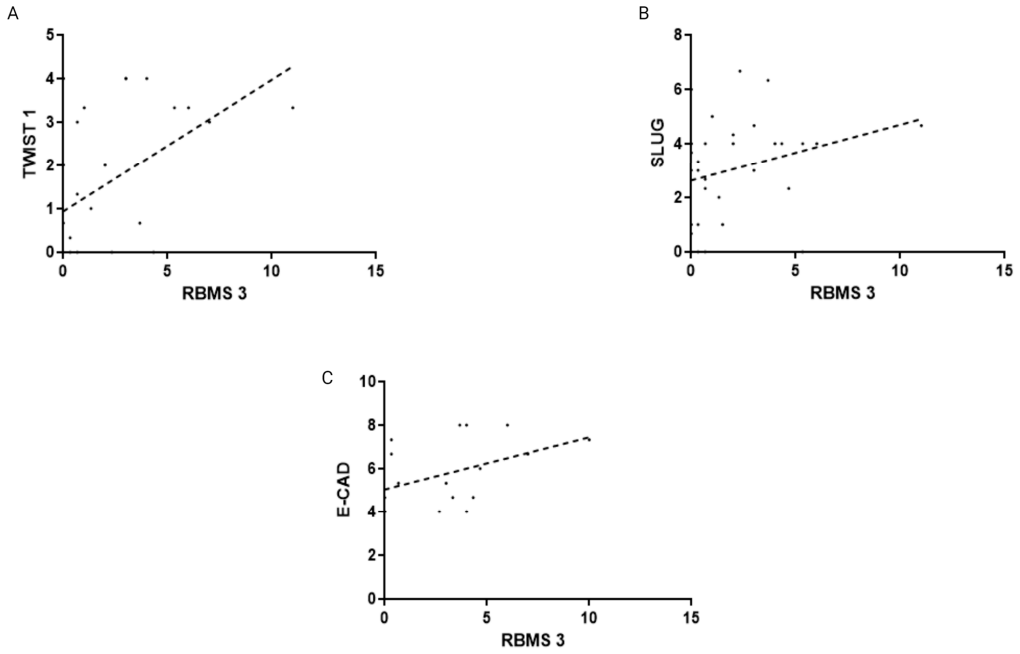


**Figure 5.** Analysis of RBMS 3 expression in TNBC IDC cases with (**A**) TWIST 1 (Spearman's Correlation Test, $p < 0.01$, r = 0.44) and (**B**) SLUG (Spearman's Correlation Test, $p < 0.09$, r = 0.30), and correlation between expression of RBMS 3 in HER-2-enriched IDC cases and (**C**) E-CAD (Spearman's Correlation Test, $p < 0.053$, r = 0.48). Created with BioRender.com.

An analysis of the IHC data in regards to histological grade unveiled that in the HER-2-enriched subtype there were significant correlation with N-CAD in grade 2. In the case of TNBC cases, we observed a significant correlation with TWIST 1 in grade 2. We also performed an analysis of the correlations between RBMS 3 and EMT markers with lymph node invasion, revealing that in the HER-2-enriched subtype there were positive correlations with TWIST 1 in tumors with lymph node metastasis, whereas in TNBC cases there were positive correlations with TWIST 1, as well as N-CAD. The results are presented in Figure 6.

### *3.3. In Vitro Models of Triple Negative and HER-2-Positive Breast Cancer Cell Lines with Overexpression of RBMS 3 Lead to Observation of Additional Specific Bands*

In order to investigate the effect of RBMS 3 expression on two of the most aggressive types of breast cancer, we have developed models of the triple negative breast cancer cell line: one MDA-MB-231 with an overexpression of RBMS 3 and one with a silenced RBMS 3 using lentiviral particles. The same goes for the HER-2-enriched cell line SKBR-3. We also created two models of this cell line, one with overexpressed and one with silenced RBMS 3. Figures 7 and 8 show data indicating this successful silencing and overexpression. As per our best knowledge, for the first time we show that the intense overexpression of RBMS 3 both in the MDA-MB-231 and SKBR-3 cell lines leads to the detection of two additional specific bands with a molecular weight of about 65 kDa and 80 kDa in MDA-MB-231 cells and 70 kDa and 100 kDa in SKBR-3 cells, in addition to the baseline protein with a molecular weight of 55 kDA.

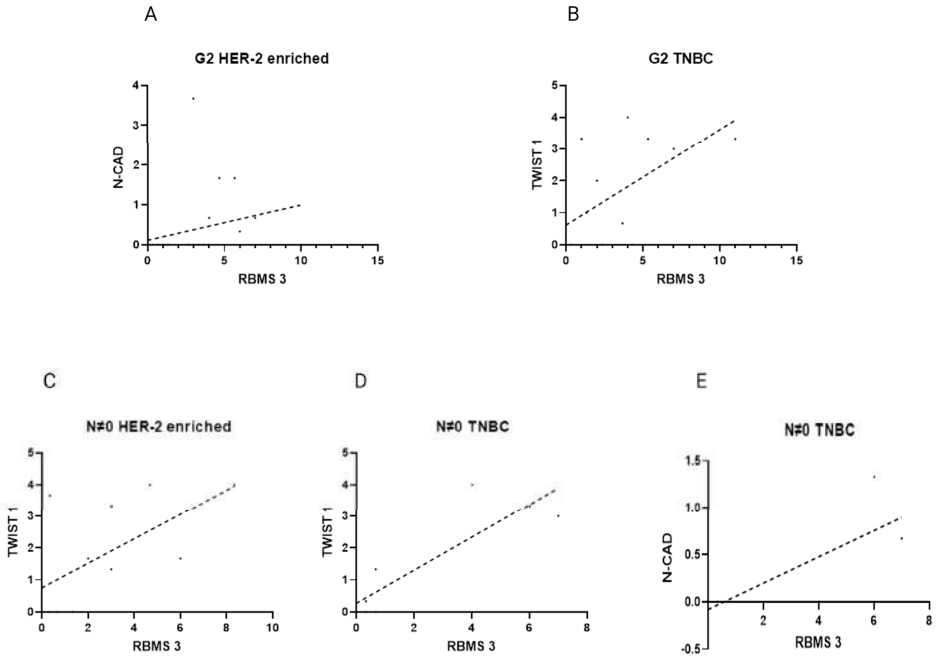


**Figure 6.** Analysis of correlation between expression of RBMS 3 and EMT makers in histological grade 2 showed positive correlations with (**A**) N-CAD in HER-2-enriched cases of IDC (Spearman's Correlation Test, $p < 0.003$, r = 0.56) and with (**B**) TWIST 1 in TNBC cases of IDC (Spearman's Correlation Test, $p < 0.025$, r = 0.58). Analysis of correlation between expression of RBMS 3 and EMT markers in cases with lymph node invasion showed positive correlations with (**C**) TWIST 1 in HER-2-enriched cases of IDC (Spearman's Correlation Test, $p < 0.035$, r = 0.65) and with (**D**) TWIST 1 (Spearman's Correlation Test, $p < 0.031$, r = 0.77) and (**E**) N-CAD (Spearman's Correlation Test, $p < 0.035$, r = 0.85).

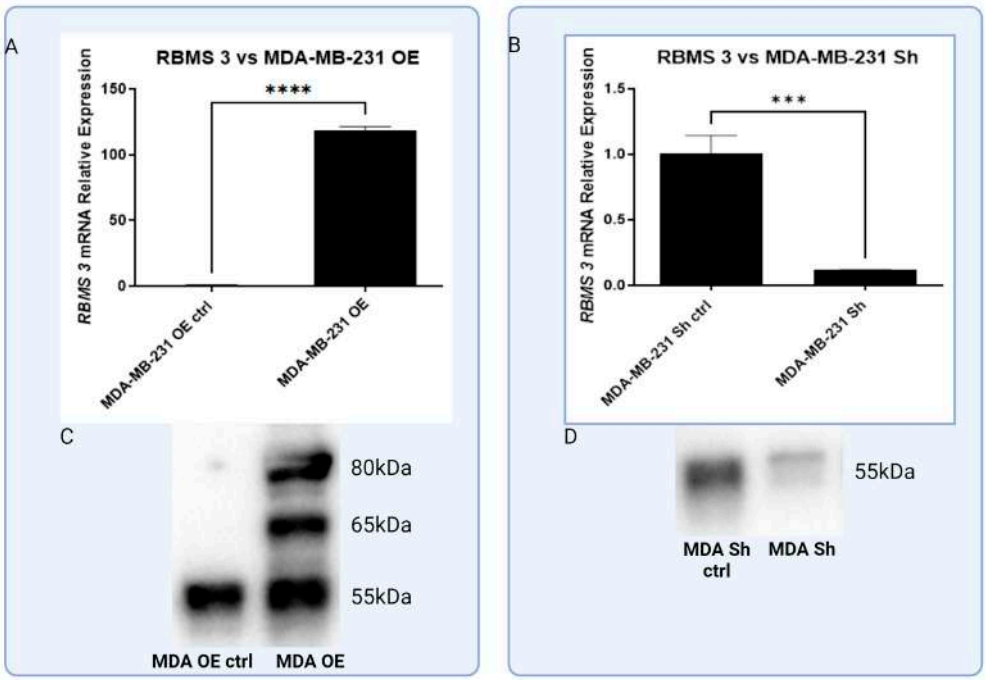


**Figure 7.** Analysis of overexpression of RBM S3 (**A**,**C**) and silencing (**B**,**D**) in MDA-MB-231 cell line on both mRNA and protein level. RT-PCR (**A**,**B**) and Western blot methods (**C**,**D**), *** $p < 0.001$, **** $p < 0.0001$. Created with BioRender.com.

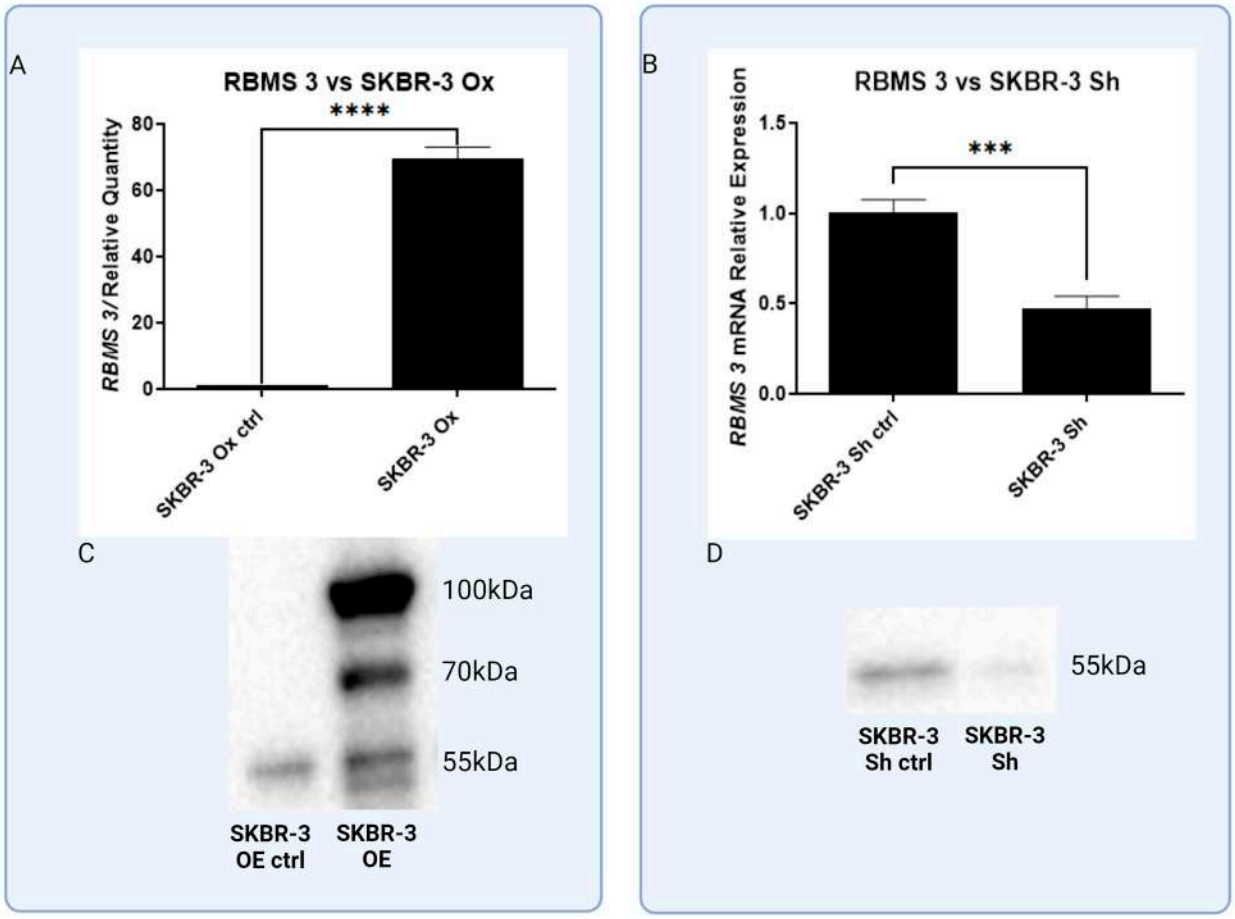


**Figure 8.** Analysis of RBMS 3 overexpression (**A**,**C**) and silencing (**B**,**D**) of RBSM 3 in SKBR-3 cell line on both mRNA and protein level. RT-PCR (**A**,**B**) and Western blot methods (**C**,**D**), *** $p < 0.001$, **** $p < 0.0001$. Created with BioRender.com.

### *3.4. In Vitro Model of RBMS 3 Overexpression Unveils Differences in RBMS 3 Impacts on EMT Markers between Molecular Subtypes*

In the following step of the study, we examined the impact of RBMS 3 overexpression in the MDA-MB-231 cell line on the expression of EMT markers used in the immunohistochemical assessment. To elucidate changes both at the mRNA and protein levels, we used RT-qPCR and Western Blot methods. On the mRNA level, all the tested markers (TWIST 1, SNAIL, SLUG, E-CAD, N-CAD and ZEB 1) were significantly elevated. At the protein level, there was an observable increase in E-CAD, N-CAD and ZEB 1 amounts. Interestingly, the TWIST 1 protein were not observed on the standard level of 28 kDa; instead of that, we observed bands at heights of about 90 kDa and 120 kDa (Figure 9).

The SKBR-3 cell line with an overexpression of RBMS 3 demonstrated a statistically significant reduction in the expression of SLUG, N-CAD and ZEB 1 mRNA compared to the negative control. No significant changes were observed in the expression of TWIST 1, SNAIL and E-CAD mRNA. On the protein level, ZEB 1 in SKBR-3 cells were below the detection threshold. No significant changes were observed in the levels of the other proteins (Figure 10).

### *3.5. In Vitro Models with Lentiviral-Silenced RBMS 3 Expression Impact Common Markers of EMT Differently Depending on the Molecular Subtype*

The silencing of RBMS 3 in triple negative breast cancer changed the expression of the studied proteins in a different way to overexpression. At the mRNA level, there is an observed significant reduction in the amount of TWIST 1, SNAIL, SLUG, and ZEB 1. The level of N-CAD does not change significantly, and in the opposition level, E-CAD

significantly increased. At the protein level, there is an observable change in the expression of SLUG and E-CAD (Figure 11).

In the HER-2-enriched model of RBMS 3 silencing, there was a significant decrease in TWIST 1 and E-CAD on the mRNA level, as well as a significant increase in N-CAD and ZEB 1. On the other hand, no observable changes were observed in the protein levels, except for SLUG, where an increase in protein level was noted. This aligned with the result of the RT-qPCR, which presented an increase in SLUG mRNA level, though not a statistically significant one (Figure 12).

### *3.6. Changes in RBMS 3 Expression Impact Motility and the Localization of E-CAD and N-CAD in Studied Cells*

In order to investigate the impact of changes in RBMS 3 expression on the migration of cells, we performed a scratch test. The results of the scratch test presented in Figures 13 and 14 unveil that in the TNBC model, the silencing of RBMS 3 impairs the migration of cancer cells more strongly than overexpression. In the HER-2-enriched model, no changes in migration were observed between the silencing and overexpression of RBMS 3.

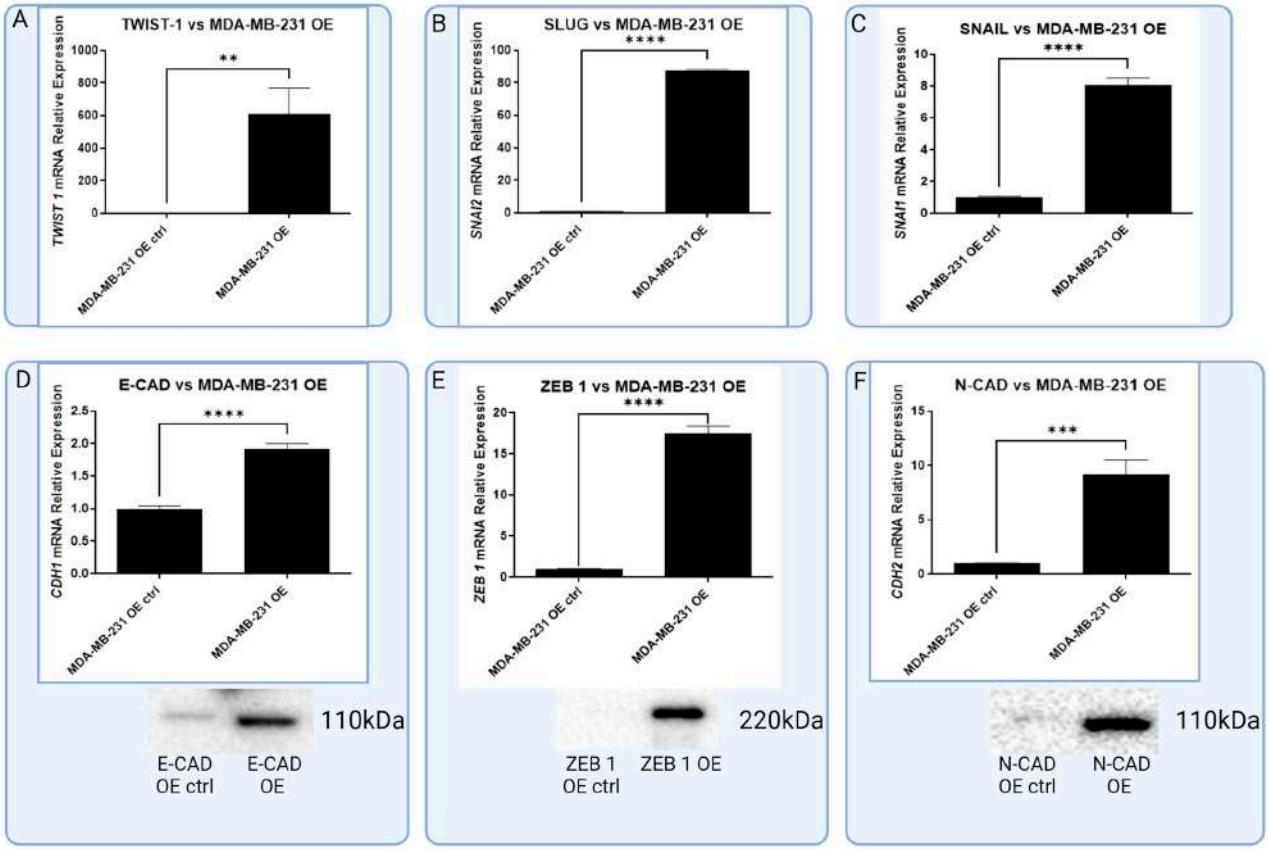


**Figure 9.** Graphic presentation of significant increase in expression of common EMT markers in RBMS 3-overexpressing TNBC cell line MDA-MB-231 on mRNA level (A–F): (**A**) TWIST 1 (T-Student test, $p < 0.002$), (**B**) SLUG (T-Student test, $p < 0.0001$), (**C**) SNAIL (T-Student test, $p < 0.0001$), (**D**) E-CAD (T-Student test, $p < 0.0001$), (**E**) ZEB 1 (T-Student test, $p < 0.0001$) and (**F**) N-CAD (T-Student test, $p < 0.0005$). Additionally in E-CAD, ZEB 1 and N-CAD (**D**–**F**), there were also observable differences in protein expression that match results of RT-qPCR, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Created with BioRender.com.

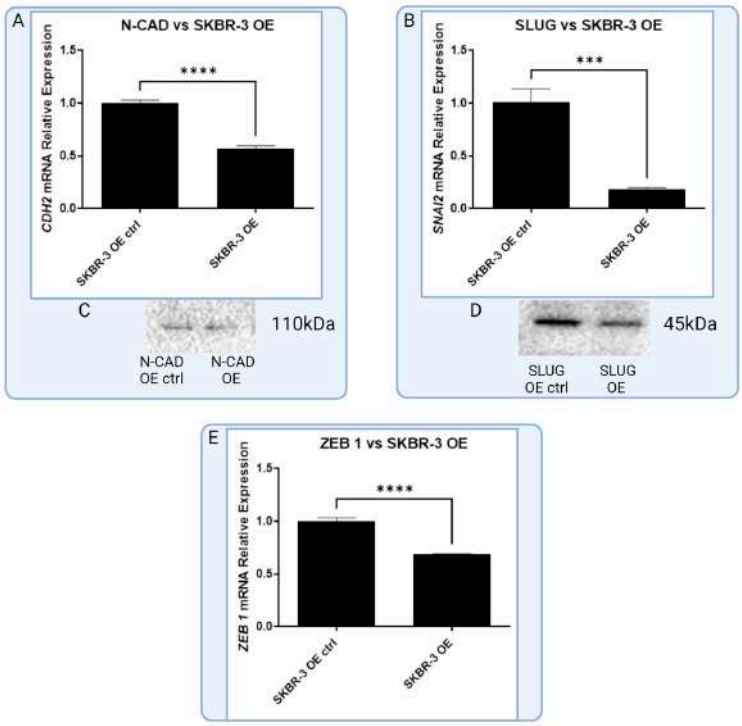


**Figure 10.** Graphic presentation of significant changes in expression of common EMT markers in HER-2-enriched cell line SKBR-3 on the level of mRNA and protein. For (**A**) N-CAD (T-Student test, $p < 0.0001$), (**B**) SLUG (T-Student test, $p < 0.0004$) and (**E**) ZEB 1 (T-Student test, $p < 0.0001$), there is statistically significant negative correlation with expression of RBMS 3. In case of N-CAD and SLUG, there are visible increases in expression of protein (**C**,**D**) in overexpressing cell line that stay in line with results of mRNA expression, *** $p < 0.001$, **** $p < 0.0001$. Created with BioRender.com.

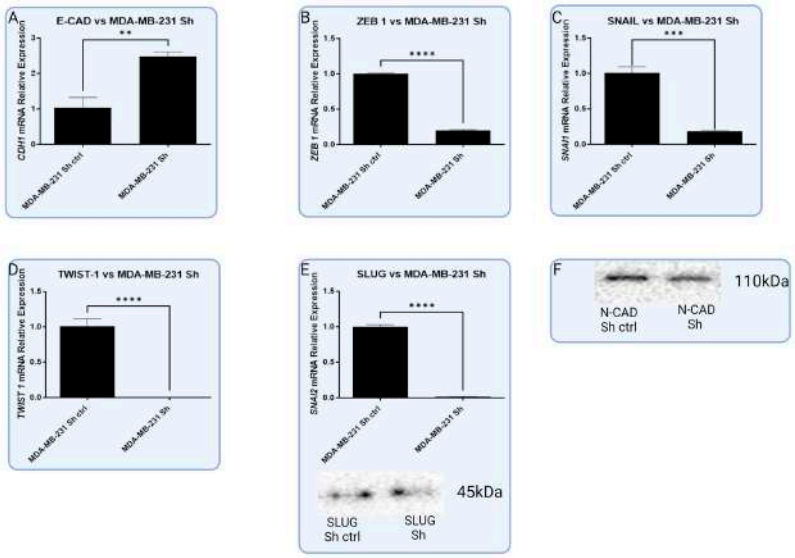


**Figure 11.** Graphic presentation of significant changes in expression of common EMT markers in RBMS 3-silenced TNBC cell line MDA-MB-231 on mRNA level (A–E) and protein level (F). Silencing of RBMS 3 lead to significant increase in expression of (**A**) E-CAD (T-Student test, $p < 0.0001$) and significant decrease in level of (**B**) ZEB 1 (T-Student test, $p < 0.0001$), (**C**) SNAIL (T-Student test, $p < 0.0001$), (**D**) TWIST 1 (T-Student test, $p < 0.0001$) and (**E**) SLUG (T-Student test, $p < 0.0001$) mRNA. In Western blot (**E**,**F**) we observed changes in (**E**) SLUG and (**F**) N-CAD protein levels between negative control and RBMS 3-silenced cells that are in line with the results of RT-qPCR, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Created with BioRender.com.

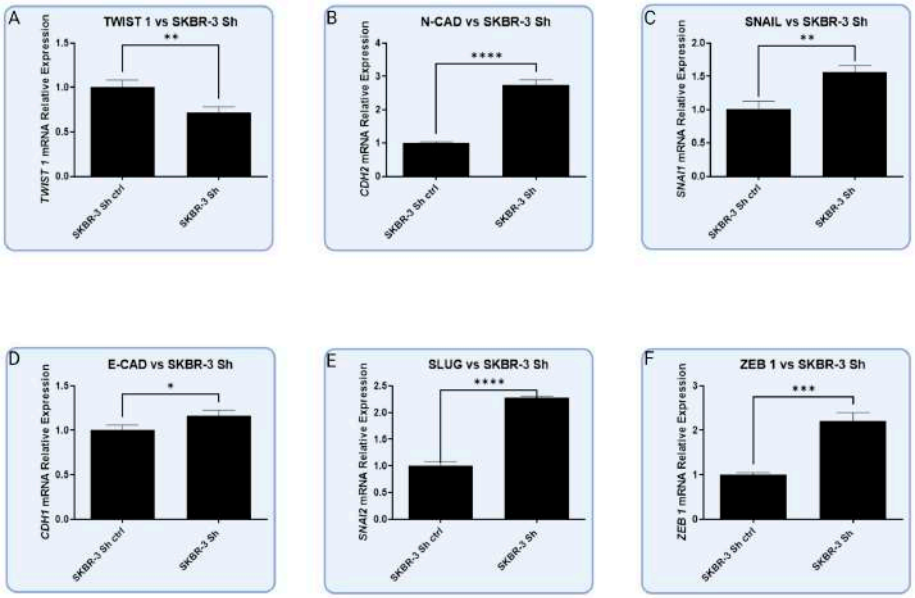


**Figure 12.** Graphic presentation of significant changes in expression of common EMT markers in HER-2-enriched cell line SKBR-3 at the level of mRNA and protein. Downregulation of RBMS 3 leads to significant decrease in level of (**A**) TWIST 1 mRNA (T-Student test, $p < 0.009$) and significant increase in all other markers: (**B**) N-CAD (T-Student test, $p < 0.0001$), (**C**) SNAIL (T-Student test, $p < 0.003$), (**D**) E-CAD (T-Student test, $p < 0.03$), (**E**) SLUG (T-Student test, $p < 0.0001$) and (**F**) ZEB 1 (T-Student test, $p < 0.0004$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Created with BioRender.com.

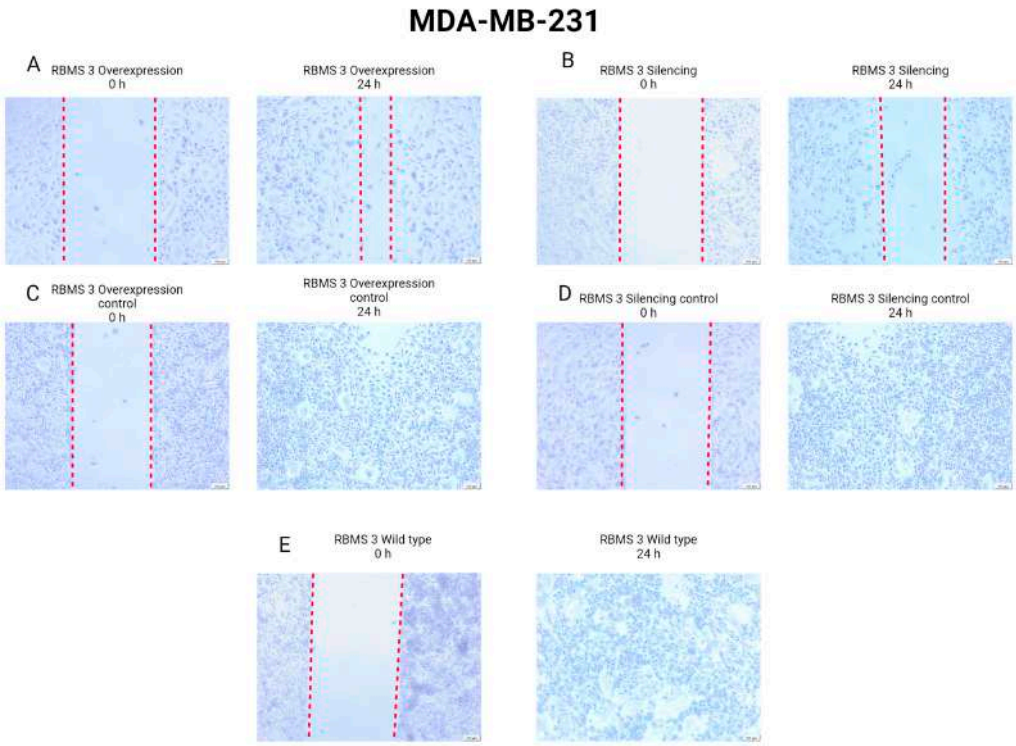


**Figure 13.** Results of scratch test performed on TNBC model (MDA-MB-231 cell line) with silenced and overexpressed RBMS 3 at the start and after 24 h. (**A**) RBMS 3 overexpression and (**C**) negative control of RBMS 3 overexpression, (**B**) RBMS 3 silencing and (**D**) negative control of RBMS 3 silencing, and (**E**) wild-type MDA-MB-231 cells. Magnification ×40. Created with BioRender.com.

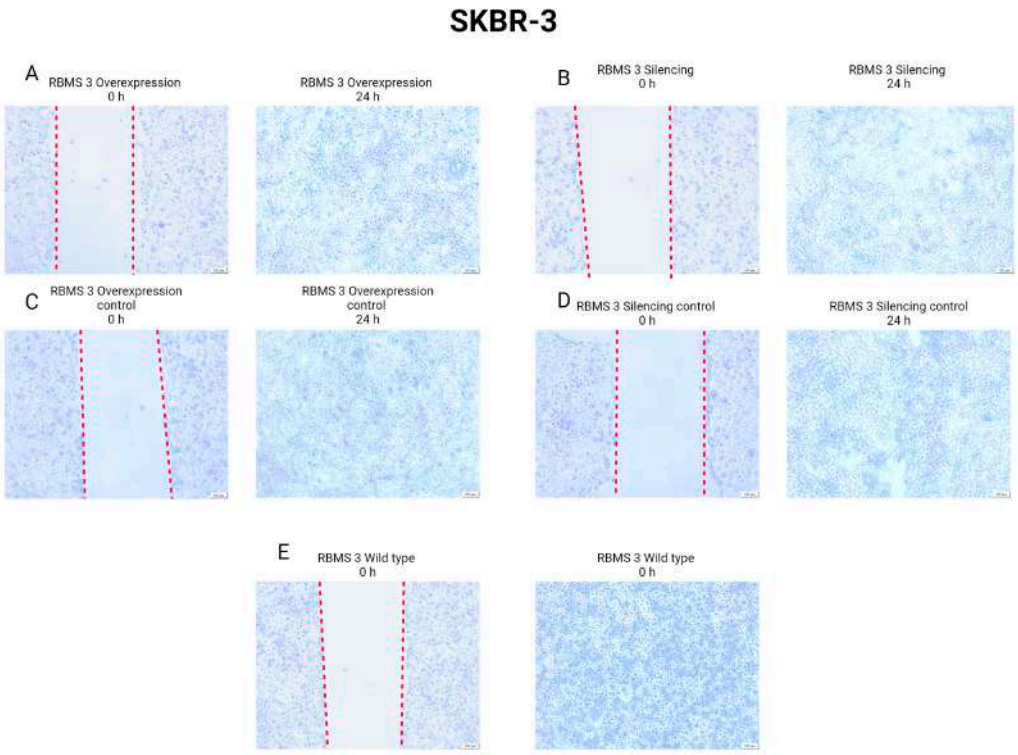


**Figure 14.** Results of scratch test performed on HER-2-enriched model (SKBR-3 cell line) with silenced and overexpressed RBMS 3 at the start and after 24 h. (**A**) RBMS 3 overexpression and (**C**) negative control of RBMS 3 overexpression, (**B**) RBMS 3 silencing and (**D**) negative control of RBMS 3 silencing, and (**E**) wild-type cells. Magnification ×40. Created with BioRender.com.

To further study the potential of cells to migrate, we conducted an immunofluorescent analysis of E-CAD and N-CAD expression in relation to RBMS 3 status. The results of this study on the TNBC model showed the presence of the cytoplasmatic expression of E-CAD and N-CAD in RBMS 3-overexpressing cells in contrast to the cells with silenced RBMS 3, where traces of E-CAD and N-CAD were observed in the cell's cytoplasm. The results of the immunofluorescent staining are presented in Figure 15.

In the case of the HER-2-enriched model, we observed the presence of E-CAD in the cytoplasm and cell membrane of both overexpressing and silenced RBMS 3. Additionally, N-CAD was detected in some cells within the HER-2-enriched model. This observation is consistent with the results of the WB analysis. Images of the immunofluorescent staining are presented in Figure 16.

To further elucidate the exact amount of cells expressing E-CAD and N-CAD, we performed an analysis of the cell count with a positive reaction using a machine learning tool. The results obtained by this analysis showed that in the case of E-CAD, in both models the expression of E-CAD was detected in significantly more cells in RBMS 3-overexpressing cells than in RBMS 3-silenced ones. The same relationship was found in the case of N-CAD in the MDA-MB-231 cell line. In the SKBR-3 cell line, with the overexpression of RBMS 3 only around 20% of the general amount of cells was identified as N-CAD-positive, which may diminish the importance of this result. These observations are consistent with the results of the WB analysis.

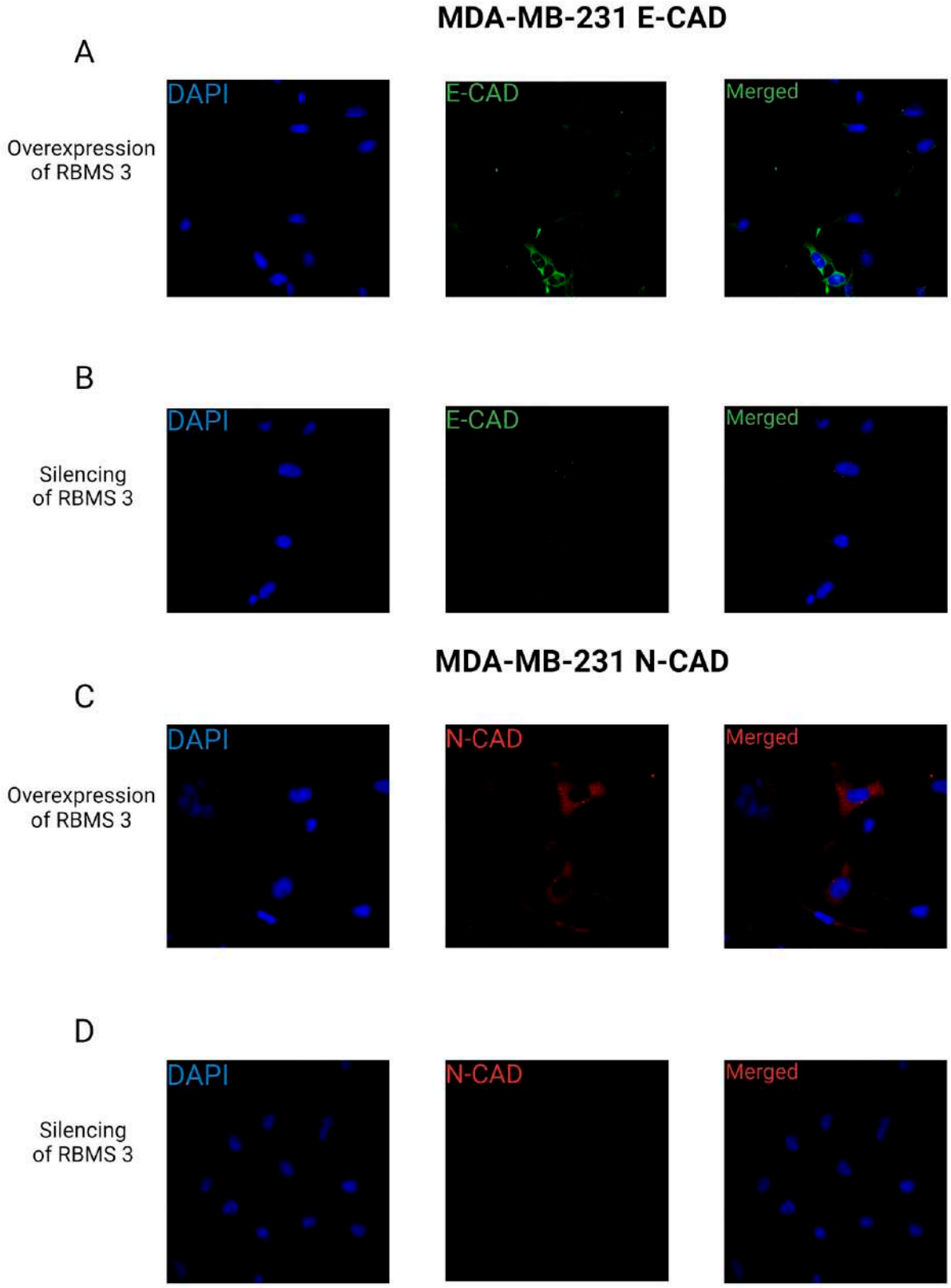


**Figure 15.** Confocal images showing expression pattern of E-CAD (**A**,**B**) and N-CAD (**C**,**D**), in MDA-MB-231 cells with silenced (**A**,**C**) and overexpressed (**B**,**D**) RBMS 3. In both cases, cytoplasmatic reactions were observed. Images were made using objective ×60. Nucleus was stained with DAPI. Created with BioRender.com.

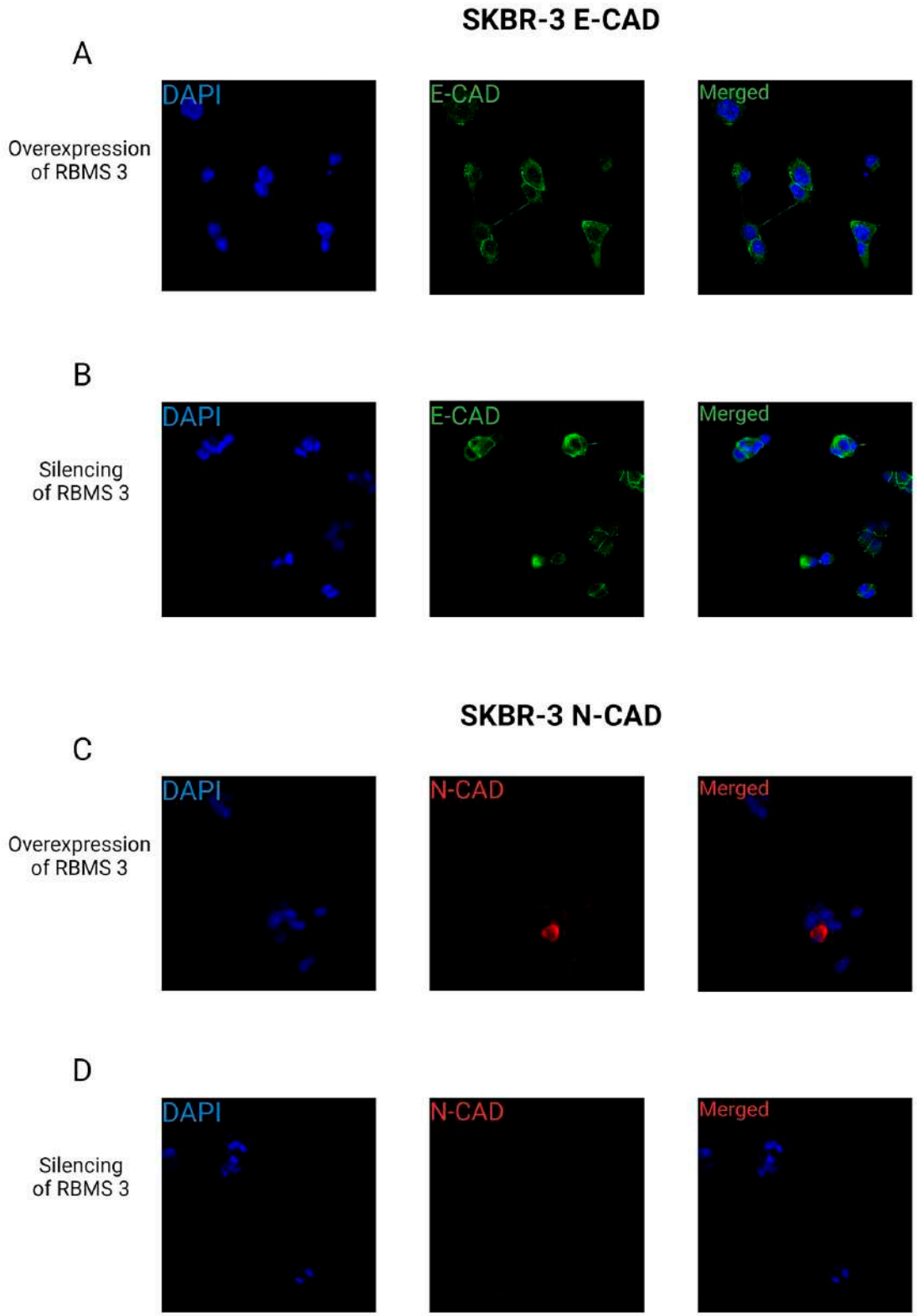


**Figure 16.** Confocal images showing membrane expression pattern of E-CAD (**A**,**B**) and N-CAD (**C**,**D**), in SKBR-3 cells with silenced (**A**,**C**) and overexpressed (**B**,**D**) RBMS 3. E-CAD expression was present in cytoplasm and cell membrane and N-CAD in cytoplasm of single cells. Images were made using objective ×60. Nucleus was stained with DAPI. Created with BioRender.com.

## 4. Discussion

A growing body of evidence suggest the RBMS 3 may potentially play a role in the progression of breast cancer and the regulation of cell adhesion genes [28,36–38]. Early studies concerning the role of RBMS 3 in breast cancer depict it as a potential tumor suppressor protein capable of inhibiting metastasis [39,40]. A previous study conducted by our group on RBMS 3 expression in IDC revealed conclusions consistent with other studies, indicating that a higher expression of RBMS 3 correlates with a higher overall survival, although in vitro studies of RBMS 3 expression in cell lines shows the highest expression of RBMS 3 in TNBC and HER-2-enriched cell lines [36]. The findings of this study provide evidence that the function of RBMS 3 in breast cancer is more intricate than previously thought. The analysis of clinical material, without the subdivision into molecular subtypes, presents RBMS 3 as a positive regulator of EMT markers, suggesting a potential pro-metastatic role of RBMS 3 in breast cancer. In a selected population of TNBC and HER-2-enriched cases, the results indicate a smaller scale of RBMS 3 impact on EMT markers, where in TNBC, RBMS 3 significantly or almost significantly positively correlated with two markers and in the case of HER-2-enriched cell lines, it did so only with one, which may indicate a more epithelial phenotype of said cells.

In the study presenting RBSM 3 as an inhibitor of metastasis [39], Yang and co-workers employed the MCF-7 cell line as model for the molecular subtype of luminal A, which is one of the most benign types of breast cancer, with a 5-year survival rate of almost 95% [41]. In contrast, in our study we focused on the most aggressive types of breast cancer, which are the TNBC and HER-2-enriched types [41], and therefore MDA-MB-231 and SK-BR-3 cells were used for a functional in vitro model This in vitro model facilitated a more profound investigation into the role of RBMS 3 in IDC subtypes. Accordingly, the results demonstrate distinctions between the function of RBMS 3 in the EMT process between the HER-2-enriched model and the TNBC model. In conclusion, RBMS 3 in the HER-2-enriched model may potentially act as a suppressor of EMT, where the overexpression of RBMS 3 leads to a decrease in the expression of several EMT markers, which may result in a potentially more epithelial phenotype of cells, indicated by a decrease in N-CAD expression. On the other hand, the silencing of RBMS 3 has been observed to result in increase in EMT marker expression, changing the cell phenotype to a more mesenchymal state by increasing the expression of N-CAD, SNAIL, SLUG and ZEB 1. Our in vitro studies are consistent with the results obtained from patient-derived IDC tumor tissues, and moreover have clarified the orchestrating role of RBMS 3 in this breast cancer subtype.

It is crucial to recognize that the EMT process is not a binary phenomenon; rather, it encompasses a spectrum of hybrid phenotypes that express both mesenchymal and epithelial markers. This may provide a potential explanation for the observed increase in E-CAD expression in both of the supposedly more aggressive types of cells, namely TNBC with an overexpression of RBMS 3 and HER-2-enriched cell lines with silenced RBMS 3 [42,43]. The results of immunofluorescent staining may shed light on the potential influence of RBMS 3 on the invasive properties of breast cancer cells. In our observations, we did not observe changes in the cell phenotype depending on the expression of RBMS 3. The cytoplasmatic expression of E-CAD and N-CAD in the TNBC model of breast cancer may indicate the destabilization of cell junctions and a potential mesenchymal type of migration of this type of cells. In the HER-2-enriched model, the presence of E-CAD in the cell membrane may imply that this type of cell can exhibit a collective type of migration [44,45]. From this perspective, the expression of RBMS 3 may regulate breast cancer cells' migration in different models, dependent on breast cancer subtypes, in relation to steroids and HER-2 receptor status.

In contrast, in the TNBC in vitro model, we observed the opposite effect to that seen in the HER-2-enriched model. The overexpression of RBMS 3 resulted in an increased expression of all the markers studied, including, interestingly, E-CAD. The increased expression of E-CAD may not necessarily imply a decrease in the potential to undergo EMT, as there is evidence that E-CAD is a necessary protein for metastasis in breast cancer [42]

and that high levels of N-CAD promote cell motility in breast cancer, despite high levels of E-CAD expression [43]. On the other hand, the silencing of RBMS 3 resulted in a decrease in the expression of all the markers studied and increased the expression of E-CAD, suggesting a more epithelial phenotype of the cells. The results of the TNBC correlations with RBMS 3 are consistent with the findings of the studies conducted by Block CJ et al. [20]. In their in vitro and in vivo studies, the authors provided evidence that silencing RBMS 3 in TNBC cell lines significantly reduced cell invasion and migration. These findings are partially complementary with the results of the migration assay. In our TNBC in vitro model, which employs MDA-MB-231 cells, the silencing of RBMS 3 mostly decreased cell migration. However, the overexpression of RBMS 3 also affects MDA-MB-231 cell migration in comparison to the controls.

Contrarily, in the case of the HER-2-positive in vitro model, which was analyzed on SKBR-3 cells, the deregulation of RBMS 3 did not affect cell migration. This result, taken together with changes in E-Cadherin localization, may suggest a role of RBMS 3 in migration patterns, depending on the breast cancer subtypes, not strictly in cell migration abilities in a 2D model.

Among the proteins investigated in this study, the relationship between the expression of TWIST 1 and RBMS 3 expression in triple negative breast cancer seems to be the one with the most contradictory results obtained by scientists to date. In the initial study on this association by Zhu L. et al., the authors found that TWIST 1 expression is significantly decrease in the RBMS 3-overexpressing MDA-MB-231 cell line [40]. However, Block CJ et al. provided evidence that they did not observe any changes in TWIST 1 expression in their experiments in the same model [20]. The results of our study provide potential evidence that TWIST 1 is indeed positively correlated with RBMS 3 expression. Our analysis conducted with the RT-qPCR method revealed a significant increase in TWIST 1 mRNA expression in the RBMS 3 overexpression model and a significant decrease in the RBMS 3 knockdown model. In the Western blot analysis, we did not observe TWIST 1 at the standard level, but specific bands were present at a higher molecular weight, which, according to the literature, may correspond to the ubiquitinous form of TWIST 1 [46]. The same conclusions can be drawn for the SNAIL and SLUG proteins, where the amount of nominal protein was low and specific bands were present at the higher levels of the molecular mass ladder [47,48]. The positive correlation of TWIST 1 with RBSM 3 in TNBC is supported by the results of the IHC analysis. It is important to note that this correlation is true not only for TNBC cases, but also for HER-2-enriched cases and for the general population of all IDC specimens studied. The differences in the results of the 2D models may stem from the fact that the model prepared by Block CJ et.al [20] was based on a human mammary epithelial cell line rather than an actual cancer cell line. Additionally, we observed multiple bands of E-CAD and ZEB 1 in WB images. The presence of multiple bands of E-CAD may be caused by the presence of the N-terminal fragment of E-CAD, which is the product of the proteolytic degradation of E-CAD that may be present in cancer cells [49,50]. In the case of ZEB 1, the additional bands may be the products of proteasomal degradation, due to the ZEB-1 protein's short half-life being only 3 h [51].

The high efficiency of RBMS 3 overexpression in both cell lines studied led to the generation of two additional heavier products of RBMS 3 mRNA. In both cell lines, the additional products were at the same molecular weight, suggesting that RBMS 3 post-translational modifications are not bound to the molecular type of breast cancer. These results may indicate that the overexpression of RBMS 3 may lead to the formation of, to our knowledge, previously undescribed forms that require further investigation.

Taking into consideration all the results presented in this study, we propose a novel multidimensional view of the role of RBMS 3 in EMT in breast cancer, suggesting that RBMS 3 plays different roles in orchestrating the protein expression levels involved in EMT depending on the molecular type of breast cancer. This hypothesis may have significant implications for the potential use of RBMS 3 as a diagnostic marker and target for therapy. The correlation between RBMS3 and the expression of EMT markers may indicate a poten-

tial role in predicting the metastatic potential of a tumor and may be used by physicians to determine the early transition from local to systemic cancer therapy. As a target for therapy, distinguishing between the effects of RBMS 3 expression in different molecular types of breast cancer may lead to the development of more specific drugs that provide a higher treatment efficacy compared to a general drug that supposedly works for all types of breast cancer. This type of approach aligns with the current trend in medicine towards a personalized approach to cancer treatment. The results of this study also open a new field of investigation into the exact molecular mechanisms of RBMS 3's role in EMT and the reasons for the differences in RBMS 3's role in different subtypes of breast cancer.

*Limitations*

The present study was conducted on 449 invasive ductal carcinoma tumors; however, investigations conducted on a higher number of TNBC and HER-2-enriched cases, which belong to the most rare subtypes, may strengthen the evidence for the role of RBMS 3 in these types of cancers. Furthermore, additional analysis in an in vivo model of TNBC and HER-2 tumors is required to fully elucidate the role of RBMS 3 in metastases occurring in these types of breast tumors.

## 5. Conclusions

The studies conducted indicate for the first time the role of RBMS 3 as an orchestrating protein in the EMT process in TNBC and HER-2-enriched IDC tumors. The results of our studies suggest that RBMS 3 may act as an EMT-promoting protein in TNBC and as a suppressor of EMT in the HER-2-enriched subtype. These findings shed light on the novel role of this protein in IDC tumors and may be useful for designing targeted therapies for specific breast cancer subtypes.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells13181548/s1, S1: IHC expression pattern, S2: Analysis of correlation between expression of RBMS 3 and common EMT markers, S3: Results of qRT-PCR statistical analysis, S4: Original WB images

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

**Funding:** This research was funded by the Ministry of Science and Higher Education grant number SKN/SP/569116/2023.

**Institutional Review Board Statement:** This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Wroclaw Medical University (decision no. KB 625/2022 25 August 2022) for studies involving humans.

**Informed Consent Statement:** All patients provided written informed consent allowing the use of their material samples for this scientific research.

**Data Availability Statement:** The raw data supporting the conclusions of this article will be made available by the authors on request.

**Acknowledgments:** The authors would like to extend special thanks to Agnieszka Barańska, Elżbieta Połejko and Bożena Przygodzka (Division of Histology and Embriology, Wroclaw Medical University) for technical assistance in the preparation of immunohistochemistry reactions.

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

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## *9. CURRICULUM VITAE*

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Kierunek Lekarski, uzyskany tytuł zawodowy: Lekarz

## Doświadczenie Zawodowe
**09.2022 - 06.2023 Uniwersytet Medyczny. Im. Piastów Śląskich we Wrocławiu, Zakład Histologii i Embriologii**
Stypendysta, badacz w projekcie OPUS DEC-2018/31/B/NZ5/02238 *„Określenie epigenetycznych mechanizmów modulacji wybranych genów z rodziny SOX oraz ich transkryptów jako potencjalnych markerów diagnostycznych i predykcyjnych w niedrobnokomórkowych rakach płuc NSCLC”*

**10.2023 – Teraz Uniwersytecki Szpital Kliniczny we Wrocławiu, ul. Borowska 2013, 55- 556 Wrocław**
Lekarz stażysta

**10.2024 – Teraz Uniwersytet Medyczny. Im. Piastów Śląskich we Wrocławiu, Zakład Histologii i Embriologii**
Asystent w grupie badawczej

## Znajomość języków obcych:
**- Angielski poziom C1 potwierdzony certyfikatem First Certificate in English (FCE), 04.2022**

## Projekty badawcze
1. *Rola białka RBMS3 w procesie przejścia epitelialno-mezenchymalnego (EMT) w progresji raka gruczołu piersiowego.*

**Projekt finansowany z grantu MNiSW SKN Tworzą Innowacje: SKN/SP/569116/2023**
**Rola: kierownik projektu**

2. Zastosowanie zdecelularyzowanej macierzy tarczycy w medycynie regeneracyjnej i onkologii

**Projekt finansowany z grantu MNiSW Perły Nauki: PN/01/0013/2022**
**Rola: kierownik projektu**

3. Nowe inhibitory VEGFR w farmakoterapii raka rdzeniastego tarczycy. Badania in vitro.

**Projekt finansowany w ramach wewnętrznej subwnecjii konkursowej Uniwersytetu Medycznego we Wrocławiu**
**Rola: kierownik projektu**

## Nagrody

**12.2023** Laur Medyczny im. Wacława Mayzla: honorowe wryróżnienie przyznawany przez Wydział Nauk Medycznych Polskiej Akademii Nauk dla studentów pracujących naukowo za publikację: Górnicki T, Lambrinow J, Mrozowska M, Romanowicz H, Smolarz B, Piotrowska A, Gomułkiewicz A, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J. Expression of RBMS3 in Breast Cancer Progression. Int J Mol Sci. 2023 Feb 2;24(3):286, doi: 10.3390/ijms24032866.

**05.2024** Laureat Stypendium w programie NAWA Walczak

## Aktywność w organizacjach naukowych

**10.2018 - 2024 SKN Histologii i Biologii Molekularnej**
Pełniona funkcja: Przewodniczący

**07.2023 – teraz TERMIS (Tissue Engineering and Regenerative Medicine International Society): San Ramon, US**
Pełniona funkcja: Członek Towarzystwa

**07.2022 – teraz Polskie Towarzystwo Histochemików i Cytochemików**
Pełniona funkcja: Członek Towarzystwa

## Działalność wdrożeniowa

Zgłoszeni patentowe: „Scaffold, sposób jego uzyskiwania oraz zastosowania” Numer zgłoszenia: P.446307 z dnia: 05.10.2023

## Wolontariat

1. *Instytut Immunologii i Terapii Doświadczalnej PAN*

Wolontariat w projekcie Gucio skierowanym do uczniów szkół średnich pod kierownictwem prof. Małgorzaty Cebrat (2017r)

# 10. DOROBEK NAUKOWY

# lek. Tomasz Stanisław Górnicki

## WYKAZ PUBLIKACJI

### 1. Publikacje w czasopismach naukowych

#### 1.1 Publikacje w czasopiśmie z IF

| Lp. | Opis bibliograficzny | IF | Punkty |
|---|---|---|---|
| 1 | Skrzypczak Tomasz, Błachnio Klaudia, **Górnicki Tomasz**, Kmieć Justyna, Ciąder Agnieszka, Biernikiewicz Małgorzata, Majchrowska Marzena, Sobieszczańska Małgorzata, Szymala-Pędzik Małgorzata, Kałka Dariusz: Association between the desire for breast augmentation and instagram engagement: a cross-sectional survey among young Polish women, International Journal of Environmental Research and Public Health, 2021, vol. 18, nr 19, art.10317 [13 s.], DOI:10.3390/ijerph181910317 | 4,614 | 140 |
| 2 | Szymonik Julia, Wala Kamila, **Górnicki Tomasz**, Saczko Jolanta, Pencakowski Bartosz, Kulbacka Julita: The impact of iron chelators on the biology of cancer stem cells, International Journal of Molecular Sciences, 2022, vol. 23, nr 1, art.89 [16 s.], DOI:10.3390/ijms23010089 | 5,6 | 140 |
| 3 | **Górnicki Tomasz**, Lambrinow Jakub, Mrozowska Monika, Podhorska-Okołów Marzena, Dzięgiel Piotr, Grzegrzółka Jędrzej: Role of RBMS3 novel potential regulator of the EMT phenomenon in physiological and pathological processes, International Journal of Molecular Sciences, 2022, vol. 23, nr 18, art.10875 [13 s.], DOI:10.3390/ijms231810875 | 5,6 | 140 |
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| 6 | Bułdyś Kacper, **Górnicki Tomasz**, Kałka Dariusz, Szuster Ewa, Biernikiewicz Małgorzata, Markuszewski Leszek, Sobieszczańska Małgorzata: What do we know about nociplastic pain?, Healthcare, 2023, vol. 11, nr 12, art.1794 [15 s.], DOI:10.3390/healthcare11121794 | 2,4 | 40 |
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| 10 | **Górnicki Tomasz**, Lambrinow Jakub, Golkar-Narenji Afsaneh, Data Krzysztof, Domagała Dominika, Niebora Julia, Farzaneh Maryam, Mozdziak Paul, Zabel Maciej, Antosik Paweł, Bukowska Dorota, Ratajczak Kornel, Podhorska-Okołów Marzenna, Dzięgiel Piotr, Kempisty Bartosz: Biomimetic scaffolds—a novel approach to three dimensional cell culture techniques for potential implementation in tissue engineering, Nanomaterials, 2024, vol. 14, nr 6, art.531 [22 s.], DOI:10.3390/nano14060531 | 4,4* | 100 |

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***IF 2023***

### 1.2 Publikacje w czasopiśmie bez IF -

## 2. Abstrakty

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**Impact Factor: 46,414**

**Punkty ministerialne: 1390,0**

**H-index według WOS: 6**

OSOBA SPORZĄDZAJĄCA: PAULINA OBUCHOWICZ
DZIAŁ BIBLIOGRAFII I BIBLIOMETRII BG UMW

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   Mój udział polegał na: konsultacjach metodologicznych dotyczących izolacji RNA oraz odwrotnej transkrypcji. Udziale przy wykonywaniu reakcji real-time PCR, a także uczestniczeniu w analizie wyników otrzymanych metodą real-time PCR.

2. Górnicki T, Lambrinow J, Mrozowska M, Krawczyńska K, Staszko N, Kmiecik A, Piotrowska A, Gomułkiewicz A, Romanowicz H, Smolarz B, Podhorska-Okołów M, Grzegrzółka J, Rusak A, Dzięgiel P, Impact of RBMS 3 Progression on Expression of EMT Markers. Cells 2024, Sep 13, 1548. https://doi.org/10.3390/cells13181548

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   Mój udział polegał na: konsultacji w zakresie hodowli komórkowych oraz mikroskopii konfokalnej, współuczestniczeniu w wykonywaniu reakcji Western Blot, uczestniczeniu w analizie wyników, poprawie pierwotnej wersji manuskryptu.

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   Mój udział polegał na: przygotowaniu mikromacierzy tkankowych inwazyjnego przewodowego raka gruczołu piersiowego oraz przeprowadzeniu reakcji immunohistochemicznych.

2. Górnicki T, Lambrinow J, Mrozowska M, Krawczyńska K, Staszko N, Kmiecik A, Piotrowska A, Gomułkiewicz A, Romanowicz H, Smolarz B, Podhorska-Okołów M, Grzegrzółka J, Rusak A, Dzięgiel P, Impact of RBMS 3 Progression on Expression of EMT Markers. Cells 2024, Sep 13, 1548. https://doi.org/10.3390/cells13181548

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   Mój udział polegał na: uczestniczeniu w przygotowaniu pierwotnej wersji manuskryptu oraz wyborze fraz kluczowych na podstawie których opracowano przegląd literatury.

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   Mój udział polegał na: uczestniczeniu w ocenie reakcji immunohistochemicznych, przygotowywaniu materiału komórkowego do analiz metodami biologii molekularnej oraz udział w przygotowywaniu pierwotnej wersji manuskryptu.

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   Mój udział polegał na: konsultacjach przy wyborze słów kluczowych użytych do przeglądu literatury oraz poprawie pierwotnej wersji manuskryptu.

2. Górnicki T, Lambrinow J, Mrozowska M, Romanowicz H, Smolarz B, Piotrowska A, Gomułkiewicz A, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J. Expression of RBMS3 in Breast Cancer Progression. Int J Mol Sci. 2023 Feb 2;24(3):2866. doi: 10.3390/ijms24032866.

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# OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

1. Górnicki T, Lambrinow J, Mrozowska M, Romanowicz H, Smolarz B, Piotrowska A, Gomułkiewicz A, Podhorska-Okołów M, Dzięgiel P, Grzegrzółka J. Expression of RBMS3 in Breast Cancer Progression. Int J Mol Sci. 2023 Feb 2;24(3):2866. doi: 10.3390/ijms24032866.

   Mój udział polegał na: zabezpieczeniu i gromadzeniu materiału klinicznego do badań oraz gromadzenia danych kliniczno-patologicznych pacjentów.

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