UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

Katedra Morfologii i Embriologii Człowieka

Zakład Histologii i Embriologii

PRACA DOKTORSKA

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Ekspresja iryzyny w rakach gruczołu piersiowego

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Promotor pomocniczy: Dr Katarzyna Nowińska

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Badania do pracy doktorskiej sfinansowano z projektu „Role of FNDC5 expression in breast cancer" uzyskanego w ramach Regionalnej Inicjatywy Doskonałości (nr grantu RID.Z501.19.015).

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Spis treści

1. Wykaz publikacji stanowiących pracę doktorską

Poniżej przedstawiono spis publikacji będących podstawą pracy doktorskiej pt.: „Ekspresja iryzyny w rakach gruczołu piersiowego”. Sumaryczny IF cyklu zgodny z rokiem publikacji jest równy 12,416, a punktacja MEiN wynosi 280.

1. Cebulski, K.; Nowińska, K.; Jablońska, K.; Romanowicz, H.; Smolarz, B.; Dzięgiel, P.; Podhorska-Okołów, M. Expression of Irisin/FNDC5 in Breast Cancer. Int. J. Mol. Sci. 2022, 23, 3530.
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2. Cebulski, K.; Piotrowska, A.; Kmiecik, A.; Haczkiewicz-Leśniak, K.; Ciesielska, U.; Grzegrzółka, J.; Jabłońska, K.; Romanowicz, H.; Smolarz, B.; Dzięgiel, P.; Podhorska-Okołów, M.; Nowińska, K. The Role of Irisin/FNDC5 Expression and Its Serum Level in Breast Cancer. Int. J. Mol. Sci. 2023, 24, 8628.
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2. Wprowadzenie

Rak gruczołu piersiowego (BC) jest jednym z najczęściej występujących nowotworów u kobiet. W 2020 roku według raportu „Global Cancer Statistics 2020" rozpoznano 2 261 419 nowych przypadków tej choroby, co stanowiło 24,5% wszystkich rozpoznanych na świecie nowotworów wśród kobiet. Rak gruczołu piersiowego był przyczyną zgonu u 684 996 pacjentek i zajął 1 miejsce wśród przyczyn zgonów. Stanowił 15,5% spośród wszystkich onkologicznych przyczyn zgonów kobiet [1]. Pomimo znacznego postępu wiedzy na temat procesu kancerogenezy i mechanizmów progresji tego raka, nadal poważnym problemem pozostaje wczesne wykrywanie oraz efektywna terapia. Mimo, że rak gruczołu piersiowego jest coraz częściej wykrywany we wczesnych stadiach choroby, a jego leczenie jest coraz bardziej efektywne, to nadal zajmuje wysokie miejsce wśród najczęstszych przyczyn zgonu pacjentek z nowotworami złośliwymi [2]. Dlatego w dalszym ciągu istnieje potrzeba poszukiwania nowych markerów prognostycznych i predykcyjnych raka gruczołu piersiowego. Badania z ostatnich lat przeprowadzone na wielu nowotworach wykazały, że jednym z takich potencjalnych markerów może być iryzyna.

FNDC5 (Fibronectin type III Domain-Containing protein 5) jest białkiem transbłonowym [3], będącym prohormonem, z którego uwalniany jest 112 aa peptyd - iryzyna. Iryzyna została wykryta po raz pierwszy w 2012 roku przez Boström i wsp. we włóknach mięśni szkieletowych myszy w odpowiedzi na wysiłek fizyczny [4]. Wykazano również, że czynnikiem transkrypcyjnym regulującym ekspresję genu *FNDC5* jest koaktywator receptora aktywowanego przez proliferatory peroksysomów. (ang. peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC1α). Do tej pory nie udało się jednak wyjaśnić mechanizmu, w wyniku którego z prohormonu zostaje uwolniona iryzyna [5–7]. Po uwolnieniu iryzyny z FNDC5 ulega ona glikozylacji, będącej jednym z najistotniejszych procesów obróbki potranslacyjnej białka, czego efektem jest znacząca zmiana jego właściwości fizykochemicznych. Zahamowanie procesu glikozylacji prowadzi do obniżenia sekrecji iryzyny. Postać nieglikozylowana posiada masę ok. 13kD, natomiast glikozylowana ok 20kD [8].

Iryzyna odgrywa kluczową rolę w procesie przemiany białej tkanki tłuszczowej w brązową poprzez zwiększenie ekspresji termogeniny (UCP1) w grzebieniach mitochondrialnych. Białko UCP1 jest kluczowe w adaptacji organizmów do niskich temperatur. Powoduje zahamowanie produkcji ATP w łańcuchu oddechowym [6]. Dotychczasowe badania

potwierdziły obecność iryzyny w wielu tkankach i narządach – m. in. także w kardiomiocytach, tkance tłuszczowej, nerkach, ścianie naczyń krwionośnych wątrobie, skórze i móżdżku [4,9]. Mało jeszcze wiadomo na temat receptorów tkankowych dla iryzyny. Jak dotychczas wykazano obecność takiego receptora (αVβ5 integryna) w osteocytach, gdzie przypuszczalnie iryzyna, po aktywacji swoich receptorów, może wpływać na osteoklastogenezę i przebudowę kości [10].

Podwyższenie ekspresji iryzyny, poza stanami fizjologicznymi, obserwowano również w różnego typu nowotworach m in. raku płuca, raku przewodu pokarmowego, raku jajnika, raku jasnokomórkowym nerki, raku krtani, kostniakomięsakach [11,12] Uważa się, że podwyższony poziom iryzyny w guzie nowotworowym może prowadzić do lokalnej hipertermii, skutkującej denaturacją białek i hamowaniem wzrostu guza przez blokowanie powstawania ATP w łańcuchu oddechowym w mitochondriach [13]. Badania na modelu *in vitro* w liniach komórkowych raka płuc wskazują, że zwiększony poziom iryzyny może prowadzić do zahamowania proliferacji, migracji i przejścia epitelialno-mezenchymalnego (EMT) przez aktywację szlaku PI3K/AKT [14]. Podobnie w kostniakomięsaku obserwowano hamujący wpływ iryzyny na proliferację, migrację i inwazję komórek nowotworowych. Jednak w tym przypadku iryzyna działa poprzez ścieżkę sygnałową STAT3–SNAIL i odwrócenie działania IL-6 na EMT [15]. Z kolei aktywacja przez iryzynę szlaku PI3K/AKT w komórkach raka wątrobowokomórkowego wywiera przeciwny, stymulujący wpływ na proliferację i inwazję komórek [16].

EMT jest procesem, w którym komórka typu nabłonkowego (z ekspresją typowego białka - E-kadheryny) ulega przekształceniu się w komórkę o typie mezenchymalnym (z ekspresją N-kadheryny). Aby do tego doszło, w komórce musi dojść do szeregu zmian. Najważniejsze z nich to zerwanie strukturalnych połączeń z innymi komórkami i utrata wyraźnej polaryzacji samych komórek. EMT obserwuje się zarówno w procesach fizjologicznych (np. gojenie ran), jak również w nowotworach. Istotna różnica jest związana z brakiem kontroli przebiegu tego procesu w przypadku progresji nowotworu [17]. Za najważniejsze czynniki transkrypcyjne uczestniczące w EMT uważa się SNAIL1 (SNAIL), SNAIL2 (SLUG), TWIST i ZEB1. Wywołane przez SNAIL EMT prowadzi do zmniejszenia poziomu E-kadheryny w komórkach i wzrostu poziomu ekspresji N-kadheryny, wimentyny, fibronektyny, czego efektem jest wzrost możliwości migracji komórek nowotworowych [17].

W dotychczasowej literaturze dostępne są pojedyncze publikacje podejmujące tematykę znaczenia iryzyny w rakach gruczołu piersiowego, w tym tylko jedna praca dotycząca jej ekspresji tkankowej w tych nowotworach [12]. Autorzy przeprowadzili badania

immunohistochemiczne (IHC) na materiale raka gruczołu piersiowego i wykazali istotnie wyższy poziom iryzyny w komórkach raka gruczołu piersiowego w porównaniu do komórek prawidłowego gruczołu piersiowego. Inne badania, w modelu *in vitro* wykazały, że iryzyna wpływała na zwiększenie efektu cytotoksycznego doxorubicyny w przypadku linii komórek raka piersi MCF-7 i MDA-MB-231 przy jednoczesnym braku wpływu na komórki linii prawidłowego gruczołu piersiowego MCF-10a [18]. Z kolei Provatopoulou i wsp. wykazali niższy poziom iryzyny w surowicy pacjentek z rakiem gruczołu piersiowego w porównaniu do jej poziomu w surowicy zdrowych kobiet [19]. Wyniku tego nie potwierdzili inni badacze, którzy zaobserwowali wyższy poziom iryzyny w surowicy pacjentek z łagodnymi i złośliwymi zmianami gruczołu piersiowego w porównaniu do poziomu w surowicy pacjentek zdrowych [20]. Autorzy tej pracy stwierdzili, że iryzyna jest niezależnym czynnikiem prognostycznym wystąpienia przerzutów. Natomiast w badaniu pacjentek z rakiem piersi z przerzutami do kości zaobserwowano obniżony poziom iryzyny w surowicy u pacjentek w porównaniu do tych bez przerzutów [21]. Jak widać z przytoczonych danych z piśmiennictwa, wyniki są niespójne. Mechanizm uwalniania iryzyny do surowicy nie jest w pełni poznany i nadal brakuje informacji dotyczących czynników wpływających na zmiany stężenia iryzyny w osoczu. Według niektórych autorów do czynników tych mogą należeć m.in. płeć, wiek, poziom aktywności fizycznej, etap cyklu miesięcznego [22]. Dodatkowo, należy pamiętać, że iryzyna może być wydzielana do osocza przez różne tkanki, niezajęte przez proces nowotworowy [4,23].

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3. Założenia i cel pracy

Celem przeprowadzonych badań było określenie zależności pomiędzy poziomem ekspresji iryzyny a rakiem gruczołu piersiowego.

Celem pierwszej pracy z cyklu publikacji (Cebulski, K.; Nowińska, K.; Jablońska, K.; Romanowicz, H.; Smolarz, B.; Dzięgiel, P.; Podhorska-Okołów, M. *Expression of Irisin/FNDC5 in Breast Cancer.* Int. J. Mol. Sci. 2022, 23, 3530. https://doi.org/10.3390/ijms23073530) było określenie lokalizacji i nasilenia ekspresji iryzyny w rakach gruczołu piersiowego, a także zbadanie jej związku z danymi kliniczno-patologicznymi (m. in. stopniem złośliwości histologicznej, stadium zaawansowania, skalą TNM, przeżyciami pacjentów) oraz markerami proliferacji (Ki-67) i biogenezy mitochondriów (PGC-1α).

Celem drugiej pracy z cyklu (Cebulski, K.; Piotrowska, A.; Kmiecik, A.; Haczkiewicz-Leśniak, K.; Ciesielska, U.; Grzegrzółka, J.; Jabłońska, K.; Romanowicz, H.; Smolarz, B.; Dzięgiel, P.; Podhorska-Okołów, M.; Nowińska, K. *The Role of Irisin/FNDC5 Expression and Its Serum Level in Breast Cancer.* Int. J. Mol. Sci. 2023, 24, 8628. https://doi.org/10.3390/ijms24108628) było zbadanie związku iryzyny z procesem przejścia epitelialno-mezenchymalnego (EMT) w raku gruczołu piersiowego oraz potwierdzenie metodami immunofluorescencji, RT-PCR i immunogold obecności iryzyny zarówno w materiale raka gruczołu piersiowego jak i na liniach komórkowych BC.

W obu pracach wykorzystano materiał archiwalny Zakładu Histologii i Embriologii Katedry Morfologii i Embriologii Człowieka Uniwersytetu Medycznego we Wrocławiu w postaci bloczków parafinowych i materiału mrożonego. Aby wykonać zaplanowane badania uzyskano zgody komisji bioetycznej (KB-726/2019 i KB-731/2019). Badania do pracy doktorskiej sfinansowane zostały z projektu „Role of FNDC5 expression in breast cancer" uzyskanego w ramach Regionalnej Inicjatywy Doskonałości (nr grantu RID.Z501.19.015).

4. Streszczenie

Rak gruczołu piersiowego (BC) jest najczęstszym nowotworem u kobiet. Od lat zajmuje wysokie miejsce wśród najczęstszych przyczyn zgonu pacjentek z rozpoznanym nowotworem złośliwym. Zachorowania na raka gruczołu piersiowego w populacji stanową duży problem diagnostyczny. Poważnym wyzwaniem pozostaje także leczenie chorych z tym rozpoznaniem, w szczególności w przypadku zaawansowanych stanów choroby. W dalszym ciągu istnieje potrzeba poszukiwania nowych markerów prognostycznych i predykcyjnych raka gruczołu piersiowego. Badania z ostatnich lat przeprowadzone na raku gruczołu piersiowego i innych typach nowotworów wskazują, że jednym z takich potencjalnych markerów może być iryzyna.

Iryzyna została wykryta po raz pierwszy w 2012 roku. Udowodniono, że odgrywa kluczową rolę w procesie przemiany białej tkanki tłuszczowej w brązową. Dotychczasowe badania potwierdziły obecność iryzyny w wielu tkankach i narządach. Podwyższenie jej ekspresji, poza stanami fizjologicznymi, obserwowano również w różnego typu nowotworach m in. raku płuca, raku przewodu pokarmowego, raku jajnika, raku jasnokomórkowym nerki, raku krtani, kostniakomięsakach. Badania na modelu *in vitro* wskazują, że zwiększony poziom iryzyny może prowadzić do zahamowania proliferacji, migracji i przejścia epitelialno-mezenchymalnego (EMT) przez szlak PI3K/AKT w komórkach raka płuc. Natomiast w raku wątroby zaobserwowano stymulowanie proliferacji i inwazji komórek przez ten sam szlak. W kostniakomięsaku zauważono, że iryzyna hamuje proliferację, migrację i inwazję poprzez odwrócenie działania IL-6 na EMT przez drogę sygnałową STAT3–SNAIL. Dane dostępne w literaturze na temat zależności pomiędzy iryzyną a EMT pozostają niejasne, a niekiedy wręcz sprzeczne. Dodatkowo, dostępne są nieliczne publikacje podejmujące tematykę znaczenia iryzyny w rakach BC. Dlatego przedmiotem mojej pracy doktorskiej zostały badania mające na celu określenie zależności pomiędzy poziomem ekspresji iryzyny a rakami BC.

W pierwszej pracy z cyklu (Cebulski, K.; Nowińska, K.; Jablońska, K.; Romanowicz, H.; Smolarz, B.; Dzięgiel, P.; Podhorska-Okołów, M. Expression of Irisin/FNDC5 in Breast Cancer. Int. J. Mol. Sci. 2022, 23, 3530. https://doi.org/10.3390/ijms23073530) przeprowadzono badania z wykorzystaniem 541 bloczków parafinowych z fragmentami guzów raka BC, oraz 61 mastopatii jako materiału kontrolnego. Za pomocą reakcji immunohistochemicznych (IHC) oceniono poziom ekspresji iryzyny, PGC1α i Ki-67. Na materiale mrożonym pod postacią 40 bloczków raka BC, 40 marginesów guza i 16 mastopatii

przeprowadzono badania metodą RT-PCR celem oceny poziomu ekspresji *mRNA* genu *FNDC5* kodującego FNDC5 – prekursor iryzyny. Otrzymane wyniki poddano analizie statystycznej.

Ekspresja iryzyny została zaobserwowana w cytoplazmie komórek raka BC, a także w komórkach mastopatii. Natomiast poziom ekspresji tego białka w BC był istotnie wyższy niż w mastopatiach. W publikacji skorelowano również poziom ekspresji iryzyny z danymi kliniczno-patologicznymi. Poziom ekspresji iryzyny był niższy u pacjentek z przerzutami do węzłów chłonnych w porównaniu do pacjentek bez przerzutów. Pomimo widocznego spadku ekspresji iryzyny wraz ze wzrostem wielkości guza (T) różnica ta nie była istotna statystycznie. Natomiast istotną statystycznie zależność zaobserwowano pomiędzy ekspresją iryzyny w guzach o rozmiarze do 10mm (T1a-b) w porównaniu do guzów o wymiarze od ponad 10mm do 20mm (T1c). Poziom iryzyny spadał w kolejnych stadiach zaawansowania klinicznego nowotworu. Różnica była istotna statystycznie pomiędzy stadium I i II. Na podstawie uzyskanych rezultatów wykazano również zależność pomiędzy poziomem ekspresji iryzyny, a całkowitym czasem przeżycia (OS) badanych pacjentek. Pacjentki z wyższym poziomem ekspresji iryzyny charakteryzowały się istotnie dłuższym czasem przeżycia. Ze względu na wpływ czynnika transkrypcyjnego PGC1α na ekspresję genu *FNDC5*, została przeanalizowana jego korelacja z poziomem iryzyny w komórkach guza. Zaobserwowano dodatnią średnią korelację pomiędzy poziomem ekspresji tych dwóch białek w rakach BC. Ponadto w celu zbadania związku ekspresji iryzyny z nasileniem proliferacji komórek raka BC oceniono jej korelację z ekspresją antygenu Ki-67. Oba białka korelowały ze sobą słabo dodatnio.

W drugiej pracy z cyklu (Cebulski, K.; Piotrowska, A.; Kmiecik, A.; Haczkiewicz-Leśniak, K.; Ciesielska, U.; Grzegrzółka, J.; Jabłońska, K.; Romanowicz, H.; Smolarz, B.; Dzięgiel, P.; Podhorska-Okołów, M.; Nowińska, K. The Role of Irisin/FNDC5 Expression and Its Serum Level in Breast Cancer. Int. J. Mol. Sci. 2023, 24, 8628. https://doi.org/10.3390/ijms24108628) również wykorzystano materiał 541 bloczków parafinowych z fragmentami guzów raka BC. Z wykorzystaniem reakcji immunohistochemicznych (IHC) oceniono poziom ekspresji markerów EMT (E-kadheryna i N-kadheryna, SNAIL, SLUG, TWIST). Otrzymane wyniki poddano analizie statystycznej.

Przedstawione w poprzedniej pracy wyniki ekspresji iryzyny na materiale raka gruczołu piersiowego porównano z wynikami oceny poziomu ekspresji E-kadheryny i N-kadheryny, SNAIL, SLUG i TWIST. Ekspresje E-kadheryny, cytoplazmatycznej i jądrowej SNAIL, wykazywały dodatnią średnią korelację z poziomem ekspresji iryzyny. Poziom ekspresji N-kadheryny nie miał związku z ekspresją iryzyny. Natomiast, w przypadku czynnika

transkrypcyjnego SLUG, zarówno dla ekspresji cytoplazmatycznej, jak i jądrowej zaobserwowano dodatnią słabą korelację poziomem ekspresji iryzyny. Podobnie jak przy wcześniej wymienionych czynnikach transkrypcyjnych, dla TWIST zaobserwowano dodatnią korelacje zarówno dla ekspresji cytoplazmatycznej, jak i jądrowej.

W celu potwierdzenia wyników badań immunohistochemicznych ekspresji iryzyny, przeprowadzono dodatkowo badania molekularne na modelu in vitro. Poziom iryzyny zbadano przy użyciu reakcji immunofluorescencyjnej (IF) w linii kontrolnej Me16c, oraz linii raków gruczołu piersiowego MCF-7, MDA-MB-231 i MDA-MB-468. Wykorzystując technikę RT-PCR oceniono również poziom ekspresji *mRNA* genu *FNDC5* kodującego FNDC5 – prekursor iryzyny.

Poziom ekspresji *mRNA FNDC5* w prawidłowych komórkach gruczołu piersiowego (Me16c) był istotnie niższy w porównaniu z poziomem obserwowanym w komórkach wszystkich badanych linii raka gruczołu piersiowego. Dodatkowo, poziom ekspresji znacząco różnił się pomiędzy liniami komórkowymi raka gruczołu piersiowego. Najwyższy poziom ekspresji genu *FNDC5* zaobserwowano w komórkach MDA-MB-468. Różnica pomiędzy poziomami *mRNA FNDC5* w pozostałych liniach MCF-7 i MDA-MB-231 była istotna. Tak jak w przypadku badań RT-PCR, w badaniach z wykorzystaniem technik immunoflurescencji (IF) wykazano, że poziom ekspresji iryzyny w prawidłowych komórkach gruczołu piersiowego (Me16c) był istotnie niższy w porównaniu z poziomem obserwowanym w komórkach wszystkich badanych linii raka gruczołu piersiowego. Różnice pomiędzy poziomami ekspresji iryzyny w liniach raka gruczołu piersiowego MDA-MB-231 a MCF-7, oraz MDA-MB-468 były istotne statystycznie.

Podsumowując, przedstawione w obu pracach badania są pierwszymi oceniającymi poziom ekspresji iryzyny na tak dużym materiale raka gruczołu piersiowego. Badania na materiale raka gruczołu piersiowego jak i na liniach komórkowych potwierdzają, że poziom ekspresji iryzyny jest istotnie wyższy w komórkach nowotworowych. Uzyskane dane sugerują również, że iryzyna może być markerem prognostycznym raka gruczołu piersiowego. Ponadto badania potwierdzają, że iryzyna może mieć wpływ na proces przejścia epitelialno-mezenchymalnego. Wskazują jednak na bardzo złożone zależności w tym zakresie, które wymagają dalszych badań.

5. Summary

Breast cancer (BC) is the most prevalent cancer in women. For many years, it has been ranked high among the most common causes of death in patients with malignant tumors. The incidence of BC in the population poses a major diagnostic problem. The treatment of BC patients also remains a major challenge, particularly in advanced disease stages. There is still a need to search for new prognostic and predictive markers for BC. Recent studies on BC and other types of cancer indicate that irisin (Ir) may be one of such potential markers.

Ir was first detected in 2012. It plays a key role in the conversion of white adipose tissue to brown adipose tissue. Previous studies have confirmed the presence of Ir in many tissues and organs. Apart from physiological conditions, the increase in its expression has also been observed in various types of cancer, including lung, gastrointestinal and ovarian cancer, as well as in clear cell renal cell carcinoma, laryngeal cancer and osteosarcoma. *In vitro* studies indicate that increased Ir levels can lead to the inhibition of proliferation, migration and epithelial-mesenchymal transition (EMT) through the PI3K/AKT pathway in lung cancer cells. In turn, stimulation of proliferation and invasion of cells by the same pathway was observed in liver cancer. In osteosarcoma, Ir was found to inhibit proliferation, migration and invasion by reversing the IL-6-induced EMT through the STAT3-SNAIL signaling pathway. The literature data on the relationship between Ir and EMT remain unclear and even contradictory. In addition, there are only several papers addressing the importance of Ir in BC. Therefore, the subject of this dissertation was related to studies aimed at determining the relationship between Ir expression and BC.

In the first paper of the series (Cebulski, K.; Nowińska, K.; Jabłońska, K.; Romanowicz, H.; Smolarz, B.; Dzięgiel, P.; Podhorska-Okołów, M. Expression of Irisin/FNDC5 in Breast Cancer. Int. J. Mol. Sci. 2022, 23, 3530. https://doi.org/10.3390/ijms23073530), investigations were conducted on 541 paraffin blocks containing sections of BC tissue, along with 61 mastopathy samples serving as controls. The expression levels of PGC1α and Ki-67 were assessed using immunohistochemical (IHC) reactions. Additionally, the *mRNA* expression level of the *FNDC5* gene, encoding FNDC5, the precursor of Ir, was evaluated using the RT-PCR technique on frozen samples from 40 BC and 40 control tissues taken from tumor margins, as well as 16 mastopathy samples. The obtained results were subjected to statistical analysis.

Ir expression was found in the cytoplasm of BC cells and mastopathy cells. However, the expression level of this protein in BC was significantly higher than in mastopathy samples. Additionally, the expression level of Ir was correlated with the clinicopathological data. The expression level of Ir was lower in patients with lymph node metastases compared to those without metastases. Despite the apparent decrease in Ir expression with increasing tumor size (T), the difference was not statistically significant. In turn, a statistically significant relationship was observed in Ir expression between tumor sizes up to 10mm (T1a-b) and those larger than 10mm but not exceeding 20mm (T1c). Irisin levels decreased in more advanced stages of tumor progression. The difference between stage I and II was statistically significant. Based on the results, the relationship was found between the expression level of Ir and overall survival (OS). Patients with higher Ir expression levels had significantly longer survival. Due to the influence of the transcription factor PGC1α on the expression of the *FNDC5* gene, its correlation with the level of Ir in tumor cells was analyzed. The moderate positive correlation was observed between the expression levels of these two proteins in BC. In addition, to examine the association of Ir expression with increased proliferation of BC cells, its correlation with the expression of Ki-67 antigen was evaluated. Both proteins were weakly positively correlated.

In the second paper of the series (Cebulski, K.; Piotrowska, A.; Kmiecik, A.; Haczkiewicz-Leśniak, K.; Ciesielska, U.; Grzegrzółka, J.; Jabłońska, K.; Romanowicz, H.; Smolarz, B.; Dzięgiel, P.; Podhorska-Okołów, M.; Nowińska, K. The Role of Irisin/FNDC5 Expression and Its Serum Level in Breast Cancer. Int. J. Mol. Sci. 2023, 24, 8628. https://doi.org/10.3390/ijms24108628), investigations were also conducted on 541 paraffin blocks containing sections of BC tissue. Expression levels of EMT markers (E-cadherin, N-cadherin, SNAIL, SLUG, TWIST) were assessed using immunohistochemical (IHC) reactions. The results of the conducted research were subjected to statistical analysis.

The previously obtained results of Ir expression from BC samples were compared with the results of expression levels of the E-cadherin, N-cadherin, SNAIL, SLUG and TWIST. The expression levels of E-cadherin and both cytoplasmic and nuclear SNAIL showed a moderate positive correlation with the expression level of Ir. The expression level of N-cadherin was not related to Ir expression. In turn, in the case of transcription factor SLUG, a positive weak correlation was found with Ir expression levels for both cytoplasmic and nuclear expression). Similar to the previously mentioned transcription factors, positive correlations were observed for TWIST for cytoplasmic and nuclear expression.

To confirm the immunohistochemical results, molecular studies were additionally performed on an *in vitro* model. The level of Ir was examined using immunofluorescence (IF) in the control line (Me16c) and BC lines (MCF-7, MDA-MB-231 and MDA-MB-468). Using the RT-PCR technique, the *mRNA* expression level of the *FNDC5* gene encoding FNDC5, which is the precursor of Ir, was also assessed.

The expression level of *FNDC5 mRNA* in normal breast cell line (Me16c) was significantly lower compared to the level in the cells of all BC lines. In addition, the expression level differed significantly between BC cell lines. The highest level of *FNDC5* gene expression was observed in MDA-MB-468 cells. The difference between *FNDC5 mRNA* levels in MCF-7 and MDA-MB-231 lines was significant. Similar to RT-PCR studies, immunofluorescence (IF) studies showed that Ir expression levels in normal breast cell line (Me16c) were significantly lower compared to those observed in the cells of all BC lines. Differences between Ir expression levels in MDA-MB-231 and MCF-7 as well as MDA-MB-468 BC cell lines were statistically significant.

In summary, the studies presented in both papers are the first to assess the level of irisin expression on such a large BC sample. Studies on BC samples and BC cell lines confirm that the level of irisin expression is significantly higher in BC cells. The obtained data also suggest that irisin may serve as a prognostic marker for BC. Additionally, the studies confirm that irisin may influence the process of EMT in BC. However, they indicate complex relationships in this regard, which require further research.

6. Publikacje

1. Cebulski, K.; Nowińska, K.; Jablońska, K.; Romanowicz, H.; Smolarz, B.; Dzięgiel, P.; Podhorska-Okołów, M. Expression of Irisin/FNDC5 in Breast Cancer. Int. J. Mol. Sci. 2022, 23, 3530. https://doi.org/10.3390/ijms23073530

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2. Cebulski, K.; Piotrowska, A.; Kmiecik, A.; Haczkiewicz-Leśniak, K.; Ciesielska, U.; Grzegrzółka, J.; Jabłońska, K.; Romanowicz, H.; Smolarz, B.; Dzięgiel, P.; Podhorska-Okołów, M.; Nowińska, K. The Role of Irisin/FNDC5 Expression and Its Serum Level in Breast Cancer. Int. J. Mol. Sci. 2023, 24, 8628. https://doi.org/10.3390/ijms24108628

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Article

Expression of Irisin/FNDC5 in Breast Cancer

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Abstract: Irisin is a myokine formed from fibronectin type III domain-containing protein 5 (FNDC5), which can be found in various cancer tissues. FNDC5 and irisin levels have been poorly studied in the tumor tissues of breast cancer (BC). The aim of this study was to determine the levels of irisin expression in BC tissues and compare them to clinicopathological factors and Ki-67 and PGC-1α expression levels. Tissue microarrays (TMAs) with 541 BC tissues and 61 samples of non-malignant breast disease (NMBD; control) were used to perform immunohistochemical reactions. *FNDC5* gene expression was measured in 40 BC tissue samples, 40 samples from the cancer margin, and 16 NMBD samples. RT-PCR was performed for the detection of *FNDC5* gene expression. Higher irisin expression was found in BC patients compared to normal breast tissue. FNDC5/irisin expression was higher in patients without lymph node metastases. Longer overall survival was observed in patients with higher irisin expression levels. FNDC5/irisin expression was increased in BC tissues and its high level was a good prognostic factor for survival in BC patients.

Keywords: irisin; FNDC5; breast cancer; cancer; Ki-67; PGC-1α; metastases

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1. Introduction

Breast cancer (BC) is one of the most prevalent malignancies in women. Despite significant advances in the knowledge of carcinogenesis and the mechanisms of cancer progression, early detection and effective therapy remain major problems. BC is often detected in its advanced stages when the available treatment is not very effective [1]. There are many studies that have searched for new methods of BC treatment, e.g., by inhibiting kinases such as MARK4 [2,3]; however, there is still a need to search for new potential drugs and prognostic and predictive markers for BC. Recent studies on BC and other cancer types have indicated that irisin could be one such potential marker.

Fibronectin type III domain-containing protein 5 (FNDC5) is a transmembrane protein [4] and a prohormone from which a 112 aa peptide (irisin) is released. The molecular weight of FNDC5 ranges from 20–32 kDa. The deglycosylated form of irisin ranges from 12 to 15k Da. N-glycosylation has a crucial role in the biological activity of irisin [5]. Irisin was first detected in mouse skeletal muscle fibers in 2012 by Boström et al. [6]. The researchers found that the release of irisin from muscles occurred in response to physical activity. However, the expression of the *FNDC5* gene is controlled by a transcription factor known as peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α). However, to date, the mechanism by which irisin is released from the prohormone has not been explained [7–9]. Irisin has been shown to play a key role in the conversion of white

adipose tissue to brown adipose tissue by increasing the expression of thermogenin (UCP1) in mitochondrial combs. The UCP1 protein is crucial in the adaptation of organisms to low temperatures. It causes the inhibition of adenosine triphosphate (ATP) production in the respiratory chain [8]. Previous studies have confirmed the presence of irisin in cardiomyocytes, adipose tissue, kidneys, blood vessel walls, and many other tissues [10]. There are also studies that have indicated the possibility of using irisin as a drug, e.g., in inhibiting the deterioration of cognitive functions in patients with Alzheimer's disease [11]. Kim et al. [12] showed that αVβ5 integrin was a receptor for irisin in osteocytes, and that the protein could be involved in osteoclastogenesis and bone remodeling. It has been suggested that irisin binds to the receptor after transitioning to a dimeric form [12]. The association of irisin with bone remodeling may be related to metastases to the bone tissue.

Further studies have shown that increased irisin levels are found in malignant lesions, such as lung cancer, gastrointestinal cancer, ovarian cancer, clear cell renal cell carcinoma, laryngeal cancer, and osteosarcoma [13–19]. In our previous paper, we observed an association of FNDC5/irisin expression with the histological malignancy grade, tumor size, and lymph node metastases in non-small cell lung carcinoma (NSCLC) [20]. It is thought that increased irisin levels in malignant tumors may lead to local hyperthermia, resulting in protein denaturation and the inhibition of tumor growth by blocking ATP formation in the mitochondrial respiratory chain [21]. Other in vitro studies have indicated that increased irisin levels could lead to the inhibition of proliferation, migration, and the epithelial–mesenchymal transition (EMT) through the PI3K/AKT pathway in lung cancer cells [15]. In turn, the stimulation of cell proliferation and invasion by the same pathway was reported in a study on liver cancer [22]. In another study involving osteosarcoma, irisin inhibited proliferation, migration, and invasion by reversing the IL-6-induced EMT through the STAT3-Snail signaling pathway [23].

To date, there has been one study on the expression and significance of irisin in BC. Kuloglu et al. [16] performed immunohistochemical (IHC) studies on BC tissue and found that irisin levels were significantly higher in BC tissue compared to normal breast tissue. In turn, Gannon et al. [24] conducted a study using an in vitro model. They found that irisin increased the cytotoxic effect of doxorubicin in MCF-7 and MDA-MB-231 cell lines. They did not observe this result in the control MCF-10a cells. Other studies have assessed plasma irisin levels in BC patients. Pravatopoulou et al. [25] showed lower serum irisin levels in women with BC compared to healthy women. Panagiotou et al. [26] reported higher serum levels of irisin in patients with benign and malignant breast lesions compared to the serum levels in healthy women. They concluded that irisin was an independent predictor of metastasis. Another study reported decreased serum irisin levels in patients with bone metastases compared to those without metastases [27]. These different results may be due to the release of irisin into the blood by various tissues, not only the tissues affected by cancer. Therefore, the careful examination of irisin levels in BC seems to be of great importance.

Due to the different results obtained by the determination of serum irisin, the main aim of this study was to investigate its levels in the cancerous tissues of BC patients. On the basis of this, we determined the location and level of irisin expression in BC cells and non-malignant breast disease (NMBD; control). Moreover, the relationship between the irisin level and clinicopathological data was examined. Another aim of this study was to determine the correlation of the expression of the transcription factor PGC-1α and cell proliferation antigen Ki-67 with the level of irisin in BC. Irisin expression levels have never been assessed in such a large cohort of BC patients.

2. Results

2.1. Immunohistochemical (IHC) Detection of Irisin Expression in Tissue Microarrays (TMAs) with Different Breast Cancer Types

Irisin expression was found in the cytoplasm of BC cells, and a significantly lower level was reported in the NMBD samples. The expression level of this protein in BC (mean

5.7 ± 0.1 SE) was significantly higher than in NMBD samples (Mann–Whitney U test; $p < 0.0001$; mean 0.9 ± 0.3 SE); Figure 1A–D and 3a). In addition, we observed increased irisin expression in the apical parts of tumor cells. We also reported the presence of irisin in vesicles secreted by tumor cells (Figure 2). However, we did not find irisin expression in the tumor stroma.

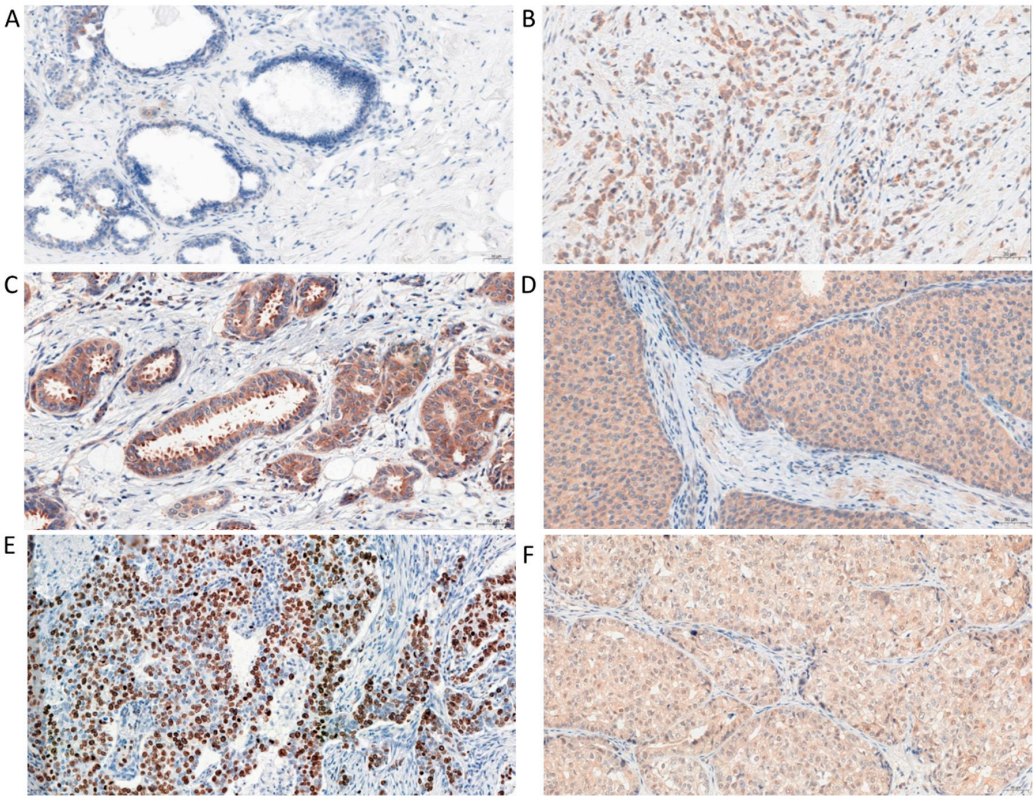

Figure 1. Comparison of irisin expression using immunohistochemistry (IHC; positive reactions—brown cell cytoplasm) in control tissue (**A**) and at different grades of malignancy (G) of breast cancer (BC; (**B**)—G1; (**C**)—G2, in apical parts of cells; (**D**)—G3) with Ki-67 (**E**) and PGC-1α (**F**); magnification x200.

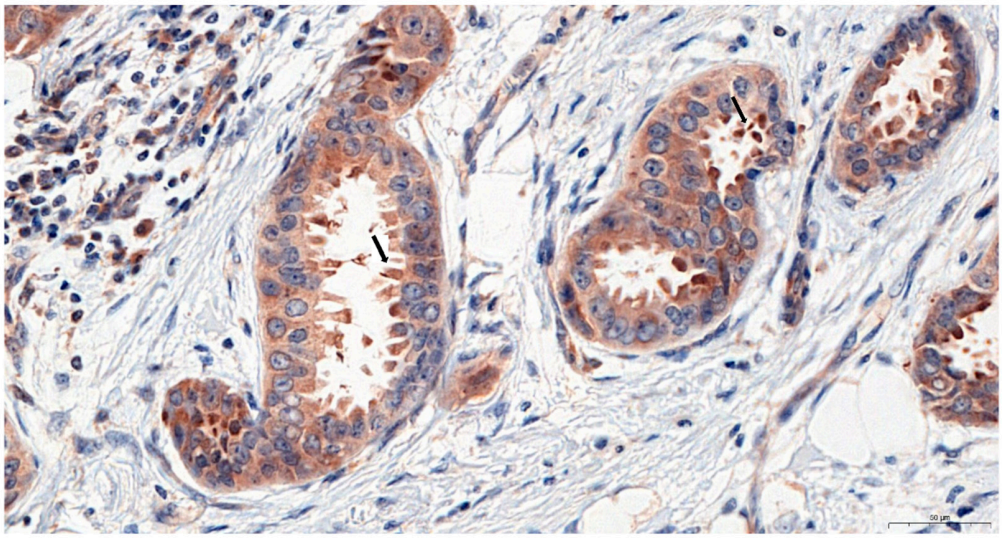

Figure 2. Positive immunohistochemical (IHC) reactions (brown color) indicating strong irisin expression in the apical parts of cancer cells and vesicles; magnification x400.

2.2. Associations between Irisin Expression in Cancer Cells and Clinicopathological Parameters

Table 1 shows the characteristics of BC patients with divided into high and low irisin expression levels according to the median. Table 2 shows the mean irisin expression levels in relation to several clinicopathological parameters. Figure 3 shows the results of the comparison of irisin expression levels in different groups of patients depending on clinicopathological features.

Table 1. Characteristics of BC patients related to low and high expression of irisin (divided according to the median value of 5.6) compared with chi-square tests.

Clinicopathological Parameter	*n* 541 (%)	Irisin Expression in BC Cells		
		Low >0–<5.6	High ≥5.6–12	Chi^2 Test *p* Value
Age ≤ 60	156 (28.8)	75 (48.1)	81 (51.9)	0.0707
>60	385 (71.2)	218 (56.6)	167 (43.4)	
Histology type IDC	521 (96.3)	281 (53.9)	240 (46.1)	0.7252
ILC	12 (2.2)	7 (58.3)	5 (41.7)	
IPC	1 (0.2)	1 (100.0)	0 (0.0)	
MC	1 (0.2)	0 (0.0)	1 (100.0)	
MetC	4 (0.7)	3 (75.0)	1 (0.0)	
MucC	2 (0.4)	1 (50.0)	1 (50.0)	
Tumour size (T) T1	344 (63.6)	183 (53.2)	161 (46.8)	**0.0414**
[T1a-b	[89 (16.5)	35 (39.3)	54 (60.7)	
T1c]	255 (47.1)]	148 (58.0)	107 (42.0)	
T2	179 (33.1)	99 (55.3)	80 (44.7)	
T3-4	18 (3.3)	11 (61.1)	7 (38.9)	
Lymph nodes (N) N0	340 (62.8)	169 (49.7)	171 (50.3)	**0.0258**
N1	193 (35.7)	119 (61.7)	74 (38.3)	
N2	8 (1.5)	5 (62.5)	3 (37.5)	
Stage I	237 (43.8)	123 (51.9)	114 (48.1)	0.5507
II	283 (52.3)	157 (55.5)	126 (44.5)	
III	21 (3.9)	13 (61.9)	8 (38.1)	
Grade of malignancy (G)				0.2929
G1	96 (17.7)	49 (51.0)	47 (49.0)	
G2	350 (64.7)	198 (56.6)	152 (43.4)	
G3	95 (17.6)	46 (48.3)	49 (51.7)	

Abbreviations: BC—breast cancer; IDC—invasive ductal carcinoma; ILC—invasive lobular carcinoma; IPC—invasive papillary carcinoma; MC—medullary carcinoma; MetC- metaplastic carcinoma; MucC—mucinous carcinoma.

Table 2. Association of irisin expression level with clinicopathological characteristics according to the World Health Organization criteria [28] in patients with BC; significance in bold.

BC	*p* Value (Mann–Whitney U Test)		Mean ± SD
Lymph Nodes (N)		**Lymph Nodes**	
N0 vs. N1	**0.0003**	**N0**	5.9 ± 2.5
N0 vs. N2	0.2259	**N1**	5.2 ± 2.4
N1 vs. N2	0.7913	**N2**	4.9 ± 1.8
N0 vs. N1-2	**0.0002**		
Tumor Size (T)		**Tumor size**	
T1 vs. T2	0.2864	**T1**	5.8 ± 2.5
T1 vs. T3–4	0.4505	**T2**	5.5 ± 2.3
T2 vs. T3–4	0.7400	**T3–4**	5.2 ± 2.0
Tumor T1 size		**Tumor T1 size**	

Table 2. *Cont.*

BC	*p* Value (Mann–Whitney U Test)		Mean ± SD
T1a–b vs. T1c	**0.0002**	**T1a–b** **T1c**	6.7 ± 2.5 5.5 ± 2.5
Stage (S)		**Stage**	
I vs. II	**0.0486**	**I**	5.9 ± 2.6
I vs. III	0.2121	**II**	5.5 ± 2.3
II vs. III	0.6178	**III**	5.2 ± 1.9
Grade of malignancy (G)		**Grade of malignancy**	
G1 vs. G2	0.0951	**G1**	6.0 ± 2.5
G1 vs. G3	0.8179	**G2**	5.5 ± 2.4
G2 vs. G3	**0.0433**	**G3**	6.0 ± 2.5

Abbreviations: vs.—versus.

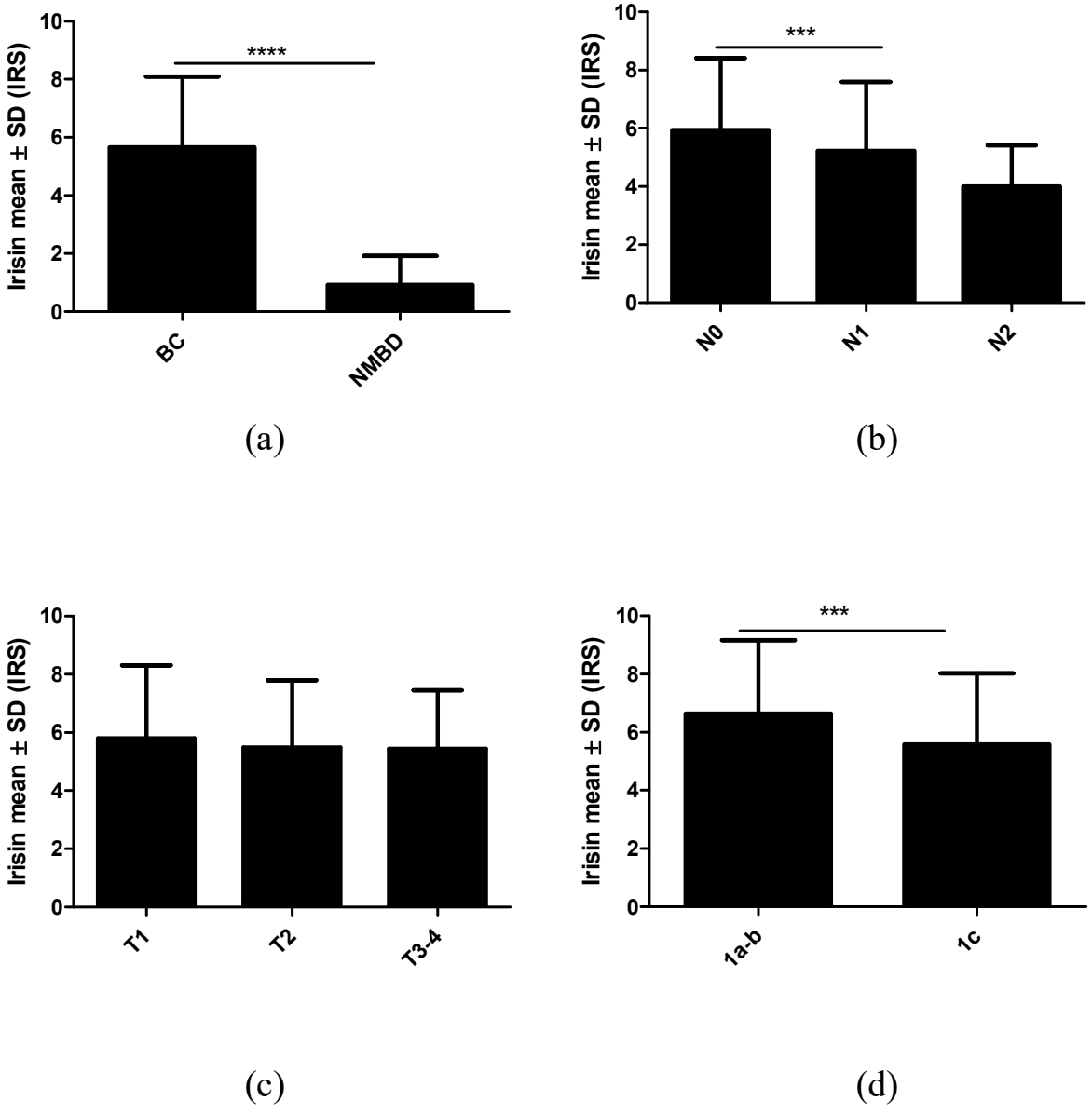

Figure 3. *Cont.*

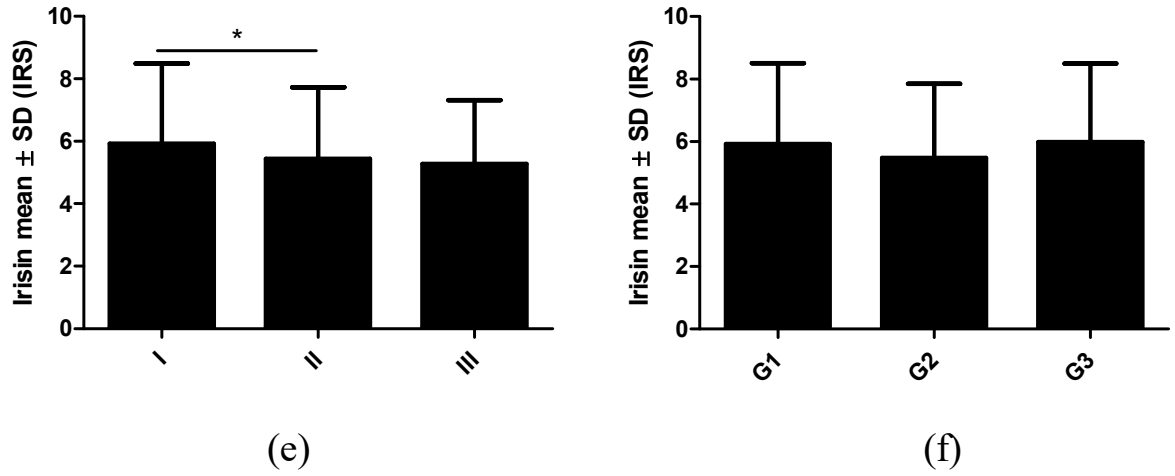

Figure 3. Comparison of irisin expression levels using immunohistochemistry (IHC) in breast cancer (BC) patients and control tissues (**a**) according to the lymph node status (**b**), tumor size (**c**,**d**), tumor stage (**e**), and malignancy grade (**f**). * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

Irisin expression levels were lower in patients with lymph node metastases compared to patients without metastases (Mann–Whitney U test; $p = 0.0002$; Table 2, Figure 3b). Despite a decrease in irisin expression level with an increase in tumor size (T), this difference was not statistically significant (Figure 3c). However, we observed a statistically significant relationship between irisin expression in T1a-b compared to T1c (Mann–Whitney U test; $p = 0.0002$; Figure 3d). We also found higher irisin expression levels in tumors with a histological grade of G3 compared to those with a grade of G2 (Mann–Whitney U test; $p = 0.0433$; Figure 3f). Irisin levels decreased in more advanced stages of tumor progression. The difference between stage I and II was statistically significant ($p = 0.0486$; Figure 3e).

2.3. Associations between Irisin Expression and Overall Survival (OS)

We found a relationship between irisin expression levels and overall survival (OS). Subjects with higher expression levels had a significantly longer survival time (Figure 4).

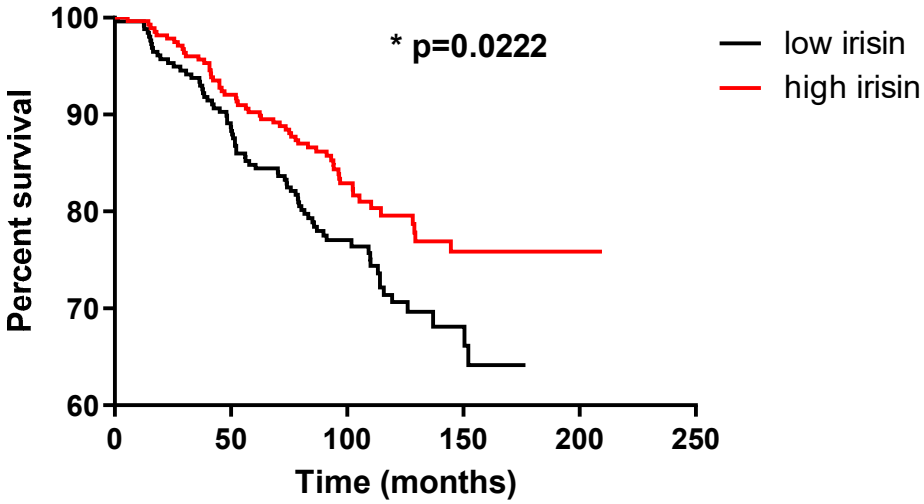

Figure 4. Kaplan–Meier survival curve presenting the prognostic impact of irisin expression levels using immunohistochemistry (IHC) on the overall survival (OS) of patients with breast cancer (BC). Patients were grouped according to the median value of irisin expression (5.3).

Overall survival time was also influenced by age, tumor size, lymph node metastases, histological grade, and stage. However, irisin expression level was not an independent prognostic factor (Table 3).

Table 3. Univariate and multivariate Cox proportional hazards analyses in 541 patients with BC.

Clinicopathological Parameter	Univariate Analysis HR (95% CI) p	Multivariate Analysis HR (95% CI) p
Age ≤**60 vs. >60**	**3.11 (2.16–4.47)** **<0.0001**	**2.45 (1.70–3.52)** **<0.0001**
pT **T1–T2 vs. T3–T4**	**10.86 (3.65–32.35)** **<0.0001**	1.67 (0.61–4.47) 0.3098
pN **N0 vs. N+**	**3.02 (2.12–4.28)** **<0.0001**	**2.10 (1.45–3.03)** **<0.0001**
Grade **G1 vs. G2–G3**	**2.08 (1.38–3.12)** **0.0005**	**2.31 (1.16–4.60)** **0.0156**
Stage **I–II vs. III–IV**	**22.44 (7.73–65.16)** **<0.0001**	2.06 (0.84–5.05) 0.1155
Irisin ≤**5.6 vs. >5.6**	**0.68 (0.48–0.95)** **0.0300**	0.88 (0.61–1.30) 0.5613
Ki-67 **<25 % vs.** ≥**25 %**	1.05 (0.51–2.21) 0.8773	
PGC1 **α** ≤**3.7 vs. >3.7**	0.83 (0.57–1.17) 0.3026	
HER2 **0 vs. 1–3**	1.56 (0.97–2.48) 0.0644	
PR **0 vs. 1–3**	0.71 (0.51–1.00) 0.0596	
ER **0 vs. 1–3**	0.76 (0.53–1.07) 0.1272	

Abbreviations: HR—hazard ratio; CI—confidence interval; BC—breast cancer. Significance in bold.

2.4. Comparison of the Expression Levels of Irisin with Ki-67 and PGC-1α

Due to the effect of the transcription factor PGC-1α on *FNDC5* gene expression, we analyzed its correlation with irisin levels in tumor cells. We found a moderate positive correlation ($r = 0.28$; $p < 0.0001$) between the expression levels of these two proteins in BC (Figure 5a). In addition, to investigate the association of irisin expression with increased BC cell proliferation, we evaluated its correlation with Ki-67 antigen expression. Both proteins were weakly positively correlated ($r = 0.23$; $p < 0.0001$; Figure 5b).

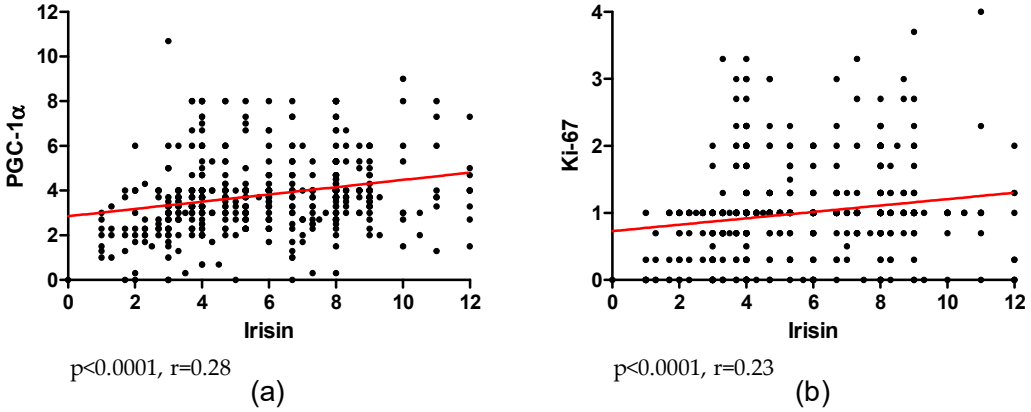

Figure 5. A moderate positive correlation of irisin expression level with the expression level of PGC-1α (**a**) and a weak correlation with Ki-67 antigen (**b**) in breast cancer (BC).

We did not find any statistically significant correlations between irisin expression levels and estrogen receptor (ER), progesterone receptor (PR), or HER2 status.

2.5. RT-PCR Detection of FNDC5 Gene Expression in BC and Control Tissue

The expression level of the *FNDC5* gene in BC was analyzed compared to the control samples (tissues from tumor margins and NMBD samples). The results indicated a significantly higher mRNA level in the tumor margin (mean 336.9 ± SD 70.12) compared to BC (mean 20.86 ± SD 22.71; Mann–Whitney U test $p < 0.0001$). No statistically significant difference was found in *FNDC5* mRNA expression in the NMBD samples compared to BC (mean 12.46 ± SD 7.1). However, *FNDC5* gene expression in the NMBD samples was significantly lower than that in the tumor margin tissue (Mann–Whitney U test $p < 0.0001$; Figure 6). As in the case of irisin levels assessed by IHC, we observed higher *FNDC5* gene expression in patients without lymph node metastases (mean 9.092 ± SD 1.7; $p = 0.0395$) compared to subjects with metastases (mean 3.679 ± SD 0.9; Figure 7a,b). We found no statistically significant difference in the *FNDC5* mRNA expression level with tumor size (T) or histological grade (G) (Figure 7c,d).

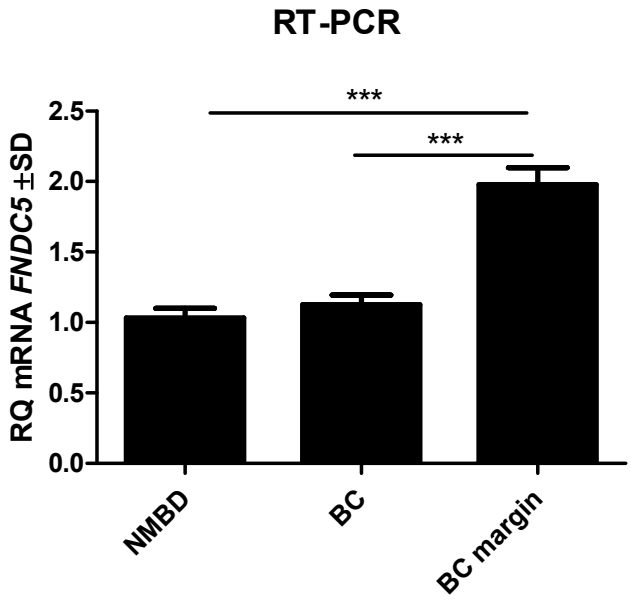

Figure 6. *FNDC5* gene expression in NMBD samples, breast cancer (BC) cells, and BC tumor margin. *** $p < 0.001$.

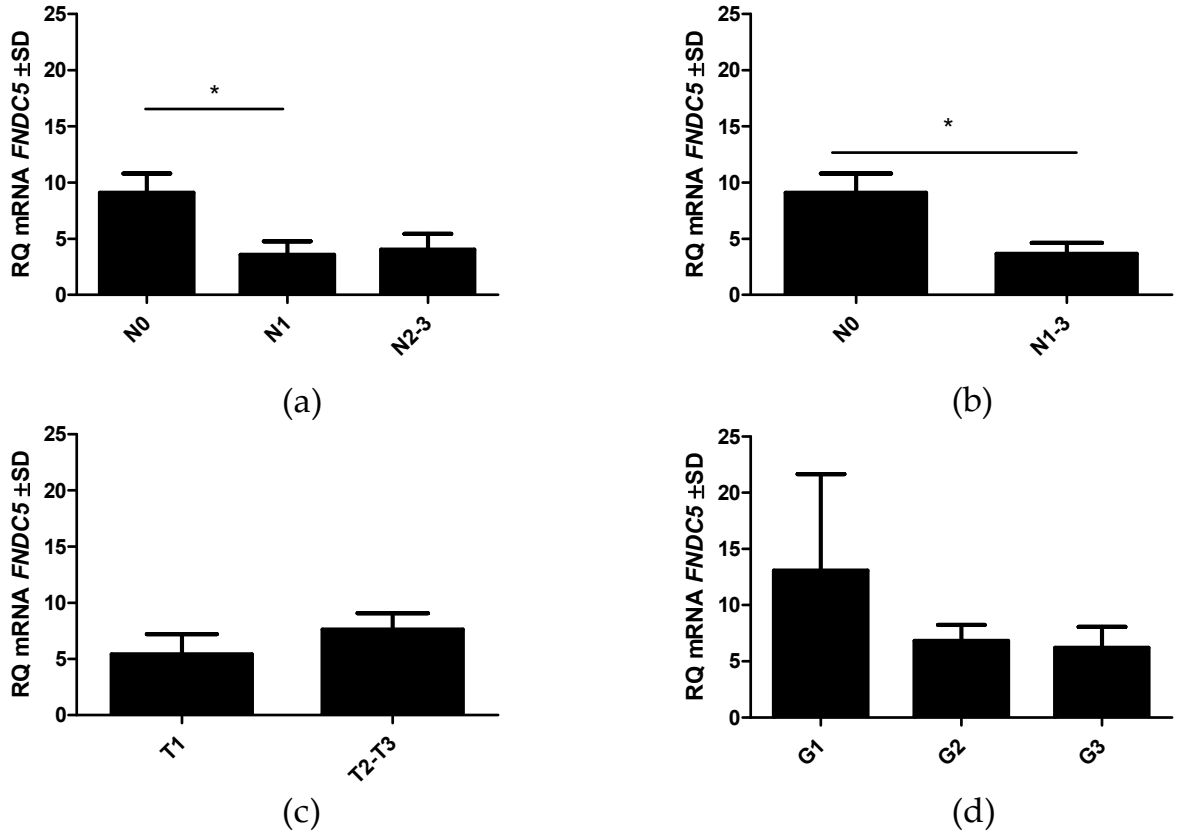

Figure 7. *FNDC5* gene expression in breast cancer (BC) cells according to the N status (**a**,**b**), tumor size (**c**), and malignancy grade (**d**). * $p < 0.05$.

3. Discussion

Our study involved an analysis of irisin expression levels in BC in a large cohort of patients ($n = 541$). We demonstrated that FNDC5/irisin levels were higher in BC than in NMBD samples. In addition, we also found higher *FNDC5* mRNA levels in BC compared to NMBD samples.

The increased expression level of mRNA encoding FNDC5 in BC cells may be related to the metabolic changes occurring in the cancer cells. At the same time, the transcriptional coactivator PGC-1α, which stimulates the expression of irisin, has been implicated in many pathways that regulate metabolism and is related to mitochondrial biogenesis and UCP1, whose expression is stimulated by irisin and affects the uncoupling of the respiratory chain [6]. Thus, increased irisin levels may be a result of an increasing energy demand in cancer cells and a switch to glycolysis instead of obtaining ATP from the respiratory chain [18]. Irisin also increases glucose uptake by cells and enhances AMP-activated kinase (AMPK) phosphorylation [29]. Therefore, higher concentrations of irisin may allow cancer cells to obtain energy through glycolysis, which could confirm our findings related to the increased expression of irisin/FNDC5 in BC compared to the controls.

In some cases, we also found an increased concentration of irisin in the apical parts of cancer cells. Small vesicles showing the presence of irisin were often seen in the lumen of abnormal tubular structures in the tumor. This may be indicative of the apocrine secretion of irisin by some BC cells. Aydin et. al. [30] found irisin in breast milk, which indicates that it is also secreted into the ductal lumen under physiological conditions. Further studies are warranted to confirm the clinical significance of irisin secretion in BC patients.

The research conducted to date has mainly focused on evaluating plasma irisin levels in BC patients [25,27] and on in vitro studies [24]. The results have indicated lower serum irisin levels in BC patients than in control groups. Some researchers have suggested that an increase in the serum irisin level by one unit results in a 90% decrease in the risk of

BC [18,25]. In our study, we obtained the opposite result. However, our study detected irisin in tissue samples and not in the serum of patients. This is probably the reason for the discrepancy between the results reported by Provatopoulou et al. [25] and our findings. Only Kuloglu et al. [16] focused on the immunohistochemical assessment of irisin in BC tissue samples. The authors found increased irisin levels in BC, ovarian, and cervical cells. Our results regarding irisin expression in BC are in line with the above studies. However, our study used TMAs, which indicated the expression level of irisin in the part of whole sections; this is a limitation of our study. However, studies comparing the results of IHC assessment on whole tissue material and TMAs indicate a very high accuracy of TMA in reflecting the whole tissue score [31,32]. In addition, compared to BC and NMBD samples, the higher *FNDC5* mRNA levels observed in normal tissue from the tumor margin are interesting. We supposed that the higher *FNDC5* mRNA level was associated with the interaction between healthy margin cells and cancer cells. In our previous study [33], we observed a higher expression of different markers in healthy tissues from the tumor margin compared to cancer cells. These changes were linked to epigenetic regulations. However, further studies are warranted to explain the cause of such a high level of *FNDC5* mRNA in the tissue from the surgical margin surrounding the tumor.

We also analyzed the association of irisin expression with clinicopathological factors. We demonstrated significantly lower levels of expression of *FNDC5* mRNA and FNDC5/irisin proteins in tumors of patients with lymph node metastases. A similar relationship was found for increasing tumor size (the difference was statistically significant only in the earliest stages, T1a–b vs. T1c) and for more advanced stages of the disease. Thus far, only Provatopoulou et al. [25] have shown a relationship between serum irisin levels and BC stage, tumor size, and lymph node metastases. However, they observed an inverse relationship compared to our findings. In their study, irisin levels increased with more advanced stages of the disease, a larger tumor size, and when lymph node metastases occurred. There are also noticeable discrepancies between our results and those of previous studies, which may be due to the fact that the mechanism of irisin secretion from FNDC5 into serum has not been understood yet. Moreover, serum irisin concentrations may be a result of irisin secretion by different tissues, such as adipose or muscle tissue. In our study, however, the level in actual BC tissue was analyzed.

We found that significantly lower irisin levels were associated with lymph node metastases (N1). In addition, lower *FNDC5* mRNA levels were observed in the group of patients with N1 cancer. This may suggest that high irisin levels prevent metastasis and cancer progression. Zhang et al. [27] analyzed BC patients without distant metastases and with spinal metastases. Their study indicated that serum irisin levels were higher in patients without metastases. They also noted that high serum irisin levels were associated with the absence of spinal metastasis [27]. Lymph node metastasis is a well-known negative prognostic factor [34]. However, there are currently no reports on the relationship between irisin levels in BC and lymph node and distant organ metastases. Research on other types of cancers has shown a similar relationship to that found in our study [18]. In our previous study [20], we observed a decrease in irisin levels in non-small cell lung carcinoma (NSCLC) cells from patients with lymph node metastases (N1) compared to those without metastases. We also reported a similar observation in laryngeal squamous cell carcinoma (LSCC) [19].

Cancer cells must undergo EMT for metastasis to occur. It has been suggested that higher irisin levels may inhibit EMT [15,18]. Our results showed higher irisin levels in the tumor tissue in the N0 group. This may indicate an inhibitory role of irisin in the EMT process of BC cells. Studies by Nowinska et al. [20], Pinkowska et al. [19], and Zhang et al. [27] support our hypothesis. Further studies are warranted to elucidate the mechanism of how irisin affects EMT inhibition and metastasis formation.

In our study, we also analyzed the survival of patients with low and high irisin levels in BC. We found that a lower irisin level was a negative prognostic factor. This finding indicates the beneficial effect of irisin in BC. In the study on NSCLC, we did not observe an association between the level of irisin expression in cancer cells and patient survival. We

observed a longer overall survival time in patients with NSCLC only when irisin expression levels were lower in the tumor stroma [20]. However, in BC, FNDC5/irisin expression was not found in the tumor stroma when IHC was used. Nowinska et al. [20] suggested that high irisin expression in cancer-associated fibroblasts (CAFs) could be associated with its effect on the proliferation and EMT of tumor cells. In addition, irisin overexpression is suspected to have an inhibitory effect on cancer tumor growth through local hyperthermia and the dysregulation of ATP synthesis [16,18,21].

By analyzing the relationship between FNDC5/irisin expression and Ki-67, we showed that there was a weak positive correlation between them. The Ki-67 antigen is a well-known and widely used diagnostic marker of tumor cell proliferation [35]. The existence of a correlation between the proteins suggests that FNDC5 and irisin may affect BC cell proliferation to a limited extent. Panagiotou et al. [26] examined serum irisin levels in patients with benign and malignant breast cancer lesions and also observed a weak positive correlation with Ki-67 antigen expression. Pinkowska et al. [19] found a moderate positive correlation of irisin with Ki-67 expression in LSCC. Irisin in LSCC was also correlated with other proliferation markers, i.e., minichromosome maintenance protein 3 (MCM3) and metallothioneins I/II (MT-I/II) [19]. However, Wozniak et al. [36] reported a weak correlation between irisin and Ki-67, MCM3 protein, and urine diphosphate-galactose ceramide galactosyltransferase (UGT8) in colorectal cancer (CRC). In addition, some studies have indicated that in some cancers (e.g., hepatocellular carcinoma), irisin can enhance tumor cell proliferation by affecting the PI3K/AKT pathway [22]. Another study showed that high irisin expression levels could lead to the inhibition of proliferation, migration, and EMT [15]. In our study on NSCLC, we reported a dual effect of irisin on proliferation, depending on whether it was present in lung cancer cells or stromal cells. However, irisin expression in NSCLC cells correlated negatively with Ki-67 levels [20].

In this study, the results did not clearly indicate whether irisin has a beneficial effect on tumor cell proliferation. Irisin expression decreased with tumor size and disease progression, which is in contrast to a positive correlation of irisin expression with Ki-67. A partial explanation could be that irisin is responsible for the increase in UCP1 expression, which in turn leads to the inhibition of mitochondrial ATP synthesis and increases heat production [8,21]. Reduced cellular ATP levels are associated with AMPK activation and the inhibition of mTOR pathways. In turn, the AMPK-mTOR pathway is known to play a key role in regulating cancer cell proliferation [18,37]. This mechanism could explain why high levels of irisin expression in cancer cells are associated with the inhibition of tumor growth. It is possible that cells have increased irisin expression in the early stages of disease, which results in metabolic changes and affects mitochondrial biogenesis. In turn, in the later stages of the disease, irisin expression is suppressed due to its effects on UCP1 expression and the inhibition of ATP synthesis. A decrease in irisin expression with disease progression was also observed in our study. The differences between irisin expression levels and tumor size, especially tumors up to 1 cm (T1a–b) or > 1–2 cm in diameter (T1c), may be related to the progressive lack of oxygen and nutrients, and thus irisin production.

In conclusion, the assessment of irisin levels in BC may be useful to determine the stage of disease progression. We observed a decrease in irisin expression levels in BC. Moreover, high irisin levels were associated with longer survival. It seems important to verify whether the level of serum irisin reflects its level in tumor cells. Further studies are warranted to explain the mechanism of the effect of irisin on proliferation, migration, and EMT, and to test the possibility of using it as a target in potential therapy.

4. Materials and Methods

4.1. Patient Cohort

The archival samples included 541 paraffin blocks with sections of BC tissue that were obtained from the Polish Mother's Memorial Hospital Research Institute between January 2004 and March 2012 in Lodz, Poland. The control samples included 61 NMBD samples obtained from the 4th Military Teaching Hospital in Wroclaw. A histological assessment

was performed according to the World Health Organization criteria and the 8th TNM edition [28]. Table 1 shows the clinicopathological characteristics of the group of patients. Subjects who had undergone surgical treatment without radiotherapy or chemotherapy were enrolled in the study. In addition, frozen samples (−80 °C), including 40 BC and 40 control tissues obtained from tumor margins and 16 NMBD samples, were also used in the study. The patients whose material was used gave written informed consent. The study was approved by the Wroclaw Medical University Institutional Review Board and the Bioethics Committee (No. 726/2019 and KB-731/2019).

4.2. Tissue Microarrays (TMAs)

Twenty tissue microarrays (TMA) were prepared from 541 paraffin blocks with BC tissue sections. Two TMAs were also prepared from NMBD samples (control). For this purpose, the histological preparations were made from archival samples which were stained with hematoxylin and eosin. The slides were scanned using a Panoramic Midi II histological scanner (3DHISTECH Ltd., Budapest, Hungary). Cancer sites with a core size of 1.5 mm were selected by the Panoramic Viewer (3DHISTECH Ltd.) and were transferred to the tissue arrays using the TMA Grand Master (3DHISTECH Ltd.).

4.3. Immunohistochemical (IHC) Reactions

IHC reactions were carried out on TMAs using primary antibodies to detect the expression of the proteins. Paraffin sections with a 4 µm thickness were prepared. After deparaffinization and hydration, thermal epitope demasking was performed using a Dako PT Link (Dako, Glostrup, Denmark) apparatus and low pH Target Retrieval Solution (Agilent Technologies, Santa Clara, CA, USA) for 30 min at 97 °C. To visualize the antigen, we used polyclonal rabbit anti-irisin (dilution 1:50; code no. NBP2-14024; Novus Biologicals, Littleton, CO, USA) with EnVision™ FLEX+ Mouse LINKER (Dako). Monoclonal mouse anti-Ki-67 antibodies (ready to use; clone MIB-1; code no. IS626; Dako) and polyclonal rabbit anti-PGC-1α antibodies (dilution 1:3200; code no. NBP1-04676, Novus Biologicals) were used to detect other markers. An automatic DAKO Autostainer Link 48 system (Dako) was used for the IHC reactions and an EnVision FLEX kit (Dako) was used to visualize the antigens. The slides were additionally stained with Mayer's hematoxylin.

4.4. Assessment of IHC

The assessment of IHC reactions was performed by two independent investigators (PD and KC). The evaluation was performed using a BX41 light microscope (Olympus, Tokyo, Japan) coupled with the CellD software (Olympus). The cytoplasmic expression of irisin and PGC-1α was assessed using the semiquantitative method immunoreactive score (IRS) according to Remmele and Stegner [38]. The final result was the product of the scores obtained by the estimation of intensity (1—weak, 2—moderate, or 3—strong) and the percentage of cancer cells with a positive reaction (0—no expression, 1 point—> 1%–≤ 10%, 2 points—>10 –≤ 50%, 3 points—> 50%–≤ 80%, 4 points—> 80%). A five-point evaluation scale was used to assess the nuclear expression of Ki-67 (0—no expression, 1 point—> 1%–≤ 10%, 2 points—> 10%–≤ 25%, 3 points—> 25%–≤ 50%, 4 points—> 50%) [20].

4.5. Real-Time PCR (RT-PCR)

Material preserved in RNAlater (Thermo Fisher Scientific, Massachusetts, WT, USA) was used for RT-PCR. The material consisted of 40 BC sections, 40 control tissues taken from the tumor margin, and 16 NMBD samples. RNA isolation was performed using an RNeasy Mini Kit (Qiagen). To obtain cDNA, a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Waltham, MA, USA) was used. The expression level of *FNDC5* (*FNDC5*; TaqMan Gene Expression Assay, Applied Biosystems) was tested using a 7900HT Fast Real-Time PCR System (Applied Biosystems) and the relative quantification (RQ) method. The analysis of *FNDC5* gene expression was performed using the RQ Manager 1.2 software (Applied Biosystems). The results were standardized in relation

to the expression of the reference gene β-actin (ACTB; TaqMan Gene Expression Assay, Applied Biosystems). The expression level of the *FNDC5* gene in BC cells was assessed in relation to its level in the normal tissue. The analysis was repeated three times.

4.6. Statistical Analysis

Kruskal–Wallis, Mann–Whitney U, and chi-square tests were used to assess the association of irisin expression level with clinicopathological factors. Patient OS was measured from the day of surgery to death or to the last follow-up. Kaplan–Meier analysis and the log-rank test were used to examine the association of patient survival with irisin expression levels. The Cox proportional hazard regression model was used to evaluate the association of OS with the clinicopathological evaluation of patients (hazard ratio and 95% confidence intervals). The statistical significance of the difference in *FNDC5* mRNA expression in BC and normal tissue was assessed using the Mann–Whitney U test. Spearman's rank correlation was used to test the correlation between the expression levels of irisin, Ki-67 antigen, PGC-1α, ER, PR, and HER2. A p value ≤ 0.05 was considered statistically significant. Statistical analysis was performed using Prism 5.0 (GraphPad, La Jolla, CA, USA) and Statistica 13.1 (StatSoft, Cracow, Poland).

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Bioethical Committee of Wroclaw Medical University (No. KB-726/2019 and No. KB-731/2019).

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

Data Availability Statement: The raw data and the analytic methods will be made available to other researchers for purposes of reproducing the results in their own laboratories upon reasonable request. To access protocols or datasets contact katarzyna.nowinska@umw.edu.pl.

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The Role of Irisin/FNDC5 Expression and Its Serum Level in Breast Cancer

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Abstract: Irisin (Ir) is an adipomyokine formed from fibronectin type III domain-containing protein 5 (FNDC5), which can be found in various cancer tissues. Additionally, FNDC5/Ir is suspected of inhibiting the epithelial-mesenchymal transition (EMT) process. This relationship has been poorly studied for breast cancer (BC). The ultrastructural cellular localizations of FNDC5/Ir were examined in BC tissues and BC cell lines. Furthermore, we compared serum levels of Ir with FNDC5/Ir expression in BC tissues. The aim of this study was to examine the levels of EMT markers, such as E-cadherin, N-cadherin, SNAIL, SLUG, and TWIST, and to compare their expression levels with FNDC5/Ir in BC tissues. Tissue microarrays with 541 BC samples were used to perform immunohistochemical reactions. Serum levels of Ir were assessed in 77 BC patients. We investigated FNDC5/Ir expression and ultrastructural localization in MCF-7, MDA-MB-231, and MDA-MB-468 BC cell lines and in the normal breast cell line (Me16c), which was used as the control. FNDC5/Ir was present in BC cell cytoplasm and tumor fibroblasts. FNDC5/Ir expression levels in BC cell lines were higher compared to those in the normal breast cell line. Serum Ir levels did not correlate with FNDC5/Ir expression in BC tissues but were associated with lymph node metastasis (N) and histological grade (G). We found that FNDC5/Ir correlated moderately with E-cadherin and SNAIL. Higher Ir serum level is associated with lymph node metastasis and increased grade of malignancy. FNDC5/Ir expression is associated with E-cadherin expression level.

Keywords: irisin; FNDC5; EMT; E-cadherin; SNAIL; SLUG; TWIST; breast cancer (BC)

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1. Introduction

Breast cancer (BC) is the most prevalent cancer in women, and it is considered the most common cause of death in cancer patients [1]. Treatment of patients with BC, particularly in the advanced stages of the disease, remains a challenge. Therefore, it seems essential to search for new treatment methods, further exploration of the pathomechanism of this cancer, and the detection of new diagnostic markers. Our previous study showed a potential protective effect of FNDC5/irisin (FNDC5/Ir) in BC. We observed a negative correlation between the expression level of FNDC5/Ir and the presence of metastases. In addition, higher expression levels were associated with longer patient survival [2]. Due to the unclear causes of the relationships that we previously reported, we decided to continue the research.

Irisin (Ir) is a transmembrane protein [3] that was first observed in the muscle fibers of mice [4]. The authors found that under the influence of physical activity, there was an

increased level of FNDC5 in muscle fibers and its further transformation into a new protein known as Ir. Next, it is secreted outside the cell and, as a hormone, affects metabolic changes in adipose tissue, which results in increased thermogenesis [4]. Expression of FNDC5/Ir has been confirmed in many other tissues, including adipose, kidney, liver, cardiomyocytes, skin, and cerebellum. It is formed as a result of the cleavage of 112 amino acids of the FNDC5 chain [5]. Next, Ir undergoes glycosylation, which is one of the most important processes of post-translational modification and significantly changes its physicochemical properties. Inhibition of glycosylation leads to decreased Ir secretion. The molecular mass of the non-glycosylated form is about 13kD and about 20kD in the case of the glycosylated form [6]. Apart from physiological conditions, increased expression of Ir was also observed in various types of cancers, e.g., in breast, prostate, digestive tract (including liver), bone, lung, and thyroid cancer [7,8]. It is believed that elevated levels of Ir in tumor tissue led to the inhibition of cell division and mitochondrial ATP synthesis [9].

It was also suggested that Ir inhibited the proliferation, migration, and EMT of cancer cells by affecting the PI3K/AKT/SNAIL pathway [10]. Such relationships were observed by Shao et al. [11] in an in vitro study on lung cancer cells. In the same study, Ir also correlated negatively with the expression level of SNAIL, which is involved in EMT. The inhibitory effect of Ir on cell proliferation (including breast, lung, bone, and prostate cancer cells) was confirmed in many other in vitro studies [10,12–15]. On the other hand, some papers did not confirm the effect of Ir on the inhibition of proliferation, adhesion, or colony formation by cancer cells [16]. Stimulation of cell invasion was also observed in liver cancer through the PI3K/AKT pathway [17]. In turn, an increased level of FNDC5/Ir expression in cancer-associated fibroblasts (CAFs) in patients with NSCLC was a negative prognostic factor [5]. CAFs participate in cancer progression by stimulating angiogenesis and EMT [18]. In vivo studies showed that Ir serum levels were decreased in various types of cancers (e.g., prostate, osteosarcoma, bladder, breast, colorectal, liver, gastric cancers) [19]. It is suspected that serum Ir levels can be used as a biomarker of different types of cancer [19].

EMT is a process during which an epithelial cell (expression of E-cadherin) transforms into a mesenchymal cell (expression of N-cadherin). The cell loses structural connections with other cells and loses its polarity. E-cadherin is one of the transmembrane proteins and also one of the most significant epithelial markers. It participates in the formation of the cytoskeleton and is responsible for maintaining normal cell polarity [20]. It plays a crucial role in maintaining intercellular connections and is necessary to preserve the stability of cell membranes. The decrease in its expression level is associated with EMT [21]. In turn, N-cadherin is one of the markers detected in mesenchymal cells. It serves as an EMT marker and is associated with the progression of many cancers. EMT is found in physiological processes (wound healing) and tumor progression. Physiological processes are strictly controlled. However, uncontrolled EMT occurs during tumor progression [22]. SNAIL, SLUG, and TWIST are considered the most important transcription factors involved in EMT. SNAIL-induced EMT leads to a decrease in the level of E-cadherin in cells and an increase in the expression levels of N-cadherin, vimentin, and fibronectin [22]. SNAIL is one of the transcription factors. Together with HDAC1, HDAC2, and mSin3A, it forms a complex that affects the downregulation of the expression of E-cadherin. SNAIL expression is controlled by many pathways, including PI3K/AKT, MAPK, Shh Notch, Wnt, and TGH-β. The increase in the expression level of SNAIL is associated with the promotion of EMT [23]. Ir is suspected of inhibiting SNAIL expression and function by inhibiting the PI3K/AKT pathway [11] and STAT3 [10]. SLUG also belongs to the SNAIL protein family and has similar properties to SNAIL [24]. It can form complexes with another protein known as TWIST. This combination inhibits the expression of many proteins, including the expression of E-cadherin. Importantly, such a complex also acts as a transcription factor for SLUG. Knockdown of SLUG completely blocks the ability of TWIST to suppress E-cadherin transcription and thus induces EMT [25]. Increased expression levels of SLUG, SNAIL, and TWIST are transcription factors that promote EMT transition [22].

The relationships between the level of Ir expression, its secretion, EMT, and cancer progression are very complex and may result in opposite effects under different conditions. Therefore, further research is warranted to explain the differences in such relationships. In this study, the level and localization of the above markers in the tissues of BC patients were determined. These markers have not yet been compared with the level of FNDC5/Ir expression in such a large cohort. We also examined the localization and expression level of FNDC5/Ir in cell lines. Furthermore, we compared serum Ir levels with FNDC5/Ir expression levels in BC tissue. To the best of our knowledge, it has never been reported before. Moreover, the ultrastructural cellular localization of Ir in BC tissues was assessed for the first time. Additionally, due to the differences in the research results related to the effect of Ir on the progression of cancer in different types, the aim of this study was to examine the levels of EMT markers, such as E-cadherin, N-cadherin, SNAIL, SLUG, and TWIST, and to compare their expression levels with the level of FNDC5/Ir expression in BC.

2. Results

2.1. The Association of FNDC5/Ir with Epithelial-to-Mesenchymal (EMT) Markers

To investigate the relationship between FNDC5/Ir expression level and EMT transition, correlations with EMT markers, such as E-cadherin and N-cadherin, SNAIL, SLUG, and TWIST, were evaluated. We compared the previously obtained results of FNDC5/Ir expression in BC tissues [2] with the level of expression of the above markers. Table 1 shows the characteristics of BC patients with the division into high and low Ir expression levels according to the median (table obtained from Cebulski et al., 2022 [2]). FNDC5/Ir expression in BC patient tissues was observed in the cytoplasm of tumor cells (Figure 1A), while E-cadherin showed membrane expression (Figure 1B). In turn, the expression of N-cadherin was noted in the cytoplasm (Figure 1C). For the transcription factors SNAIL (Figure 1D,E), SLUG (Figure 1F), and TWIST (Figure 1G,H) in BC tumors, we observed cytoplasmic and nuclear expression. Therefore, the levels of cytoplasmic and nuclear expression of each protein were evaluated.

Table 1. Characteristics of BC patients related to low and high expression of Ir (divided according to the median) compared with the Chi^2 test (from Cebulski et al., 2022 [2]).

Clinicopathological Parameter	*n* 541 (%)	Irisin Expression in BC Cells		
		Low >0–5.6	High ≥5.6–12	Chi^2 Test *p*
Age				
≤ 60	156 (28.8)	75 (48.1)	81 (51.9)	0.0707
>60	385 (71.2)	218 (56.6)	167 (43.4)	
Histological type				
IDC	521 (96.3)	281 (53.9)	240 (46.1)	
ILC	12 (2.2)	7 (58.3)	5 (41.7)	
IPC	1 (0.2)	1 (100.0)	0 (0.0)	0.7252
MC	1 (0.2)	0 (0.0)	1 (100.0)	
MetC	4 (0.7)	3 (75.0)	1 (0.0)	
MucC	2 (0.4)	1 (50.0)	1 (50.0)	
Tumor size (T)				
T1	344 (63.6)	183 (53.2)	161 (46.8)	
[T1a-b	[89 (16.5)	35 (39.3)	54 (60.7)	**0.0414**
T1c]	255 (47.1)]	148 (58.0)	107 (42.0)	
T2	179 (33.1)	99 (55.3)	80 (44.7)	
T3-4	18 (3.3)	11 (61.1)	7 (38.9)	
Lymph nodes (N)				
N0	340 (62.8)	169 (49.7)	171 (50.3)	**0.0258**
N1	193 (35.7)	119 (61.7)	74 (38.3)	
N2	8 (1.5)	5 (62.5)	3 (37.5)	

Table 1. *Cont.*

Clinicopathological Parameter	*n* 541 (%)	Irisin Expression in BC Cells		
		Low >0–5.6	High ≥5.6–12	Chi^2 Test *p*
Stage				
I	237 (43.8)	123 (51.9)	114 (48.1)	0.5507
II	283 (52.3)	157 (55.5)	126 (44.5)	
III	21 (3.9)	13 (61.9)	8 (38.1)	
Histological grade (G)				
G1	96 (17.7)	49 (51.0)	47 (49.0)	0.2929
G2	350 (64.7)	198 (56.6)	152 (43.4)	
G3	95 (17.6)	46 (48.3)	49 (51.7)	

BC—breast cancer; IDC—invasive ductal carcinoma, ILC—invasive lobular carcinoma, IPC—invasive papillary carcinoma, MC—medullary carcinoma, MetC—metaplastic carcinoma, MucC—mucinous carcinoma.

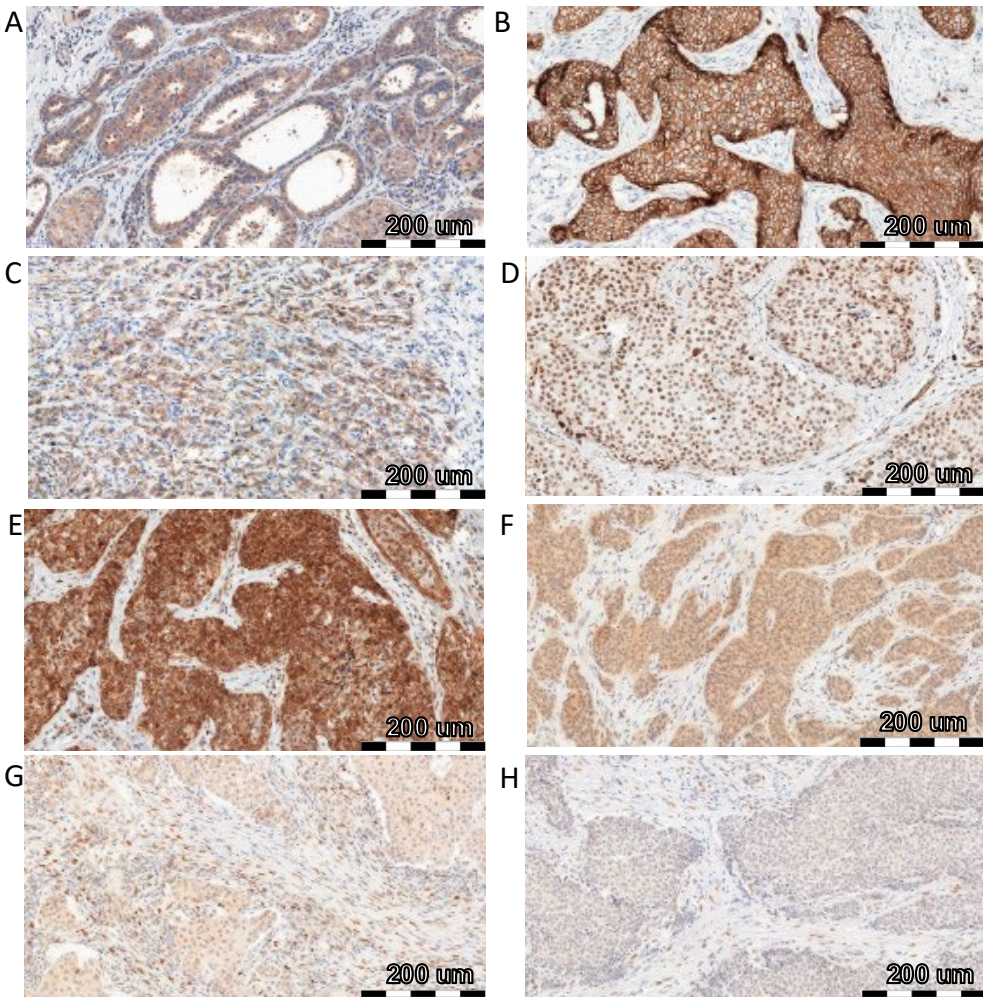

Figure 1. Comparison of FNDC5/Ir ((**A**)—IRS score 8) expression with E-cadherin ((**B**)—IRS score 12), N-cadherin ((**C**)—IRS score 8), SNAIL ((**D**)—IRS score 3 and % of nuclear expression 4, (**E**)—IRS score 12 and % of nuclear expression 4), SLUG ((**F**)—IRS score 8 and % of nuclear expression 3) and TWIST ((**G**)—IRS score 4 and % of nuclear expression 2, (**H**)—IRS score 4 and % of nuclear expression 2) using immunohistochemistry (IHC) (positive reactions—brown cell cytoplasm) in breast cancer (BC), magnification ×200.

For each protein, we analyzed the correlation of its expression level with the level of FNDC5/Ir in tumor cells. E-cadherin showed a moderate positive correlation with the FNDC5/Ir expression level (r = 0.31, $p < 0.0001$) (Figure 2A). The expression level

of N-cadherin was not related to FNDC5/Ir expression (Figure 2B). Cytoplasmic and nuclear SNAIL expression correlated moderately positively with FNDC5/Ir expression level (r = 0.35; $p < 0.0001$; r = 0.29, $p < 0.0001$, respectively) (Figure 2C,D). In contrast, in the case of the transcription factor SLUG, we observed a weak positive correlation with the expression level of FNDC5/Ir for cytoplasmic and nuclear expression (r = 0.18, $p < 0.0001$; r = 0.23, $p < 0.0001$, respectively) (Figure 2E,F). We observed a positive moderate correlation for cytoplasmic expression of TWIST (r = 0.31; $p < 0.0001$) and a weak positive correlation for nuclear (r = 0.22; $p < 0.0001$) expression of TWIST (Figure 2G,H).

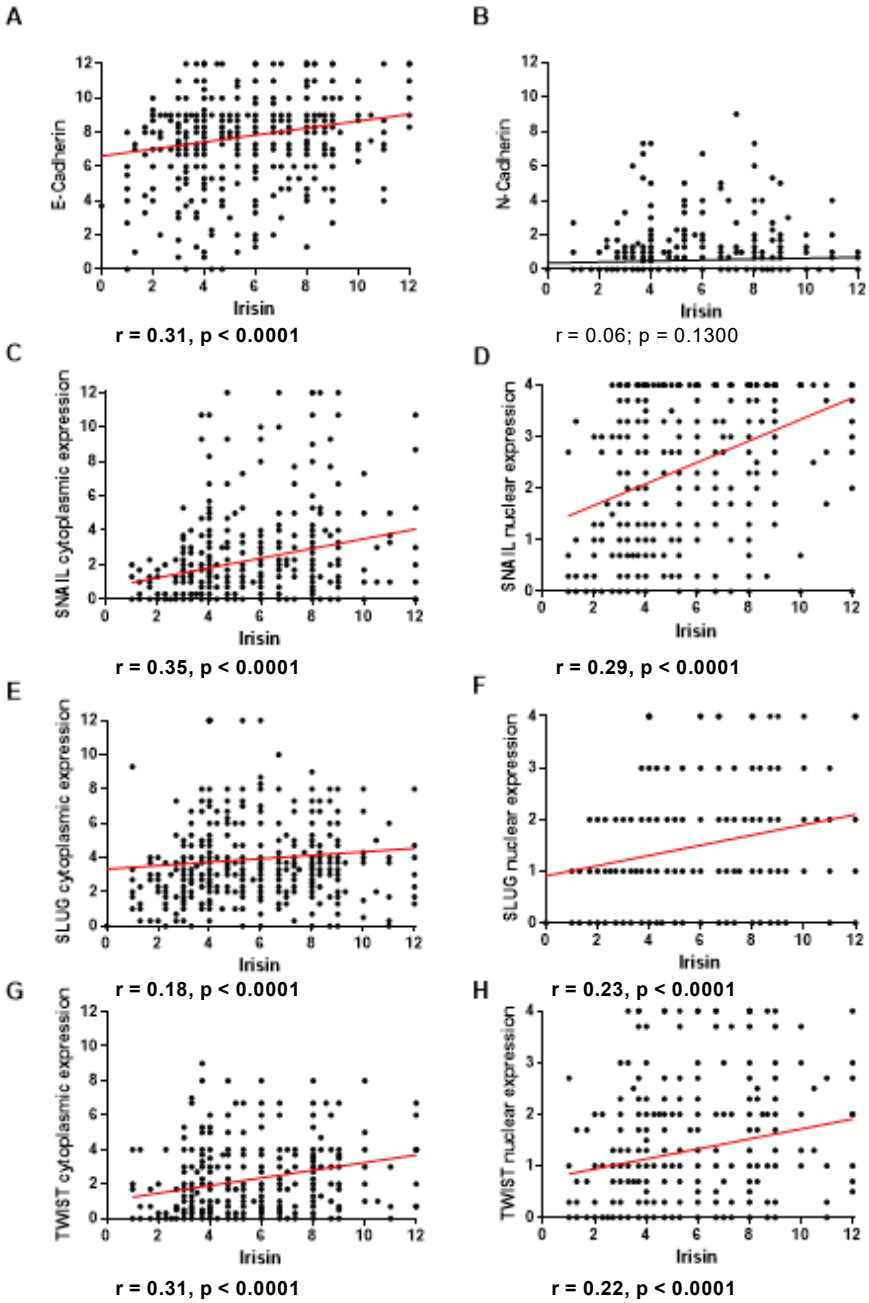

Figure 2. Correlation of FNDC5/Ir expression level with E-cadherin (**A**) and N-cadherin (**B**), cytoplasmic (**C**) and nuclear (**D**) SNAIL expression levels, cytoplasmic (**E**) and nuclear (**F**) SLUG expression levels, cytoplasmic (**G**) and nuclear (**H**) TWIST expression levels in breast cancer (BC) (sample size $n = 541$).

2.2. Comparison of Ir Levels in Tissues and Serum of Breast Cancer Patients

Serum Ir concentrations were evaluated by ELISA. The association of the results of serum Ir levels in patients with the clinicopathological data, as well as with the results of the FNDC5/Ir expression level in the BC tissue were investigated.

We did not observe an association between the expression level of FNDC5/Ir in BC tissue examined by IHC and serum concentrations of Ir (Figure 3A). However, we observed higher serum concentrations of Ir in patients with a large number of lymph node metastases (N2, mean 20.86 ± 23.7 SD) compared to patients without metastases (N0, mean 9.25 ± 2.1 SD) and with single lymph node metastases (N1, mean 10.33 ± 7.7 SD) (Mann-Whitney U test; $p = 0.0055$, $p = 0.0072$, respectively) (Figure 3B). We observed higher concentrations of Ir in N1 patients compared to N0 subjects. However, the difference was not statistically significant. In addition, serum concentrations of Ir were higher with the increase in tumor malignancy (mean G1—8.02 ± 1.4 SD; G2—8.96 ± 2.2 SD; G3—9.86 ± 1.4 SD). We observed higher serum Ir concentrations in patients with G3 compared to G1 ($p = 0.0166$) (Figure 3C). We found no statistically significant differences in Ir levels for the increasing tumor size (Figure 3D). Table 2 shows the characteristics of BC patients with the division into low and high serum Ir concentration levels according to the median. The analysis with the Chi^2 test showed a difference between the groups with high and low Ir expression (division according to the median value, Table 2) only in different age groups of patients (≤60 and >60 years of age). Table 3 shows the associations of Ir serum level with the clinicopathological characteristics in patients with BC.

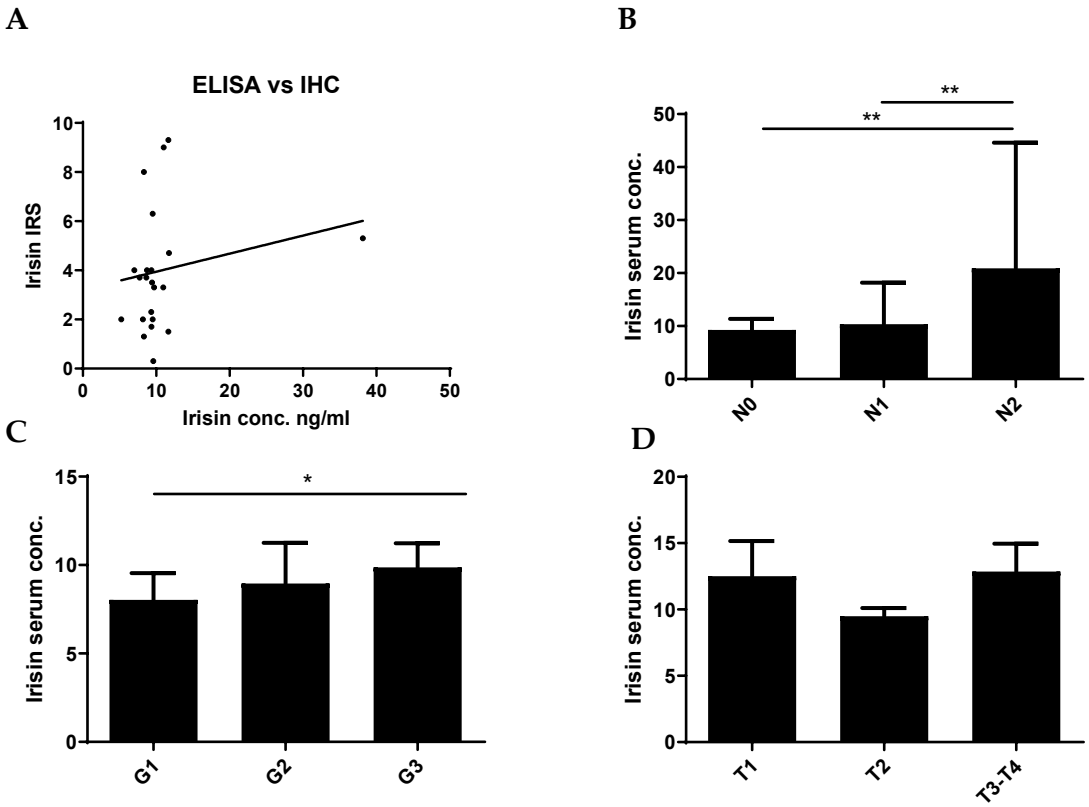

Figure 3. Comparison of serum Ir concentrations in breast cancer (BC) patients with expression levels in BC tissue (**A**). Comparison of Ir concentrations in BC patients according to the lymph node status [N] (**B**), histological grade [G] (**C**), and tumor size [T] (**D**), * $p < 0.05$ ** $p < 0.01$ (sample size $n = 77$). The lymph node status, histological grade, and tumor size were evaluated based on the World Health Organization Criteria and the 8th TNM edition.

Table 2. Characteristics of BC patients related to low and high serum Ir levels (divided according to the median) compared with the Chi^2 test. Statistically significant results are marked in bold.

Clinicopathological Parameter	*n* 77 (%)	Irisin Serum Concentrations in BC Patients		
		Low <8.9 ng/mL	High ≥8.9 ng/mL	Chi^2 Test *p*
Age				
≤60	51 (66.2)	20 (39.2)	31 (60.8)	**0.0127**
>60	26 (33.8)	18 (69.2)	8 (30.8)	
Tumor size (T)				
T1	41 (53.2)	18 (43.9)	23 (56.1)	0.1816
T2	27 (35.1)	17 (63.0)	10 (37.0)	
T3-T4	9 (11.7)	3 (33.3)	6 (66.7)	
Lymph nodes (N)				
N0	36 (46.8)	17 (47.2)	19 (52.8)	0.6469
N1	33 (42.9)	18 (53.3)	15 (46.7)	
N2	8 (10.4)	3 (23.1)	5 (76.9)	
Histological grade (G)				
G1	19 (24.7)	14 (73.7)	5 (26.3)	0.1194
G2	49 (63.6)	23 (46.9)	26 (53.1)	
G3	9 (11.7)	4 (44.4)	5 (55.6)	

Table 3. Associations of Ir serum level with the clinicopathological characteristics in patients with BC. Statistically significant results are marked in bold.

Comparison of BC Groups	*p* (Mann–Whitney U Test)	BC Groups	Mean Value ± SD
Tumor size (T)		**Tumor size (T)**	
T1 vs. T2	0.6348	**T1**	12.50 ± 13.3
T1 vs. T3-4	0.2273	**T2**	9.50 ± 2.4
T2 vs. T3-4	0.2618	**T3-T4**	12.85 ± 6.3
Lymph nodes (N)		**Lymph nodes (N)**	
N0 vs. N1	0.4937	**N0**	9.25 ± 2.1
N0 vs. N2	**0.0055**	**N1**	10.33 ± 7.7
N1 vs. N2	**0.0072**	**N2**	20.86 ± 23.7
Histological grade (G)		**Histological grade (G)**	
G1 vs. G2	0.3224	**G1**	8.02 ± 1.4
G1 vs. G3	**0.0166**	**G2**	8.96 ± 2.2
G2 vs. G3	0.2268	**G3**	9.86 ± 1.4

2.3. FNDC5/Ir Expression in Breast Cancer Cell Lines

Some examinations were conducted on BC cell lines (MCF-7, MDA-MB-231, MDA-MB-468). The normal breast cell line (Me16c) was used as the control. We estimated the FNDC5/Ir expression level in cells by measuring the level of immunofluorescence using confocal microscopy. In addition, we examined the expression level of *FNDC5* mRNA in cells using RT-PCR.

FNDC5 mRNA expression levels in normal breast cells (Me16c) were significantly lower (mean 1.03 ± 0.05 SD) compared to the levels found in the cells of all BC lines (MCF-7, mean 30.71 ± 8.43 SD, $p = 0.0037$; MDA-MB-231, mean 7.58 ± 4.05 SD, $p = 0.0492$; MDA-MB-468, mean 111.60 ± 41.93 SD, $p = 0.0103$). In addition, the expression levels were significantly different between BC cell lines. The highest level of expression of the *FNDC5* gene was observed in MDA-MB-468 cells. The difference between the *FNDC5* mRNA levels in MCF-7 and MDA-MB-231 lines was statistically significant ($p = 0.0306$; $p = 0.0129$, respectively) (Figure 4A).

Additionally, the measurement of immunofluorescence (IF) showed that the level of FNDC5/Ir expression in normal breast cells (Me16c) was significantly lower (mean 18.54 ± 4.50 SD) compared to the level in the cells of all BC lines (MCF-7—mean 125.60 ± 36.63 SD, $p < 0.0001$; MDA-MB-231 mean 37.81 ± 20.47 SD, $p = 0.0327$; MDA-MB-468 mean 74.13 ± 15.40 SD, $p < 0.0001$). The difference between the FNDC5/Ir levels in MDA-MB-231 and MCF-7, MDA-MB-468 lines was statistically significant ($p = 0.0014$;

$p = 0.0094$, respectively) (Figure 4B). FNDC5/Ir expression by confocal microscopy in the normal breast cell line (Me16c) and different BC cell lines are given in Figure 5.

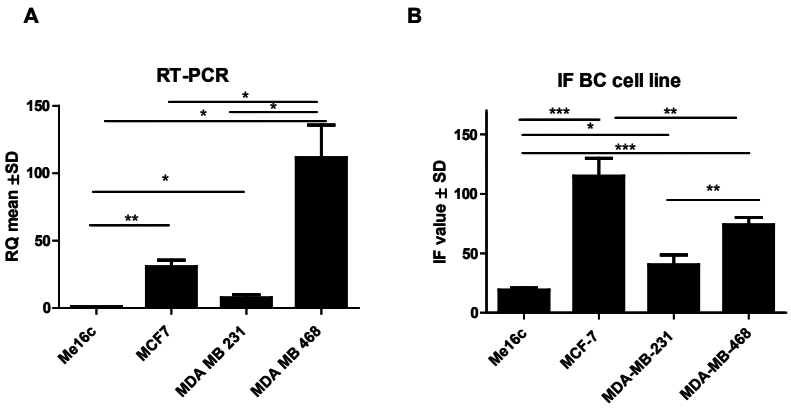

Figure 4. Comparison of mRNA FNDC5 expression levels detected by RT-PCR (**A**) and FNDC5/Ir levels (**B**) in the normal breast cell line (Me16c) and different types of BC cell lines (MCF-7, MDA-MB-231, MDA-MB-468) * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

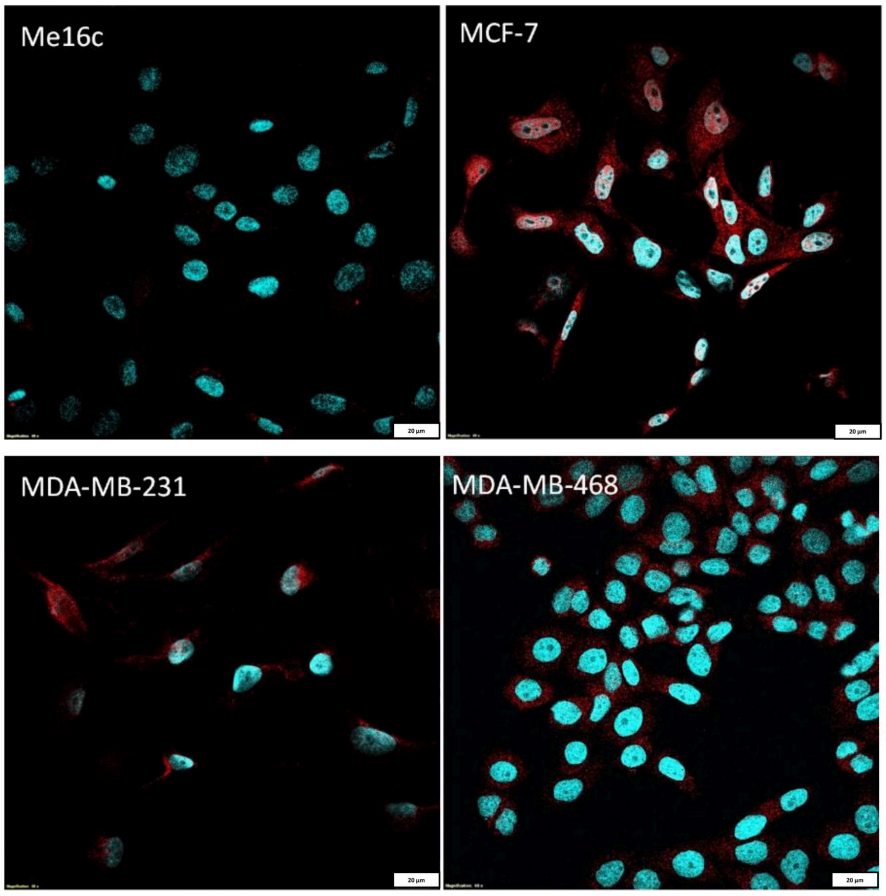

Figure 5. Comparison of FNDC5/Ir expression by confocal microscopy in the normal breast cell line (Me16c) and different BC cell lines (MCF-7, MDA-MB-231, MDA-MB-468).

2.4. Ultrastructural Localization of Ir in Breast Cancer Cells

Immunolocalization of FNDC5/Ir at the ultrastructural level was performed after the application of the post-embedding method. First observations were made on invasive ductal carcinoma (IDC) tumors at different stages of malignancy (G2 and G3) (Figure 6A–D

and Figure 7). In both stages, Ir was visible in the tumor cells, the extracellular matrix, and stromal cells, particularly in fibroblasts (Figure 7). Fibroblasts were recognizable by accompanying collagen, elastic fibers, and extracellular fluid. These cells were elongated, with the abundant, slightly dilated rough endoplasmic reticulum, which suggests an active release of proteins and stacks of Golgi cisternae. However, in BC cells the strongest immunogold reaction was found in the mitochondria, cell membranes of the neighboring cells, near the intermediate filaments, and the rough endoplasmic reticulum. Moreover, we also found some inflammatory cells and many capillaries in the stroma of breast tumors. We also analyzed MDA-MB-468 BC cell lines embedded in the form of cell pellets entrapped in the fibrin clot and post-stained with osmium tetroxide (Figure 6E,F). The most striking features were microvilli-like structures formed by the neighboring cancer cells and long cytoplasmic extensions. Colloidal gold nanoparticles were detected in these structures or the cytoplasm.

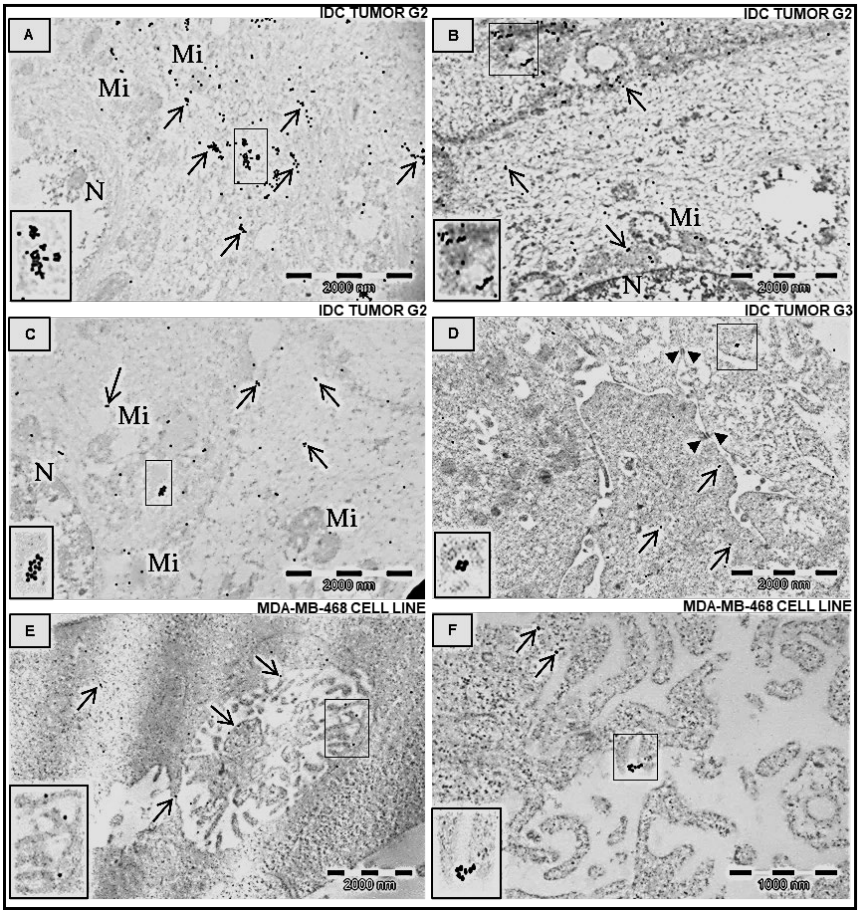

Figure 6. Immunolocalization of Ir in transmission electron microscopy. Ultrathin section examination of human adenocarcinoma BC cells (from tumors, (**A**–**D**) and MDA-MB 468 cell line, (**E**,**F**)). The specific primary antibody against FNDC5/Ir was applied. Next, the ultrathin sections were labeled with the secondary antibody conjugated with the 20 nm-colloidal gold nanoparticles, which shows the antigen distribution in the cells. Arrows indicate positive gold nanoparticles. A strong reaction was detected in the cytoplasm of cancer cells, in the mitochondria, and at the border of the cell membranes of neighboring cells. Note the localization of Ir near the specific microvilli–like structure (**E**) and at the cytoplasmatic processes of BC cells (**F**). Brief double staining with UranyLess solution and lead citrate (3%). IDC—invasive ductal carcinoma at different grades of malignancy, N—nucleus, Mi—mitochondrion.

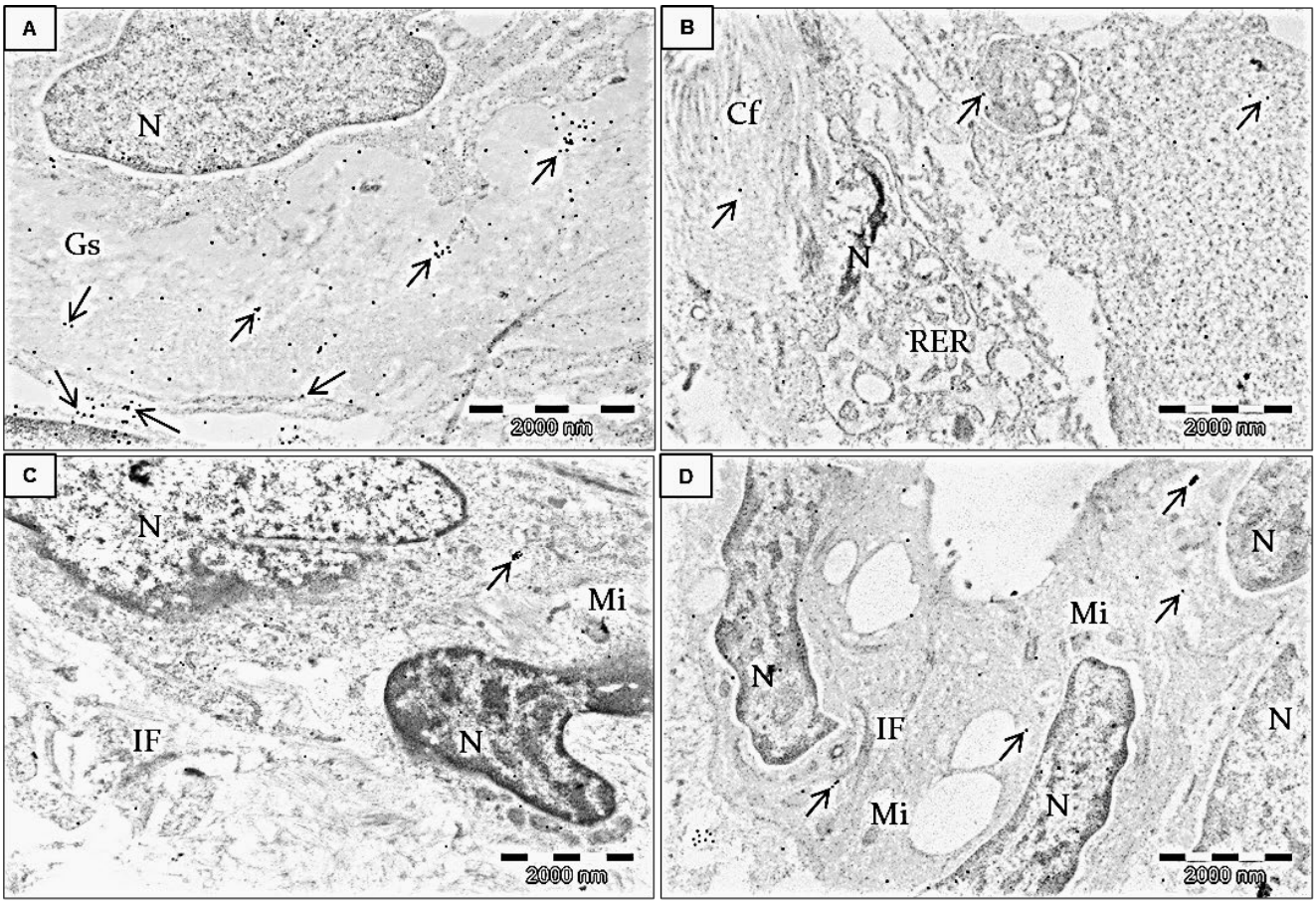

Figure 7. Immunolocalization of Ir in transmission electron microscopy. Ultrathin section examination of human adenocarcinoma BC cells from the breast tumor microenvironment (stroma). All electronograms show invasive ductal carcinoma G2. The specific primary antibody against FNDC5/Ir was applied as previously described, followed by applying the species-specific secondary antibody conjugated with 20 nm-colloidal gold nanoparticles. Arrows indicate positive gold nanoparticles. Strong immunogold reaction was detected in the extracellular matrix and cancer-associated fibroblasts (in the cytoplasm and at the border of the cell membrane). Brief double staining with UranyLess solution and lead citrate (3%). N—nucleus, Gs—ground substance of the extracellular matrix (**A**), Cf—collagen fibers, RER—rough endoplasmic reticulum in the fibroblast (**B**). IF—intermediate filaments (**C**), Mi—mitochondrion (**D**).

3. Discussion

This study is a continuation of our previous studies evaluating the expression level of FNDC5/Ir in BC tissue [2]. In this study, we assessed the association of serum levels of Ir with clinicopathological factors and IHC tissue expression, ultrastructural localization of FNDC5/Ir expression, and their correlation with EMT markers in a group of 541 BC patients.

For metastasis to occur, the EMT process must occur in the cancer cells [26]. The most important change is the disappearance of E-cadherin expression and the appearance of N-cadherin, which is characteristic of the mesenchymal phenotype [21,27]. In our study, the level of FNDC5/Ir expression positively correlated with the level of E-cadherin expression. However, we did not notice a relationship between FNDC5/Ir levels and N-cadherin expression. The results suggest that FNDC5/Ir may be associated only with the pathway of E-cadherin expression. Retaining a high level of E-cadherin expression in cancer cells maintains the epithelial phenotype of the cell and blocks EMT progression. We found a positive correlation between FNDC5/Ir and E-cadherin expression levels, which

is consistent with other studies. In their research on lung cancer cell lines, Shao et al. [11] showed that cell exposure to Ir caused an increase in the level of E-cadherin expression. Similarly, Zhu et al. [28] observed an increase in E-cadherin expression after the administration of Ir to ovarian cancer cells (SKOV3 and A2780). They also observed that the amount of phosphorylated AKT decreased when Ir was administered to cells. This suggests that Ir influences EMT suppression of ovarian and pancreatic cancer cells through the PI3/AKT pathway by inhibiting AKT phosphorylation [28,29]. However, in the case of osteosarcoma, FNDC5/Ir inhibited STAT3 phosphorylation in the STAT3/SNAIL signaling pathway, which is also associated with the EMT process [10,11,15,28]. Our finding of a positive correlation between FNDC5/Ir and E-cadherin is consistent with previous studies indicating its inhibitory effect on EMT. This is also confirmed by the results of our earlier study on the level of FNDC5/Ir expression in BC tissues [2]. The higher the FNDC5/Ir expression level in BC cells, the lower the risk of metastases and the longer the patient s survival. These findings are consistent with the positive correlation between FNDC5/Ir and E-cadherin. Perhaps the lack of a relationship with N-cadherin indicates that Ir affects EMT only by increasing the expression of E-cadherin. On the other hand, Hollestelle et al. [30] observed in a large group of BC cell lines that the loss of E-cadherin expression was not necessary for cell reshaping to spindle shape and EMT. In both BC cell lines and tissue material, they observed that the loss of E-cadherin expression in cells that underwent EMT was not necessary. Moreover, the loss of E-cadherin was demonstrated in only half of metaplastic BC. Hollestelle et al. investigated whether the CpG1 and CpG3 hypermethylation of the E-cadherin gene in BC cell lines had an impact on EMT. They indicated that CpG3 hypermethylation was a prerequisite for the complete loss of E-cadherin. Interestingly, re-upregulation of E-cadherin expression did not restore the cells to their typical epithelial shape. Multiple BC cell lines showed high E-cadherin expression with the simultaneous expression of EMT markers. In addition, Hollestelle et al. [30] indicated that the expression of E-cadherin gene repressors, such as *SNAI1*, *SNAI2*, and *TWIST1*, was also observed in non-spindle cell lines. Additionally, the positive correlation between FNDC5/Ir and E-cadherin did not preclude the occurrence of EMT in BC cells, which is in line with the findings of Hollestelle et al. This indicates a high complexity of interactions between E-cadherin repressors.

In our study, we observed weak and moderate positive correlations between SLUG, TWIST, SNAIL, and FNDC5/Ir levels. Such correlations were found in the cytoplasm and the nucleus of BC cells. SLUG, TWIST, and SNAIL are transcription factors that induce EMT in the cell by modifying the expression of many proteins, including the inhibition of E-cadherin expression [22]. The positive correlations of SNAIL, SLUG, and TWIST transcription factors with FNDC5/Ir seem to contradict the positive correlation between E-cadherin expression and FNDC5/Ir. However, these correlations were weak and positive for SLUG and nuclear TWIST. On the other hand, a moderate correlation between SNAIL and FNDC5/Ir was observed for cytoplasmic and nuclear expression of this transcription factor and also between TWIST and FNDC5/Ir for cytoplasmic expression. This may indicate that higher FNDC5/Ir concentrations affect EMT in a more complex manner.

FNDC5/Ir may not only influence the levels of the transcription factors (SNAIL, SLUG, and TWIST) in the tissue but also their activity in the cell. Wang et al. [31] indicated that the expression, transport, and function of SNAIL were controlled in the cells also at the stage of post-translational modification by β-TrCP-induced ubiquitination and GSK3β-dependent phosphorylation. SNAIL in the cytoplasm is rapidly degraded. In turn, SNAIL in the nucleus is characterized by greater stability and is functionally active [31]. SNAIL phosphorylated by GSK3β is transported from the nucleus to the cell cytoplasm, where after ubiquitination by β-TrCP it is degraded inside the peroxisomes [31]. In our study, we observed a moderate correlation between FNDC5/Ir and SNAIL for cytoplasmic and nuclear expression. FNDC5/Ir could lead to a lack of SNAIL activity and could promote SNAIL transportation to the cytoplasm, but IHC only indicates a relationship and is not sufficient enough to prove it. Further studies are warranted to clarify the functional

relationship of these proteins and whether FNDC5/Ir may affect the activity of the SNAIL transcription factor.

The high complexity of the effect of FNDC5/Ir on EMT was demonstrated by various studies [5,11,16,17]. Although most studies showed the inhibitory effect of Ir on the proliferation and migration of BC cells [10,12–15], some research did not confirm such findings. Moon et al. [16] did not observe a significant effect of Ir on the proliferation and migration of endometrial, colorectal, thyroid, and esophageal cancer cell lines. In the case of hepatocellular carcinoma, Shi et al. [17] found that FNDC5/Ir had a positive effect on the proliferation and invasion of cancer cells also by affecting the PI3K/Akt signaling pathway. Perhaps, the differences in the influence of Ir on EMT are specific to different types of cancer and are the result of the influence of Ir on particular metabolic pathways.

In our study, we also analyzed and compared serum and tumor levels of Ir from BC patients. We noticed a tendency for a positive relationship between the expression level of FNDC5/Ir in BC tissue and serum concentrations of Ir in patients. However, this relationship was not statistically significant. The lack of statistical significance may result from various reasons. First, newly synthesized FNDC5 in the cell must undergo a complex pathway before it is converted to glycosylated Ir and released into plasma. The release of Ir into the serum is still not fully understood. The factors affecting changes in plasma concentrations of Ir are still not clear. Such factors may include gender, age, the level of physical activity, or the stage of the menstrual cycle [32]. In addition, Ir can be secreted into the plasma by various tissues [3,4]. The complexity of the whole process indicates that plasma Ir concentrations do not necessarily correlate with the levels found in cancer cells. In our study, we used a relatively small group to compare Ir levels in serum and tissue.

However, when the relationship between plasma Ir levels and the clinicopathological data was analyzed, we observed significantly higher concentrations of Ir in patients with many lymph node metastases (N2) compared to patients without metastases (N0). So far, several studies on evaluating plasma concentrations of Ir in BC patients have been conducted. However, only two of them investigated the relationship between serum levels of Ir and the clinicopathological data of the patients. Our results are consistent with those presented by Provatopoulou et al. [33]. They also assessed plasma Ir levels in BC patients and showed that Ir levels were higher in patients with lymph node metastases, distant metastases, or high stages of cancer. However, Zhang et al. [34] examined Ir levels in the plasma of patients with BC without distant metastases and with spinal metastases. They found that high Ir levels in the serum were associated with the absence of spinal metastases. In our study, we did not check the relationship between Ir levels and distant metastases. However, Zhang et al. focused only on the presence or absence of distant metastases. They did not include comparisons of Ir levels with the clinical stage of the disease, lymph node metastases, or tumor size. The increase in plasma concentrations of Ir may occur in patients in advanced stages when the destruction of the body is observed. The increase in Ir expression in the N2 group may be related to the further progression of neoplastic disease. The literature suggests that in patients in more advanced stages of cancer, Ir is involved in developing cachexia, thus accelerating the destruction of the patient's body [9].

In the immunogold study, we confirmed that FNDC5/Ir was present in the cytoplasm of BC cells in tissue as well as in cell lines. FNDC5/Ir was observed in the mitochondria, cell membranes, near the intermediate filaments, and the rough endoplasmic reticulum. This confirms FNDC5/Ir expression observed by IHC and IF. Additionally, we observed Ir in the extracellular matrix and the cells of stromal fibroblasts. Nowinska et al. [35] also detected Ir in the mitochondria, endoplasmic reticulum, and cytoplasm in the ultrastructure of A549, NCI-H522, and NCI-H1703 lung cancer cells. In addition, Ir has previously been observed in NSCLC stromal fibroblasts [5].

We also conducted research on BC cell lines. We examined the FNDC5 expression level and the FNDC5/Ir protein level. Studies on an in vitro model confirmed a higher level of FNDC5/Ir expression in BC tissues compared to NMBD tissues, which we previously observed [2]. As in the tissue material, we detected significantly lower FNDC5 mRNA

expression and FNDC5/Ir expression in normal Me16c cells compared to MCF-7, MDA-MB-231, and MDA-MB-468 cells. These findings were also confirmed by studies on other types of cancer. Similarly, Pinkowska et al. [6] observed a higher level of FNDC5/Ir in HEp-2 laryngeal carcinoma lines compared to normal cells. Higher levels of Ir were also detected in colon cancer [36] and lung cancer lines compared to normal cells [5]. Differences in FNDC5/Ir levels might be the result of metabolic changes in BC cells and their progesterone (PR), estrogen (ER), and HER receptor status. We observed the highest expression of mRNA *FNDC5* in the MDA-MB-468 cell line which is the most aggressive of the tested cell lines because of the triple-negative receptor status (HER-, ER-, PR-). Cancer cells show accelerated metabolism associated with the intensification of glycolysis or mitochondrial biogenesis [37]. The above processes result in changes in the expression of many proteins, including peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) [38]. PGC1α is a transcription factor that controls the expression of many proteins, including FNDC5 [4]. The increased level of FNDC5/Ir expression in cancer cells may result from the increasing energy demand of the cancer cell and the modification of its metabolic processes. They lead to the intensification of glycolysis and obtaining energy mainly in the anaerobic mechanism. This process is commonly observed in cancer [39].

In conclusion, the expression level of FNDC5/Ir in BC cell lines was higher than in normal BC epithelial cells. Ultrastructural studies showed the presence of FNDC5/Ir in the cytoplasmic structures of BC tumor cells and stromal fibroblasts. Higher Ir serum levels are associated with lymph node metastasis and a higher grade of malignancy. However, serum Ir levels in patients did not reflect its levels in BC tumor cells. Serum Ir levels in patients might also be affected by its secretion by other tissues, e.g., muscle and adipose. Further research is warranted to clarify the mechanisms of Ir release from cells to plasma and the regulation of this process. Additionally, FNDC5/Ir expression is associated with the expression level of E-cadherin and correlates with EMT marker expression in BC tissues. However, the results are contradictory and need to be confirmed in further studies. Therefore, further studies are warranted to explain the mechanism by which FNDC5/Ir influences E-cadherin upregulation and EMT transition.

4. Materials and Methods

4.1. Patient Cohort

The study was conducted on archival material consisting of 541 tumor tissues (patient characteristics in Table 1) and 77 serum samples from BC patients. As the control, 61 cases of NMBD were used. The material was collected from January 2004 to December 2012. All BC patients were diagnosed and treated at the Polish Mother's Memorial Health Institute in Łódź. The control samples of NMBD were obtained from the 4th Military Teaching Hospital in Wroclaw. The study was approved by the Bioethics Committee of Wroclaw Medical University (No. 726/2019 and KB-731/2019). The mean age of patients during the treatment period was 56 years (24–86 years). The treatment and follow-up were based on the World Health Organization criteria and the 8th TNM edition [40]. The histological grade (G) and the clinical stage of BC patients were determined.

4.2. Cell Line Culture

Molecular biology studies were conducted using the adherent BC cell lines (MCF-7, MDA-MB-231, MDA-MB-468) obtained from The American Type Culture Collection (Manassas, VA, USA). The Me16c cell line was used as the control. MCF-7 cells were cultured in an EMEM medium (Lonza, Basel, Switzerland). Leibovitz's L-15 Medium (Sigma-Aldrich, St. Louis, MO, USA) was used to culture MDA-MB-231 and MDA-MB-468. Me16c was cultured in MEBM medium (Lonza, Basel, Switzerland) supplemented with insulin, hydrocortisone, bovine pituitary extract (BPE), and the human epidermal growth factor (hEGF). All media were supplemented with 10% fetal bovine serum (FBS) (Merck, Darmstadt, Germany), 1% penicillin/streptomycin (Merck, Darmstadt, Germany), and L-glutamine

(Merck). The HERA cell incubator (Heraeus, Hanau, Germany) was used to maintain constant cell culture conditions (37 °C, 5% CO_2 concentration, and a 95% humidity level).

4.3. Immunohistochemistry (IHC) on Tissue Microarrays (TMAs)

Tissue microarrays (TMAs) were performed on 541 BC and 61 NMBD sections. Slides with the whole BC or NMBD tissue sections were stained with hematoxylin and eosin and scanned with the Pannoramic Midi II (3D HISTECH Ltd., Budapest, Hungary) histological scanner. Three demonstrative sites with cancer were selected by the Pannoramic Viewer (3D HISTECH Ltd., RRID: SCR_014424, Budapest, Hungary). Subsequently, the selected cancer sites were transferred with a core of 1.5 mm to the tissue arrays using the TMA Grand Master (3D HISTECH Ltd.).

Immunohistochemical reactions were performed on each TMA section. Deparaffinization, hydration, and thermal epitope demasking were completed using a low pH Target Retrieval Solution (Agilent Technologies, Santa Clara, CA, USA) for 20 min at 97 °C in a Dako PT Link (Dako, Glostrup, Denmark) apparatus. The expression of proteins was detected by specific primary antibodies, i.e., polyclonal rabbit: anti-irisin/FNDC5 (dilution 1:50; code no. NBP2-14024; Novus Biologicals, Littleton, CO, USA) and anti-SNAIL (1:400, Clone, code 13099-1-AP, Proteintech, Rosemont, IL, USA), monoclonal mouse: anti-E-cadherin antibody (ready to use, Clone NCH-38, code IR059; Dako, Glostrup, Denmark), anti-N-cadherin (1:50, Clone 6G11, code M3613; Dako), anti-SLUG (1:50, clone A-7, sc-166476, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and monoclonal mouse anti-TWIST (dilution 1:50, clone Twist2C1a, code ab-50887, Abcam, Cambridge, UK). DAKO Autostainer Link48 (Dako) automated system and an EnVision FLEX kit (Dako) were used to visualize the antigens.

At least two independent pathologists-researchers conducted the evaluation of the IHC reactions at ×200 magnification. The assessment was performed using a BX41 light microscope (Olympus, Tokyo, Japan) coupled with a visual circuit and the Cell D (Olympus) software. The nuclear expression levels of SNAIL, TWIST, and SLUG were determined using a five-point evaluation scale (% of nuclear expression) (0—no expression, 1 point—>0–10%, 2 points—>10–25%, 3 points—>25–50%, 4 points—>50%) [5,41]. To estimate the cytoplasmic expression levels of SLUG, TWIST, SNAIL, FNDC5/Ir, and N-cadherin, we used the semiquantitative method immunoreactive score (IRS) according to Remmele and Stegner [42]. The final result of the estimation was the outcome of the multiplication of the obtained points from the intensity of the color reaction (1—weak, 2—moderate, 3—strong) and the percentage of IRS-positive cancer cells (0 point—lack of expression, 1 point—>1–10%, 2 points—>10–50%, 3 points—>50–80%, 4 points—>80%). The expression of the E-cadherin antigen was evaluated quantitatively by estimating the percentage of positive tumor cells (0–5% = no reaction (0 points), 6–25% = weak reaction (1 point), 26–50% = moderate reaction (2 points), above 50% = intense reaction (3 points)).

4.4. Real-Time PCR (RT-PCR) Analysis

RT-PCR reactions were performed for BC cell lines and the control cell line. The RNeasy Mini Kit (Qiagen) was used for RNA isolation. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) with RNase Inhibitor (Applied Biosystems) was used to perform the reverse transcription reaction. The 7900HT Fast Real-Time PCR System (Applied Biosystems) and the relative quantification (RQ) method were used to analyze mRNA *FNDC5* expression (FNDC5; TaqMan Gene Expression Assay, Applied Biosystems) in the cell lines. The RQ Manager 1.2 software (Applied Biosystems) was used for the analysis. The results were standardized by the reference gene for β-actin expression (ACTB; TaqMan Gene Expression Assay, Applied Biosystems). Changes in the mRNA *FNDC5* level in BC cells were assessed in relation to normal cells. RT-PCR was repeated three times.

4.5. Immunofluorescence (IF)

For 24-h microculture, 600 µL of 20 × 10^4 cells/mL suspension of cells was instilled into each well of Millicell EZ 8-well glass slides (Merck). Microcultures with cells were incubated at 37 °C for 24 h. Then, the cells were fixed using 4% formaldehyde. Subsequently, the fixed cells were incubated with the specific polyclonal rabbit anti-irisin/FNDC5 antibody (dilution 1:50; code no. NBP2-14024; Novus Biologicals) at 4 °C overnight. The slides with the fixed cells were rinsed and incubated for 1 h with donkey anti-rabbit secondary AlexaFluor 568 conjugated antibody (dilution 1:2000; code no. A10042; Invitrogen, Waltham, MA, USA). The secondary antibody was diluted in a background-reducing reagent (Agilent Technologies, Santa Clara, CA, USA). The Prolong DAPI Mounting Medium (Invitrogen) was used to stain the cell nucleus and mount the slides. The observations were made at ×600 magnification using Olympus Fluoview FV3000 confocal microscopy coupled with CellSense (Olympus) software.

4.6. Enzyme-Linked Immunosorbent Measurement (ELISA) Tests

The enzyme-linked immunosorbent assay (ELISA) tests were performed on serum samples from BC patients. Serum samples were obtained from venous blood after centrifugation at 3000 rpm for 10 min and were preserved at −80 °C for further analysis. Serum Ir levels were examined using commercially available ELISA recombinant irisin kit test Irisin, Recombinant Elisa kit (Cat No. EK-0670-29, Phoenix Pharmaceuticals, CA, USA). The ELISA tests were performed according to the manufacturer's protocol. For each sample, 50 µL of serum was used. The results were verified by microplate reader ELX-800 (BIO-TEK, VT, USA). The examination was performed in two repetitions.

4.7. Transmission Electron Microscopy (TEM)

After the application of TEM, the subcellular localization of Ir was examined in two types of samples. Breast tumors sections were cut into small pieces and in vitro culture of the MDA-MB-468 cell line representing metastatic adenocarcinoma of the breast was immersed in a 4% solution of PBS and paraformaldehyde (Boster Bio, Pleasanton, CA, USA) and fixed overnight at 4 °C (for tumors) or 25 min at room temperature (for the cell line). Subsequently, the fixed cells were gently removed with a cell scraper and placed in conical tubes (15 mL). After three cycles of centrifugation (1800 rpm for 8 min), the fixative was washed with PBS and distilled water. The cell pellets were entrapped in a fibrin clot formed from bovine thrombin (lyophilizate reconstituted with PBS; Biomed, Lublin, Poland) and fibrinogen (1 mg/mL; Merck KGaA, Darmstadt, Germany). To preserve cellular ultrastructure, each sample was post-fixed for 7 min in a 0.25% (w/v) solution of PBS and osmium tetroxide (Serva Electrophoresis, Heidelberg, Germany). Following washing with PBS (3 × 5 min), dehydration of the specimens was completed in a graded series of ethanol (2 × 50%, 2 × 70%, 1 × 96%, 4 × 100%; Stanlab, Lublin, Poland). The samples were incubated with a mixture of 99.8% ethanol and LR White resin (medium catalyzed) (Polysciences, Inc., Warrington, PA, USA) combined in appropriate proportions, i.e., 2:1 (20 min), 1:1 (1 h), and 1:2 (1 h), respectively. Next, the material labeled with the corresponding number was placed at the bottom of the gelatine capsule (flat embedding molds; Pelco, Ted Pella, Redding, CA, USA), embedded in pure resin, and underwent the hardening process in the laboratory incubator (55 °C for 48 h). Resin blocks were sectioned using the RMC ultramicrotome (Power Tome XL; Tucson, AZ, USA) into 700-nm-thick semithin sections. These sections, which were mounted onto the slide on the heating plate (at 100 °C), were stained with 1% aqueous toluidine blue (Serva Electrophoresis, Heidelberg, Germany) mixed with sodium carbonate (Alchem, Toruń, Poland). This staining is dedicated to semithin sections and allows obtaining different intensities of tissue fragments, which facilitates trimming of the area, cancer cells, and stroma with the exclusion of fat tissue. After trimming, serial 80-nm-thick ultrathin sections were obtained using the ultra-diamond knife and placed on the grids (nickel-made; Ted Pella, Redding, CA, USA). The immunogold reaction was performed to trace the distribution of Ir. All

steps were performed in the Petri dish. The first step was based on blocking residual aldehyde groups by placing the sections in 0.02 M glycine solution (BioShop Canada Inc., Burlington, ON, Canada) in PBS for 10 min. Next, the samples were immersed in triton X-100 (0.1% in PBS; Bioshop, Burlington, ON, Canada) to increase membrane permeability and then washed with PBS (5 min, 3 times). The sections were subsequently blocked for 1 h in a 1% bovine serum albumin (BSA; Carl Roth, Mannheim, Germany) to inhibit the nonspecific antigen-binding sites and quickly washed with PBS.

Next, ultrathin sections were incubated in the appropriate antibodies diluted in 0.1% BSA in PBS—primary polyclonal antibody against Ir (1:10 dilution, code no. NBP2-14024; Novus Biologicals, Littleton, CO, USA) and then anti-rabbit secondary antibody tagged with colloidal gold particles (1:10 dilution, code no. ab27237, goat anti-rabbit IgG H&L, 20 nm gold; Abcam, Cambridge, UK) for 1 h in a dark chamber. The steps were as follows: grids were rinsed in PBS and distilled water, post-fixed in 1% glutaraldehyde (Serva Electrophoresis, Heidelberg, Germany), and post-stained with the UranyLess solution and Reynolds lead citrate 3% (Electron Microscopy Sciences, Hatfield, PA, USA) to preserve the ultrastructure quality and washed thoroughly in distilled water. JEM-1011 (Jeol, Tokyo, Japan) transmission electron microscope operating at the accelerating voltage of 80 kV was used. Electronograms were obtained using the TEM imaging platform (iTEM1233) equipped with a Morada Camera (Olympus, Tokyo, Japan).

4.8. Statistical Analysis

The distribution of the results was checked by the Kolmogorov–Smirnov test. The differences in the serum levels of markers or tissue expression in BC and NMBD and their association with the clinicopathological factors were analyzed by the Kruskal–Wallis test or the Mann–Whitney U test. Correlations between Ir and E-cadherin, N-cadherin, SNAIL, and SLUG proteins were evaluated by the Spearman rank test. The unpaired t-test was used to assess the differences between the Ir level in BC and the control cell lines. Statistical analysis was performed using Prism 5.0 (GraphPad, La Jolla, CA, USA). P values below 0.05 were considered statistically significant.

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

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Bioethics Committee of Wroclaw Medical University (No. KB-726/2019 and No. KB-731/2019).

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

Data Availability Statement: The raw data and the analytic methods will be made available to other researchers for the purposes of reproducing the results in their own laboratories upon reasonable request. To access protocols or datasets contact katarzyna.nowinska@umw.edu.pl.

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7. Podsumowanie i wnioski

Przeprowadzone i przedstawione w cyklu publikacji badania wykazały potencjalną rolę iryzyny w progresji raka gruczołu piersiowego (BC). Wykazano, że iryzyna jest obecna w cytoplazmie komórek raka gruczołu piersiowego. Ponadto poziom iryzyny w wycinkach raka gruczołu piersiowego jest istotnie wyższy w porównaniu do poziomu w mastopatiach. Tę obserwację potwierdzają również dane uzyskane z wykorzystaniem modelu *in vitro*. Poziomy iryzyny i ekspresji *mRNA FNDC5* były istotnie wyższe w liniach raka gruczołu piersiowego w porównaniu do linii kontrolnej. Sugeruje to, że w przypadku BC iryzyna może być pomocnym markerem w różnicowaniu zmian łagodnych od złośliwych, może też służyć jako wskaźnik predykcyjny w rakach gruczołu piersiowego.

Porównanie poziomu ekspresji iryzyny oraz danych kliniczno-patologicznych pacjentek z rakiem gruczołu piersiowego wskazało na jej istotne znaczenie w tym nowotworze. Analiza przeżyć pacjentek z niskim i wysokim poziomem iryzyny wykazała, że jest ona pozytywnym czynnikiem rokowniczym. Ponadto wysoki poziom ekspresji iryzyny w guzach raka gruczołu piersiowego był związany z brakiem przerzutów do węzłów chłonnych oraz z mniejszym stadium zaawansowania choroby. Immunohistochemiczne oznaczenia poziomu iryzyny mogłoby być potencjalnie przydatne do oceny prognostycznej w raku gruczołu piersiowego.

Poziom ekspresji iryzyny w guzach BC koreluje słabo dodatnio z antygenem Ki-67, który jest znanym powszechnie markerem proliferacji komórek nowotworowych. Istnienie korelacji pomiędzy białkami sugeruje, że iryzyna może wpływać na pobudzanie proliferacji komórek raka gruczołu piersiowego. Ponadto zaobserwowano związek wzrostu poziomu ekspresji iryzny ze wzrostem ekspresji PGC1α, który uznawany jest za jeden z czynników transkrypcyjnych dla FNDC5 i iryzyny.

W przeprowadzonych badaniach nie udało się jednoznacznie stwierdzić, jaki jest wpływ poziomu ekspresji iryzyny na proces przejścia epitelialno-mezenchymalnego (EMT) w guzach raka gruczołu piersiowego. Zaobserwowana została dodatnia korelacja pomiędzy poziomem iryzyny, a poziomem E-kadheryny w komórkach nowotworowych raka gruczołu piersiowego. Jednak odnotowane zostały także dodatnie korelacje z czynnikami transkrypcyjnymi SNAIL, SLUG, TWIST. Wskazuje to na bardzo złożony charakter tych zależności. Wyjaśnienie wpływów iryzyny na proces EMT wymaga dalszych badań.

Wnioski:

1. Poziom iryzyny w guzie może być pomocnym markerem w różnicowaniu zmian łagodnych od raka gruczołu piersiowego.

2. Poziom iryzyny w guzie może być markerem predykcyjnym raka gruczołu piersiowego.

3. Poziomu iryzyny w guzach może być potencjalnym markerem prognostycznym u pacjentek z rakiem gruczołu piersiowego.

4. Iryzyna może wpływać na proces przejścia epitelialno-mezenchymalnego w raku gruczołu piersiowego.

8. Załączniki

8.1.Oświadczenia współautorów publikacji stanowiących podstawę pracy doktorskiej.

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Wrocław, 12 maja 2023

OŚWIADCZENIE

Oświadczam, że w pracy "Expression of Irisin/FNDC5 in Breast Cancer"; Kamil Cebulski,Katarzyna Nowińska, Karolina Jablońska, Hanna Romanowicz, Beata Smolarz, Piotr Dzięgiel, Marzenna Podhorska-Okołów; 2022; Internationa Journal of Molecular Sciences;; mój udział polegał na opracowaniu koncepcji pracy, wykonaniu oceny IHC materiału badanego, współwykonaniu badań RT-PCR, analizie statystycznej wyników, przygotowaniu manuskryptu, oraz korekcie pracy przed złożeniem do druku.

Oświadczam, że w pracy "The Role of Irisin/FNDC5 Expression and Its Serum Level in Breast Cancer"; Kamil Cebulski, Aleksandra Piotrowska, Alicja Kmiecik, Katarzyna Haczkiewicz-Leśniak, Urszula Ciesielska, Jędrzej Grzegrzółka, Karolina Jabłońska, Hanna Romanowicz, Beata Smolarz, Piotr Dzięgiel, Marzenna Podhorska-Okołów, Katarzyna Nowińska; 2023; Internationa Journal of Molecular Sciences; mój udział polegał na opracowaniu koncepcji pracy, wykonaniu oceny IHC materiału badanego, współwykonaniu badań molekularnych, analizie statystycznej wyników, przygotowaniu manuskryptu, oraz korekcie pracy przed złożeniem do druku..

Podpis

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.....

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Wrocław, 12 maja 2023

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Podpis

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Aleksandra Piotrowska
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Katarzyna Nowińska
Podpis

Katarzyna Haczkiewicz-Leśniak

Zakład Badań Ultrastrukturalnych
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