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AGNIESZKA PINKOWSKA

ROLA IRYZYNY W RAKACH PŁASKONABŁONKOWYCH KRTANI

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Pragnę wyrazić wdzięczność mojej Promotor

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Spis treści

1. Wykaz publikacji stanowiących rozprawę doktorską

I. **Agnieszka Pinkowska**, Marzenna Podhorska- Okołów, Piotr Dzięgiel, Katarzyna Nowińska. The Role of Irisin in Cancer Disease. Cells 2021, 10, 1479.
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II. **Agnieszka Pinkowska**, Katarzyna Nowińska, Urszula Ciesielska, Marzenna Podhorska- Okołów. Irisin Association with Ki-67, MCM3 and MT-I/II in Squamous Cell Carcinomas of the Larynx. Biomolecules 2022, 12, 52.
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2. Wstęp

Rak płaskonabłonkowy krtani jest obecnie najczęstszym nowotworem okolicy głowy i szyi [1]. Typowo występuje on w populacji palących papierosy mężczyzn po 50 roku życia, z podstawowym wykształceniem i niskim statusem socjoekonomicznym. Obserwuje się 8- krotnie częstsze zachorowania wśród mężczyzn niż u kobiet. Rak krtani zajmuje 7 miejsce w grupie najczęściej rozpoznawanych nowotworów złośliwych w populacji męskiej. Współczynniki 5- letnich przeżyć i zgonów są również mniej korzystne w grupie mężczyzn [2,3]. Najnowsze wyniki badań wykazują, że poza typowym obrazem epidemiologicznym, coraz częściej rak krtani diagnozowany jest u pacjentów młodszych, poniżej 40 roku życia, nie palących, u których czynnikiem rozwoju nowotworu jest zakażenie wirusem brodawczaka typu ludzkiego HPV (ang. human papilloma virus) [4].

Wybór metody leczenia raka krtani jest uwarunkowany wieloma czynnikami, do których należą: pierwotna lokalizacja guza, występowanie przerzutów odległych, złośliwość histologiczna guza, wiek pacjenta i jego stan ogólny, a także doświadczenie zespołu chirurgicznego w konkretnych technikach zabiegowych [5,6]. We wczesnych stadiach zaawansowania nowotworu (T1-2, N0, M0) stosuje się radioterapię i leczenie chirurgiczne, obejmujące częściową laryngektomię endoskopową lub laryngektomię z dostępu otwartego [6]. W rakach, o wyższym stopniu zaawansowania narządowego wykonuje się laryngektomię całkowitą [7,8]. W Polsce blisko 60% przypadków raka krtani jest diagnozowanych w stadium T3-4 i ok. 50 % pacjentów w tej grupie ma przerzuty do regionalnych węzłów chłonnych [9]. Brak objawów patognomonicznych raka krtani i późne rozpoznanie są czynnikiem znacznie ograniczającym wybór metody leczenia i jej skuteczności. Laryngektomia całkowita pozostaje nadal złotym standardem postępowania terapeutycznego [7,8]. Zabieg radykalny odbiera pacjentowi zdolność porozumiewania się za pomocą głosu, co ma negatywny wpływ na jakość życia ale także utrudnia jego pełen powrót do zdrowia [10,11]. Opracowanie metod wczesnej diagnostyki raka płaskonabłonkowego krtani, opartych na swoistych markerach związanych z kancerogenezą, jest wyzwaniem stawianym przed współczesną onkologią. Ostatnie wyniki badań wskazują, że iryzyna, stosunkowo niedawno

odkryta adipomiokina, jest zaangażowana w procesy kancerogenezy wielu nowotworów, między innymi gruczołu tarczowego i płuc [12–14].

Iryzyna (Ir) została opisana po raz pierwszy jako hormon uwalniany do krwi przez tkankę mięśniową pod wpływem wysiłku fizycznego. Ir powstaje z prohormonu FNDC5, który u ludzi kodowany jest przez gen FNDC5. Wzrost ekspresji białka błonowego FNDC5 i dalsze modyfikacje posttranslacyjne domeny zewnątrzkomórkowej prohormonu FNDC5, skutkują uwolnieniem Ir. Wydzielona Ir ulega dimeryzacji lub występuje w postaci homodimeru [15]. Dalsze badania wykazały, że ekspresję Ir zaobserwowano także w innych tkankach i narządach, takich jak tkanka tłuszczowa, mięsień sercowy, nerki, wątroba, skóra oraz móżdżek [16–19]. Ponadto, obecność Ir wykazano także w guzach nowotworowych takich jak: rak gruczołu piersiowego, narządu rodnego, kości, płuc, i nowotworach przewodu pokarmowego [20–23], a także w surowicy krwi pacjentów z chorobami nowotworowymi [22,24–28]. Dodatkowo, obserwowano ekspresję Ir w komórkach podścieliska niedrobnokomórkowego raka płuc [12]. Obecnie prowadzone są liczne badania, które mają na celu określenie związku Ir z procesem transformacji nowotworowej.

W ostatnim czasie pojawiły się badania wykazujące związek między poziomem ekspresji Ir w komórkach nowotworowych, a parametrami kliniczno-patologicznymi. Zaobserwowano, że w komórkach niedrobnokomórkowego raka płuc ekspresja Ir spada w wyższych stopniach histologicznej złośliwości guza (G) i w guzach o większych rozmiarach (T). Natomiast w trakcie powstawania przerzutów do węzłów chłonnych, poziom ekspresji Ir jest wyższy w guzach z przerzutami odległymi niż w guzach z przerzutami do węzłów chłonnych węzłach regionalnych oraz w guzach bez przerzutów. U pacjentów z przerzutami odległymi wykazano wyższą ekspresję Ir niż u pacjentów bez przerzutów w podścielisku guzów NSCLC [12]. Powyższe badania wskazują na potencjalną rolę iryzyny w procesie diagnostyczno- terapeutycznym nowotworów.

Jak dotąd zespoły badawcze zajmujące się związkiem Ir z kancerogenezą uzyskały rozbieżne wyniki dotyczące jej wpływu na proliferację. Część badań wykazała, że Ir hamuje proliferację, migrację i inwazję komórek raka gruczołu piersiowego [29], płuc

[13], kości [30], gruczołu krokowego [31] w warunkach in vitro. Podczas gdy, w komórkach raka wątroby Ir stymuluje proliferację poprzez aktywację szlaku PI3K/Akt [32]. Z kolei, w raku endometrium, okrężnicy, tarczycy i przełyku nie wykazano wpływu Ir na proliferację [33]. W badaniu na rakach niedrobnokomórkowych płuc zaobserwowano odmienny wpływ Ir na proliferację w zależności od tego, w których komórkach była ekspresjonowana. Poziom Ir w komórkach raka płuc korelował negatywnie z poziomem uznanego markera proliferacji antygenu Ki-67. Natomiast poziom Ir w komórkach podścieliska guza korelował pozytywnie z antygenem Ki-67 [12]. Ponadto w raku piersi i raku okrężnicy także odnotowano słaba pozytywną korelację z markerami proliferacji Ki-67 i MCM3 [34,35]. Brak jednoznacznych danych o związku Ir z proliferacją nowotworową skłoniło mnie do zbadania tej zależności w płaskonabłonkowych rakach krtani.

Obiecujące wyniki badań dotyczące udziału Ir w procesie kancerogenezy i brak danych określających jej ekspresję w rakach płaskonabłonkowych krtani, były inspiracją do badań wykonanych przeze mnie w ramach pracy doktorskiej. Uzyskane wyniki nie wskazują jednoznacznie Ir jako predyktora w procesie diagnostyczno-terapeutycznym raka płaskonabłonkowego krtani. Jednakże korelacje Ir, z uznanymi markerami proliferacji nowotworowej, takimi jak Ki-67, MCM3 and MT-I/II, wskazują na jej potencjalną rolę w procesie transformacji nowotworowej tego typu guzów.

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3. Założenia i cel pracy doktorskiej

Najnowsze wyniki badań wspierają hipotezę o roli Ir w procesach proliferacji, migracji i przejścia epitelialno- mezenchymalnego w wielu typach nowotworów. Dotychczas nie pojawiły się badania oceniające ekspresję Ir w tkankach krtani i jej potencjalnej roli w kancerogenezie raka płaskonabłonkowego krtani (LSCC). Celem badań było określenie lokalizacji i nasilenia ekspresji Ir w LSCC oraz określenie związku jej poziomu z danymi kliniczno- patologicznymi (m. in. stopniem złośliwości histologicznej, stadium zaawansowania choroby, klasyfikacją TNM, długością przeżyć pacjentów). Wyniki uzyskanych badań miały pozwolić na określenie ewentualnego znaczenia ekspresji Ir w ocenie prognostycznej raka płaskonabłonkowego krtani.

Celem pierwszej publikacji cyklu- pracy poglądowej, był przegląd dostępnego piśmiennictwa, który pozwolił na opisanie budowy cząsteczki Ir, jej roli w procesach metabolicznych związanych z kancerogenezą wielu nowotworów, a także wpływu jaki Ir wywiera na proces nowotworzenia. Ponadto, w pracy dokonano usystematyzowanego przeglądu dostępnych danych literaturowych, dotyczących znaczenia Ir w poszczególnych typach nowotworów, w których badano poziom jej ekspresji i stężenia w surowicy pacjentów. Podsumowując, wyniki prac dotyczące poszczególnych typów nowotworów, zostały opisane w aspekcie: czy i w jaki sposób wykazują ekspresję Ir, jakiej metodologii użyto do wykonania badań, jaki materiał został wykorzystany do badań. W publikacji zaprezentowałam także wnioski wyciągnięte przeze mnie na podstawie rezultatów badań poszczególnych zespołów badawczych.

Wyniki badań i wnioski wyciągnięte w trakcie powstawania pracy poglądowej, a także brak doniesień literaturowych opisujących ekspresję Ir w rakach krtani, skłoniły mnie do wykonania eksperymentów, które byłyby próbą określenia potencjalnej roli Ir w procesie kancerogenezy raka płaskonabłonkowego krtani.

Badania przedstawione w drugiej publikacji cyklu- pracy oryginalnej, miały na celu ocenę lokalizacji i nasilenia ekspresji Ir w komórkach raka płaskonabłonkowego krtani, brodawczakach krtani i łagodnych zmianach przerostowych krtani. Do badań

wykorzystano materiał archiwalny Zakładu Histologii i Embriologii Katedry Morfologii i Embriologii Człowieka Uniwersytetu Medycznego we Wrocławiu, zgromadzony w postaci bloczków parafinowych LSCC. W celu oceny nasilenia ekspresji białek Ki-67, MCM3,5,7 oraz MT-I/II w komórkach raka krtani przeprowadzono reakcje immunohistochemiczne (IHC). Następnie dokonano oceny reakcji IHC, przy użyciu skali Remmele- Stegner oraz skali procentowej. Uzyskane wyniki określające nasilenie ekspresji Ir w komórkach LSCC, skorelowano z dostępnymi danymi kliniczno- patologicznymi (wiek, płeć, stopień złośliwości histologicznej i zaawansowania klinicznego TNM oraz czasem przeżycia pacjentów). Miało to na celu ocenę potencjału prognostycznego Ir. Wykonano także badania w modelu in vitro, wykorzystując linie komórkowe raka krtani HEp-2 oraz prawidłowych keratynocytów HaCaT. Nasilenie ekspresji Ir na poziomie białka oszacowano za pomocą techniki Western blot i reakcji immunofluorescencyjnej, co miało potwierdzić wyniki badań przeprowadzonych na materiale archiwalnym LSCC.

4. Streszczenie

Po upływie dekady od odkrycia i opisania iryzyny (Ir), nasza wiedza o jej roli w procesie kancerogenezy pozostaje nadal niepełna. W chwili obecnej prowadzonych jest wiele badań, mających na celu określenie wpływu Ir na proliferację, migrację i przejście epitelialno- mezenchymalne komórek raka, leżące u podstawy przerzutowania nowotworów. W literaturze dostępne są wyniki badań immunohistochemicznych, molekularnych i w modelu in vitro dotyczących roli Ir w najczęściej występujących w populacjach nowotworów (rak płuca, rak gruczołu piersiowego i wielu innych).

Przegląd piśmiennictwa dotyczący budowy Ir, funkcji jaką pełni w procesach metabolicznych, a zwłaszcza rolę jej ekspresji w procesach nowotworzenia został przedstawiony w pracy poglądowej opublikowanej w czasopiśmie Cells (Pinkowska i wsp. The Role of Irisin in Cancer Disease. Cells 2021, 10, 1479).

Ir jest adipomiocytokiną, zaangażowaną w regulację procesów metabolicznych. Wpływa także na procesy związane ze stanem zapalnym, obserwowanym w wielu chorobach przewlekłych, w tym nowotworowych. Obecność Ir wykazano w komórkach nowotworowych oraz surowicy krwi pacjentów, w różnych typach nowotworów. Wyniki wielu badań wskazują na związki Ir z kancerogenezą nowotworów (m.in.: raka gruczołu piersiowego, narządu rodnego, kości, płuc, gruczołu krokowego czy nowotworów przewodu pokarmowego). Ponadto, dowiedziono, że Ir hamuje proliferację, migrację i inwazję komórek raka gruczołu piersiowego, płuc, kości, gruczołu krokowego w warunkach in vitro. Jest także zaangażowana w hamowanie przejścia epitelialno- mezenchymalnego (EMT), które jest związane z powstawaniem przerzutów nowotworowych.

W dostępnej literaturze pojawiło się, jak dotąd, niewiele badań wykazujących związek ekspresji Ir z parametrami kliniczno- patologicznymi w nowotworach człowieka. Ponadto, brakowało danych z badań prowadzonych na materiale raka płaskonabłonkowego krtani. Dlatego przedmiotem mojej pracy doktorskiej była próba określenia roli Ir w procesach kancerogenezy raka płaskonabłonkowego krtani.

Do badań opublikowanych w pracy oryginalnej (Pinkowska i wsp. Irisin Association with Ki-67, MCM3 and MT-I/II in Squamous Cell Carcinomas of the Larynx. Biomolecules 2022, 12, 52) zebrano materiał od pacjentów, diagnozowanych i leczonych na oddziale laryngologii Oddziału Patomorfologii Wojewódzkiego Szpitala im. J. Babińskiego we Wrocławiu i Katedry i Kliniki Otolaryngologii, Chirurgii Głowy i Szyi, Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu w latach 1997-2003. Materiał do badań obejmował:

1) 140 przypadków guzów raka płaskonabłonkowego krtani

2) 57 przypadków brodawczaków krtani

3) 14 przypadków łagodnych zmian przerostowych (guzy śpiewacze i obrzęki Reinkego)- materiał kontrolny.

Wycinki pobrane od pacjentów zostały utrwalone w postaci bloczków parafinowych. Na podstawie klasyfikacji TNM zatwierdzonej przez Międzynarodową Unię do Walki z Rakiem (ang. UICC- The International Union Against Cancer) został określony stopień złośliwości (G) oraz stadium zaawansowania klinicznego LSCC.

Z bloczków parafinowych wykonano mikromacierze tkankowe (ang. TMA - Tissue microarrays). W celu oceny poziomu ekspresji badanych białek w komórkach raka krtani i zmian łagodnych przeprowadzono na skrawkach parafinowych reakcje immunohistochemiczne (IHC) z wykorzystaniem poliklonalnych przeciwciał króliczych skierowanych przeciwko Ir i mysich monoklonalnych skierowanych przeciwko markerom proliferacji: Ki-67, MT-I/II, MCM2, 3, 5 i 7.

Na wykonanych preparatach obserwowano obecność pozytywnej reakcji IHC przy użyciu mikroskopu świetlnego BX41 (Olympus, Tokyo, Japan). Obecność Ir i MT-I/II zaobserwowano w cytoplazmie. Poziom białek został oceniony przy użyciu metody półilościowej IRS wg. Remele i Stegner. Natomiast ekspresję markerów proliferacji Ki-67, MCM2, 3, 5 i 7 obserwowano w jądrze komórkowym. Do oceny ww. markerów proliferacji użyto skali procentowej. Otrzymane wyniki poddano analizie statystycznej i korelacji z danymi kliniczno- patologicznymi.

W celu potwierdzenia wyników badań IHC przeprowadzono badania molekularne w modelu in vitro. Badania ekspresji Ir wykonano przy użyciu:

1) komercyjnej linii komórek raka płaskonabłonkowego krtani 2 (HEp-2),

2) linii komórkowej ludzkich keratynocytów (HaCaT) - materiał kontrolny.

W celu określenia poziomu ekspresji Ir w wyżej wymienionych komórkach wykorzystano metodę Western blot oraz reakcje immunofluorescencjne (IF) z wykorzystaniem mikroskopu konfokalnego

Ocena reakcji IHC wykazała ekspresję Ir w obrębie łagodnych zmian przerostowych nabłonka krtani, brodawczaków, a także w cytoplazmie komórek raka płaskonabłonkowego krtani. Poziom ekspresji Ir był wyższy u pacjentów z LSCC w porównaniu do grupy kontrolnej ($p=0.001$). Ponadto, zaobserwowano także wyższą ekspresję Ir w LSCC niż w brodawczakach ($p<0.0001$). Otrzymane wyniki badań skorelowano z danymi kliniczno-patologicznymi. Średni poziom ekspresji Ir był wyższy w stadium I zaawansowania niż w stadium II guza i ponownie rósł w stadiach III-IV. Jednakże, istotna statystycznie różnica była obserwowana tylko między stadium II, a III-IV ($p=0.0083$). Zaobserwowano również podwyższony poziom ekspresji Ir wraz ze wzrostem wielkości guza (T), a różnica między T1-2 i T3-4 była istotnie statystyczna ($p=0.0348$). Poziom ekspresji Ir w LSCC nieznacznie rósł wraz ze wzrostem złośliwości nowotworu (G). Jednakże, obserwowane różnice nie wykazywały istotności statystycznej. Zauważono także związek między poziomem ekspresji Ir, a przerzutami do węzłów chłonnych. Najwyższą średnią wartość ekspresji Ir zaobserwowano w grupie pacjentów bez przerzutów do węzłów chłonnych (N0). W przypadkach z przerzutami do węzłów chłonnych wnękowych i śródpłucnych (N1) była ona najniższa i ponownie rosła w przypadkach z przerzutami do węzłów śródpiersiowych (N2-3). Istotne statystycznie były różnice między poziomami Ir w N0 i N1 ($p =0.0031$), a także pomiędzy N0 i N2-3 ($p=0.0457$). Nie zaobserwowano związku między długością przeżycia całkowitego (OS), a poziomem Ir u pacjentów z LSCC. W jądrach komórek LSCC wykazano także ekspresję markerów proliferacji Ki-67, MCM2, 3, 5 i 7, a ekspresję MT-I/II w cytoplazmie komórek LSCC. Średnią dodatnią korelację zaobserwowano pomiędzy poziomem ekspresji Ir, a

antygenu Ki-67 (r=0.36; p<0.0001). Ponadto, słabą dodatnią korelację wykazano między poziomami ekspresji Ir i MCM3 (r=0.25; p=0.0033). Średnią dodatnią korelację zaobserwowano również między poziomem ekspresji Ir, a MT-I/II (r = 0.35; p<0.0001) w LSCC. Natomiast, prezentowane w pracy badania przeprowadzone w modelu in vitro, za pomocą metody Western blot i reakcji immunofluorescencyjnej, wykazały obecność ekspresji Ir w obu liniach komórkowych. Wyższy poziom ekspresji Ir odnotowano w komórkach raka krtani HEp-2 niż w kontrolnych komórkach HaCat. Różnica nie była istotna statystycznie.

Przedstawione wyniki badań są pierwszymi badaniami wykazującymi obecność Ir w LSCC. Pokazują wyższą ekspresję Ir u pacjentów z LSCC w porównaniu do grupy kontrolnej (bez zmian nowotworowych). Wykazują także jej związek ze wzrostem guza i proliferacją komórek raka. Ponadto, niezwykle interesujący jest obserwowany wyższy poziom Ir w grupie pacjentów N0 niż w grupie N1. Może to wskazywać na potencjalną rolą Ir w procesie przerzutowania komórek nowotworowych. Porównanie ekspresji Ir z czynnikami kliniczno-patologicznymi oraz uznanymi markerami proliferacji wskazuje, na możliwą rolę Ir zarówno w procesie transformacji nowotworowej, jak również progresji choroby LSCC.

5. Summary

One decade after the discovery and description of irisin (Ir), the knowledge of its role in carcinogenesis has still been incomplete. Currently, many studies are conducted to determine the effects of Ir on proliferation, migration and epithelial-mesenchymal transition (EMT) of cancer cells underlying metastasis. Immunohistochemical, molecular and *in vitro* model study results connected with the role of Ir in the most prevalent cancers (i.e. lung, breast and many other cancers) are available in the literature.

A review of the literature on the structure of Ir, the function it plays in metabolic processes, and particularly the role of its expression in carcinogenesis was presented in a review paper published in *Cells* (Pinkowska et al., The Role of Irisin in Cancer Disease. Cells 2021, 10, 1479).

Irisin is an adipomyokine involved in the regulation of metabolic processes. It also influences processes related to inflammation in many chronic diseases, including cancer. The presence of Ir has been demonstrated in cancer cells and the blood serum of patients in various types of cancer. The results of many studies indicate that Ir is associated with the carcinogenesis of cancers (including breast, reproductive organ, bone, lung, prostate or gastrointestinal cancers). In addition, Ir has been proven to inhibit proliferation, migration and invasion of breast, lung, bone and prostate cancer cells under *in vitro* conditions. It is also involved in the inhibition of epithelial-mesenchymal transition (EMT), which is associated with metastasis.

In the available literature, there have been only some studies demonstrating the association of Ir expression with clinical and pathological parameters in human cancers. In addition, there was no data from studies on laryngeal squamous cell carcinoma (LSCC). Therefore, the subject of the dissertation was an attempt to determine the role of Ir in the processes of carcinogenesis of LSCC.

For the original paper, the material was collected from patients diagnosed and treated at the Department of Pathomorphology of the J. Babinski Regional Hospital of Wroclaw and the Department and Clinic of Otolaryngology, Head and Neck Surgery, Wroclaw Medical University between 1997 and 2003 (Pinkowska et al., Irisin Association with Ki-67, MCM3 and MT-I/II in Squamous Cell Carcinomas of the Larynx. Biomolecules 2022, 12, 52).

The study material included:

1) 140 cases of LSCC

2) 57 cases of laryngeal papillomas

3) 14 cases of benign hypertrophic changes (vocal cord nodules and Reinke's edema) -control material.

The specimens obtained from patients were fixed in paraffin blocks. Based on the TNM classification approved by the International Union Against Cancer (UICC), the grade (G) and the clinical stage of LSCC were determined.

Tissue microarrays (TMAs) were performed from paraffin blocks. To assess the expression levels of the proteins in laryngeal cancer cells and benign changes, immunohistochemical (IHC) reactions were performed on paraffin sections using polyclonal anti-irisin rabbit antibodies and mouse monoclonal antibodies against proliferation markers, such as Ki-67, MT-I/II, MCM2, 3, 5 and 7.

A positive IHC reaction of Ir was observed using the BX41 light microscope (Olympus, Tokyo, Japan). The presence of Ir and MT-I/II was observed in the cytoplasm. Protein levels were assessed using the semi-quantitative IRS method according to Remele and Stegner. In turn, the expression of proliferation markers, such as Ki-67, MCM2, 3, 5 and 7, was observed in the cell nucleus. A percentage scale was used to evaluate the above proliferation markers. The obtained results underwent statistical analysis and correlation with clinicopathological data.

To confirm the IHC results, molecular studies were performed in an *in vitro* model. Ir expression studies were performed using:

1) a commercial laryngeal cancer cell line Hep-2

2) human keratinocyte cell line (HaCaT) - control.

Western blot and immunofluorescence (IF) reactions using confocal microscopy were used to determine the level of Ir expression in the above-mentioned cells.

Evaluation of IHC reactions showed Ir expression in benign hypertrophic changes of laryngeal epithelium, papillomas, as well as in the cytoplasm of LSCC cells. The level of Ir expression was higher in LSCC patients compared to controls ($p=0.001$). In addition,

higher Ir expression was also observed in LSCC than in papillomas ($p<0.0001$). The obtained findings were correlated with clinicopathological data. The mean level of Ir expression was higher in stage I than in stage II tumors and increased in stages III-IV. However, a statistically significant difference was observed only between stage II and III-IV ($p=0.0083$). Elevated Ir expression levels were also found with increasing tumor size (T), and the difference between T1-2 and T3-4 was statistically significant ($p=0.0348$). The level of Ir expression in LSCC slightly increased with increasing tumor malignancy (G). However, the differences did not show statistical significance. An association was also found between the level of Ir expression and lymph node metastasis. The highest mean value of Ir expression was noted in the group of patients without lymph node metastasis (N0). It was lowest in cases with hilar and mediastinal lymph node metastasis (N1) and increased again in cases with mediastinal node metastasis (N2-3). There were statistically significant differences between Ir levels in N0 and N1 ($p=0.0031$), as well as between N0 and N2-3 ($p=0.0457$). No association was observed between overall survival (OS) and Ir levels in LSCC patients. The nuclei of LSCC cells also showed expression of the proliferation markers, such as Ki-67, MCM2, 3, 5 and 7, and expression of MT-I/II in the cytoplasm of LSCC cells. The mean positive correlation was observed between the expression level of Ir and Ki-67 antigen ($r=0.36$; $p<0.0001$). In addition, a weak positive correlation was shown between Ir and MCM3 expression levels ($r=0.25$; $p=0.0033$). A moderate positive correlation was also found between Ir expression levels and MT-I/II ($r = 0.35$; $p<0.0001$) in LSCC. In turn, the study conducted in an *in vitro* model using Western blot and immunofluorescence reaction showed the presence of Ir expression in both cell lines. Higher levels of Ir expression were noted in HEp-2 laryngeal cancer cells than in control HaCaT cells. The difference was not statistically significant.

The presented results are the first studies showing the presence of Ir in LSCC. They demonstrate higher Ir expression in LSCC patients compared to controls (without cancer). They also show its association with tumor growth and cancer cell proliferation. In addition, compared to the N1 group, a higher level of Ir in the N0 patient group is extremely interesting. This may indicate a potential role for Ir in the process of cancer cell metastasis. The comparison of Ir expression with clinicopathological factors and proliferation markers indicates a possible role of Ir in the process of tumor transformation and progression of LSCC

6. Publikacje

Publikacje będące podstawą rozprawy doktorskiej

I. **Agnieszka Pinkowska**, Marzenna Podhorska- Okołów, Piotr Dzięgiel, Katarzyna Nowińska. The Role of Irisin in Cancer Disease. Cells 2021, 10, 1479. https://doi.org/10.3390/cells10061479

II. **Agnieszka Pinkowska**, Katarzyna Nowińska, Urszula Ciesielska, Marzenna Podhorska- Okołów. Irisin Association with Ki-67, MCM3 and MT-I/II in Squamous Cell Carcinomas of the Larynx. Biomolecules 2022, 12, 52. https://doi.org/10.3390/biom12010052

cells

MDPI

Review

The Role of Irisin in Cancer Disease

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Abstract: Irisin (Ir) is an adipomyokine that is involved in the regulation of metabolic processes. It also influences processes related to inflammation, including cancer. Initially, Ir was considered a hormone secreted by skeletal muscles in response to physical exercise. Further studies showed that Ir is also present in other healthy tissues, organs, and plasma. It influences the change in phenotype of white adipose tissue (WAT) into brown adipose tissue (BAT). It increases mitochondrial biogenesis and affects the expression of thermogenin (UCP1). This adipomyokine has also been found in many tumor tissues and in the serum of cancer patients. Studies are underway to determine the association between Ir and carcinogenesis. It has been confirmed that Ir inhibits in vitro proliferation, migration, and invasion. It is involved in the inhibition of epithelial–mesenchymal transition (EMT). Additionally, Ir affects the expression of the transcription factor Snail, which is involved in EMT, and inhibits transcription of the gene encoding E-cadherin, which is characteristic of epithelial-derived cells. Many studies have been performed to determine the role of Ir in physiological and pathological processes. Further detailed studies should determine more precisely the effect of Ir on the body in health and disease.

Keywords: Irisin; FNDC5; cancer; proliferation; migration; epithelial–mesenchymal transition

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1. Introduction

Irisin (Ir) was first described in 2012 as a hormone released into the blood by skeletal muscles in response to physical exercise. In their study, Boström et al. [1] found that an increase in the expression of fibronectin type III domain-containing protein 5 (FNDC5, a membrane protein) occurs under the influence of physical exercise in the muscle tissue. Further transformation of FNDC5 results in the formation of a new protein, known as Ir. This process is controlled by the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α). Boström et al. [1] assumed with high probability that other tissues could also be involved in the secretion of Ir. Multidirectional studies on Ir confirmed the primary assumptions of its discoverers. In addition to the primary localization, the expression of Ir has been found in other tissues and organs, i.e., in the adipose tissue [2,3], cardiomyocytes [4], kidney [4], liver [4], skin [4], and cerebellum [5].

FNDC5 is the precursor of Ir. In humans, this prohormone is encoded by the *FNDC5* gene, which is located on chromosome 1 at position 35.1 (1p35.1). The *FNDC5* gene consists of six exons and five introns and spans 8.47 kbp [6]. Expression of this gene occurs under the influence of peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α), and the FNDC5 protein (also known as Frcp2 and PEP) is the product of its expression (Figure 1). The mass of the FNDC5 protein ranges from 20 to 32 kDa, and this difference is related to post-translational modification [1]. FNDC5 is composed of a 29-amino-acid signal peptide, a 94-amino-acid fibronectin type III domain, a 28-amino-acid portion of unknown

function (most likely Ir proteolytic cleavage site), a 19-amino-acid transmembrane domain, and a 39-amino-acid cytoplasmic domain [6,7].

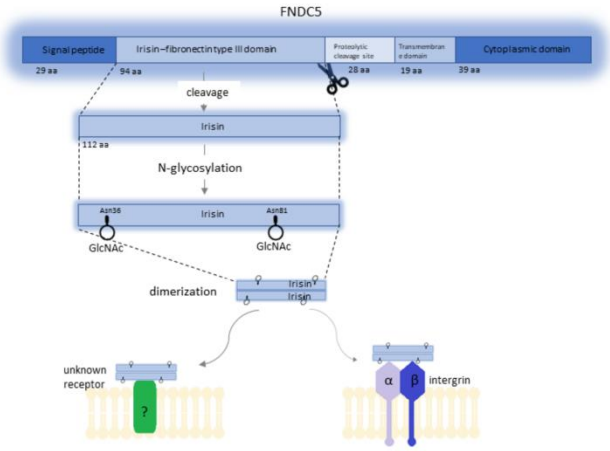

Figure 1. Structure of FNDC5 and Irisin. FNDC5 is composed of a 29-amino-acid signal peptide, a 94-amino-acid fibronectin type III domain, a 28-amino-acid domain (potential site of proteolytic cleavage), a 19-amino-acid transmembrane domain, and a 39-amino-acid cytoplasmic domain. The diagram also shows the glycosylation site of Irisin by the formation of a bond between *N*-acetylglucosamine and the nitrogen originating from the amide group of asparagine (Asn36 and Asn81) and subsequent dimerization of Irisin molecules. Irisin is a ligand for the integrin receptor. Perhaps, it also works by attaching to another unknown membrane receptor.

Post-transcriptional processing of FNDC5 results in the formation of four Ir isoforms via alternative splicing. Isoform 1 (Q8NAU1-1) is used as a canonical sequence. The other three isoforms, isoform 2 (Q8NAU1-2), isoform 3 (Q8NAU1-3), and isoform 4 (Q8NAU1-4), have missing sequences of amino acids at positions 182–212, 1–75, and 1–75, respectively [8]. In addition, biochemical studies have shown that Ir can exist as a homodimer, and the FNIII-like domain forms a continuous intersubunit β-sheet dimer [9]. Ir is formed as a result of further modifications due to cleavage and glycosylation of the extracellular domain of FNDC5 and contains 112 amino acids [1]. The molecular mass of Ir is estimated at 12 kDa, although studies suggest that glycosylated Ir ranging from 20 to 32 kDa is also secreted [3]. Initial studies showed remarkable conservation of Ir in mammals, which, according to Boström et al. [1], implied its conserved function mediated by a cell surface receptor. Further studies showed that human *FNDC5* is a gene with an atypical start codon to ATA (encoding isoleucine) instead of ATG (encoding methionine), which is present in the gene in animals. According to these researchers, using this noncanonical start site is associated with the generation of full-length protein, whereas the start of translation at the ATG start codon results in the formation of a truncated isoform of Ir [10]. The occurrence of Ir, which is secreted by skeletal muscles into human and mice plasma as a result of physical effort, and its potential influence on metabolism have generated much controversy. The criticism was mostly related to the research methods. Erikson [7] indicated that several studies used antibodies that were irrelevant to Ir. Boström et al. [1] used a polyclonal antibody against a peptide corresponding to C-terminal amino acids of the human FNDC5 (transmembrane segment) with no sequence from the Ir peptide. Similar objections were reported by Albrecht et al. [11] who paid attention to the variety of available assays for detecting and quantifying serum Ir. Due to their use, many studies were published in which

serum Ir levels ranged from 0.01 to over 2000 ng/mL. Albrecht et al. [11] demonstrated that the antibodies they analyzed in their study showed clear cross-reactions with proteins other than Ir. Considering these concerns, some doubts occurred whether Ir was released into the plasma following physical effort and whether it could be assigned physiological functions. Jędrychowski et al. [12] performed the identification and quantitative assessment of human Ir. Studies using mass spectrometry (MS) showed that human Ir was present and was secreted into the circulation in association with physical effort. The quantitative levels of human Ir circulating in the blood were also determined. Consideration was given to the differences in concentration levels related to sedentary individuals (~3.6 ng/mL) and those who underwent training (~4.3 ng/mL). The analysis using mass spectrometry was also performed by Albrecht et al. [11] who detected a peptide that corresponded to FNDC5 or Ir. Of note, its apparently low level at the detection limit of the tested antibodies makes a physiological role of Ir very unlikely

In addition to the reservations of Albrecht et al. [11], our doubts were also raised by the presentation of the study results. Serum Ir levels of healthy controls and cancer patients were presented in different units, i.e., μg/mL [13], pg/mL [14], and ng/mL [15]. Such inconsistency makes it difficult to compare the results of different research teams. The significant distribution of Ir levels may be influenced by the selection of the study group in terms of the physical activity of the subjects, type, body shape, comorbidities, and the way of sample collection and storage, which could be an additional factor affecting the stability of serum Ir.

Many papers showed that Ir level increases with physical effort [1,16–18]. However, other studies did not show a strong relationship between physical effort and the level of circulating Ir [19–21]. There are also papers showing a baseline difference in Ir levels between physically active and inactive subjects [12], as well as between trained and untrained individuals [22]. The form and the duration of physical exercise are also of significance. Significant increases in serum Ir were found in response to vigorous physical effort after 1 week of training. However, no effect of vigorous physical exercise was reported after 8 weeks of training [16]. The analysis of 74 studies from the MEDLINE database conducted by Fatouros [23] showed that strength and endurance exercise represented a potent stimulus for release of Ir if this exercise was characterized by adequate intensity and duration. Animal studies suggested that Ir levels could also increase in response to systematic training of low intensity.

However, most human studies have produced contradictory results. According to the above research, the results might also be affected by the methodology of Ir measurement, age of subjects, their conditioning status, and exercise intensity. The half-life and physicochemical properties of Ir are determined by post-translational modifications, including glycosylation. Glycosylation is one of the most common post-translational modifications, and most membrane and secretory proteins undergo such modification. It involves the enzymatic attachment of sugar residues via a glycosidic bond [24]. Tissue-specific and cell-specific enzymes of the endoplasmic reticulum and the Golgi apparatus participate in the glycosylation process. Proteins can be modified in the process of *N*-glycosylation and/or *O*-glycosylation [25]. The *N*-glycans and *O*-glycans formed by glycosylation affect the physicochemical properties of proteins, which determines their role in metabolic processes [26]. *N*-Linked glycoproteins are created by the formation of a glycosidic bond between *N*-acetylglucosamine (GlcNAc) and the nitrogen originating from the amide group of asparagine (Asn) in the sequence Asn–X–Ser/Thr (X being any amino acid except proline) [24,25]. In oncogenesis, abnormal glycosylation is one of the key factors in tumor development. Glycopeptides are involved in cancer cell signaling, migration, invasion, and metastasis. In addition, they are involved in the relationship between the cell and the extracellular matrix. They participate in angiogenesis and influence immune cells [27]. The process of glycosylation of Ir is poorly understood. Nie et al. [28] showed that FNDC5 is an *N*-glycan with two potential glycosylation sites (Asn36 and Asn81). In the same study, they observed that inhibition of glycosylation decreased Ir secretion, reducing the stability

of FNDC5 and its half-life [28]. Glycosylation also increases the molecular mass of Ir [29]. Many research teams attempted to determine the role of Ir in metabolic processes, its impact on tissues, and its potential influence on carcinogenesis. These processes may depend on the course of Ir glycosylation. Different and sometimes contradictory study results related to the effect of Ir on cells in the vitro model may also depend on whether glycosylated or non-glycosylated Ir was used in studies. The comparison of study results of selected research teams (Table 1) shows that researchers did not always accurately determine the form of Ir. Ganon et al. [30] and Shi et al. [31] indicated that such a distinction is essential for interpreting study results.

2. Irisin as a Ligand for Integrins

Due to the short half-life of Ir in the serum (about 20 min), Boström et al. [1] suggested that Ir could interact through a cell surface receptor. The ability of Ir to form homodimers [9] supports this hypothesis. Many studies show Ir as a protein that transfers information between the muscle tissue and other tissues. Its role ideally corresponds to its name, which is derived from the Greek goddess Iris, who was the messenger of the gods. Ir was shown to increase the mass of bone [32]. It prevents bone mass loss and influences bone healing in mice [33]. It also positively affects the mechanisms responsible for bone metabolism in mice [34]. Research showing the effects of Ir on bone tissue resulted in a study on its receptor.

Integrins are transmembrane proteins composed of α/β heterodimers, which are bidirectionally activated by the cell membrane. Physiological processes involved in integrin activation and ligand attachment determine cellular homeostasis. Abnormal activation under pathological conditions allows cell migration to tissues and the extracellular matrix, which initiates inflammatory processes and carcinogenesis [35].

Kim et al. [36] described the Ir receptor as a subset of integrin complexes. Ir was bound to several integrin complexes and showed the highest affinity for $\alpha V/\beta 5$ integrin, which was also confirmed by hydrogen–deuterium exchange/mass spectrometry (HDX/MS). Furthermore, Ir activated integrin receptor-specific signaling, including focal adhesion kinase (FAK), within 1 min of Ir being added to osteocytes. Moreover, integrin inhibitors or antagonistic antibodies directed against integrin $\alpha V/\beta 5$ inhibited Ir signaling and its further gene expression. Kim et al. [36] demonstrated that the Ir receptor formed a complex of integrins, especially those containing αV integrin, at least in osteocytes and the adipose tissue. Estel et al. [37] also showed that $\alpha_V\beta_5$ integrins acted as a receptor for Ir on osteocytes. The expression of $\alpha_V\beta_5$ subunits increased in osteoclast cultures after administration of Ir, and blocking the integrin complex with a neutralizing antibody completely suppressed the activating effect of Ir on osteoclastogenesis. Oguri et al. [38] showed that CD81 formed complexes with $\alpha_V\beta_1$ and $\alpha_V\beta_5$ integrins, mediating the activation of integrin–FAK signaling in response to Ir. CD81 molecules are markers of adipocyte progenitor cells (APCs) and are involved in cold-induced brown adipose tissue lipogenesis, and their loss causes glucose intolerance and insulin resistance. Bi et al. [39] found that Ir restored the intestinal barrier function, which is lost due to ischemia, by binding to the $\alpha_V\beta_5$ integrin receptor and activating the AMPK–UCP2 pathway. In the same study, immunofluorescence staining revealed the colocalization of Ir and $\alpha_V\beta_5$ integrin after administration of Ir to cells under hypoxia/reoxygenation conditions. According to Park et al. [35], the above studies demonstrate that Ir is an $\alpha_V\beta_5$ ligand and, thus, exerts its effects on tissues. However, further studies are warranted to identify other membrane receptors for Ir.

3. Irisin as a Coordinator of Metabolic Processes

Exercise-induced Ir results in changes in the white adipose tissue (WAT) and induces browning [1]. WAT, as an energy reservoir, is mainly a store of triglycerides, whereas brown adipose tissue (BAT) is responsible for energy expenditure [26]. Uncoupling is a process in which energy is released during the oxidation of respiratory substrates in mitochondria. The uncoupling protein (UCP), also known as thermogenin, is a specific marker of BAT.

UCPs are present in the inner mitochondrial membrane of all eukaryotic cells and are protein complexes that function as proton pumps. By dissipating energy and releasing it in the form of heat, UCP1 participates in the control of cell energy metabolism [40]. The function of BAT is based on the conversion of energy provided with food into heat energy.

Heat generation, known as adaptive non-shivering thermogenesis, is controlled by the adrenergic system and is related to the adaptation to life in cold climate conditions in the case of hibernating and newborn mammals, including human neonates. Under certain conditions (e.g., reduced temperature), it may also serve as a tool for regulating metabolism. In active BAT, large amounts of glucose and lipids are combusted, and the energy is dissipated in the form of heat. This is of great physiological importance to the body due to its potential role as a natural mechanism for weight control [41]. BAT has a beneficial effect on metabolism, whereas the traditionally perceived role of WAT is related to energy storage and fatty-acid release. However, its metabolic function in the body is more complex. WAT is essential for normal glucose homeostasis. It is involved in producing proinflammatory cytokines, some of which are involved in lipid metabolism, while others are involved in vascular homeostasis. Its hormonal activity (secretion of leptin, adiponectin, angiotensin, IL-6, resistin, and TNF-α) induces insulin resistance, which is responsible for the development of type II diabetes, thus linking diabetes to obesity. It promotes the development of polymetabolic syndrome, hypertension, and hypercholesterolemia, which results in cardiovascular complications, and it promotes cancer formation [42,43].

Transcription factors of the peroxisome proliferator-activated receptor (PPAR) family are involved in adipose tissue differentiation. The participation of PGC1α (a coactivator of PPARγ-1 α), which is a transcription factor that controls UCP1 expression, is required for BAT formation. PGC1α is induced in the muscle tissue by physical exercise. Boström et al. [1] conducted a study that showed WAT browning (increase in UCP1 mRNA expression of adipose cells) in mice subjected to physical activity. The results were confirmed in vitro using media conditioned by myocytes expressing PGC1α. Further studies showed an increase in FNDC5 mRNA expression, which was induced by exercise and a significant increase in UCP1 mRNA induction in BAT cell cultures under the influence of FNDC5 compared to the osteogenic protein (BMP-7), which was previously considered an inducer of browning. Physical exercise also affects the hippocampus by regulating the expression of the brain-derived neurotrophic factor (BDNF). This factor is a regulator of neuronal survival and neurogenesis in adults [44].

Wrann et al. [45] showed that PGC1α overexpression increased *FNCD5* gene expression in neurons. The increase in *FNDC5* expression depends on the formation of the PGC1α transcriptional complex with estrogen-related receptor alpha (ERRα). The researchers found that the expression of *BDNF*, *FNDC5*, and *ERRα* increased in the hippocampus due to exercise and showed that FNDC5 was a regulator of *BDNF* gene expression in neurons. Furthermore, they showed that BDNF in a feedback loop negatively regulated *FNCD5* expression. Immunohistochemical (IHC) studies also showed an increase in UCP1-positive adipocytes due to FNDC5 [1].

WAT browning induces the formation of the beige adipose tissue, which shows UCP1 expression and phenotypically and functionally resembles BAT. It is involved in controlling body temperature. It influences glucose and lipid metabolism, as well as energy homeostasis. Additionally, it has endocrine functions [46]. Ir, which is secreted in response to physical effort and stimulates an increase in UCP1 expression and its metabolic sequelae, may play an essential role in maintaining metabolic homeostasis of the body. It improves glucose homeostasis, lipid profile, and metabolic parameters. It is a promising predictive marker of insulin resistance [47]. The effect of Ir on diseases associated with polymetabolic syndrome has been widely studied, and its detailed presentation is beyond the scope of this paper.

4. Irisin in Cancer Proliferation Process

The aim of cancer cells is to form a tumor and create expansive forms capable of metastasizing to other areas of the body. This is possible due to the potential of tumor cells for unlimited growth, angiogenesis, or the inhibition of apoptosis. Many changes occur in tumor cells during neoplastic transformation. These changes determine growth and division and occur in the tumor microenvironment [48]. Tumor cells are characterized by increased metabolism. Rapidly proliferating normal cells and cancer cells prefer anaerobic harvesting of energy, converting glucose to lactate, even in the presence of oxygen. This phenomenon is known as the Warburg effect [49]. An alternative way of obtaining energy is provided by activated carcinoma-associated fibroblasts (CAFs) in the tumor stroma. Under oxidative stress, they provide tumor cells with the necessary substrates for anabolic processes using the Warburg effect. Due to the supplied substrates, cancer cells produce energy, mainly through aerobic respiration. This phenomenon is known as the reverse Warburg effect [50]. Ir influences cancer cell proliferation. Nowinska et al. [51] showed that Ir expression was higher in stromal cells of non-small-cell lung cancer (NSCLC) and increased in tumors with higher malignancy and higher staging, which could affect the proliferation of NSCLC cancer cells.

The energy that is produced in the process of cellular respiration is necessary for cell growth, migration, and differentiation, as well as the maintenance of constant body temperature. Glucose is the essential substrate for these processes. Due to proto-oncogene mutations and altered signaling pathways, tumor cells inhibit differentiation and use increased glucose requirements mainly for survival, growth, and proliferation [50]. The anaerobic respiration and high energy demand of tumor cells result in enhanced glycolysis and increased glucose uptake by the cells, which is mediated by glucose transporters known as GLUTs. Overexpression of membrane glucose transporters, including GLUT1, has been observed in many malignancies, including breast, colorectal, salivary, and gastric cancers [52]. Serine threonine kinase Akt, which is the main effector of phosphatidylinositol 3-kinase PI3K, plays an essential role in modifying cancer cell metabolism. Akt affects GLUT1 expression through activation of mammalian target of rapamycin kinase (mTOR kinase) [53]. Previous studies on mice showed that Ir increased glucose tolerance and uptake, as evidenced by GLUT4 translocation in skeletal muscle of diabetic mice. Ir enhanced glucose utilization by increasing 5'AMP-activated protein kinase (AMPK) phosphorylation in myocytes and hepatocytes of diabetic mice in in vitro and in vivo studies [54]. Uncontrolled tumor growth results in impaired blood supply and causes hypoxia. Hypoxia-induced factors are produced under hypoxic conditions, including the protein HIF-1α, which mediates many adaptive responses aimed at cell survival. HIF-1α increases vascular endothelial growth factor (VEGF) expression by affecting the intensification of neoangiogenesis and increased vascular permeability in the tumor. Under anaerobic conditions, HIF-1α promotes the activation of oxygen-independent metabolic pathways, including glycolysis by stimulating the expression of glucose transporters, which are crucial for increased glucose uptake [55].

Activation of the PI3K/Akt pathway in an mTOR-dependent or -independent manner influences an increase in HIF-1α expression [53]. Gaggini et al. [56] showed that FNDC5 mRNA overexpression in hepatocellular carcinoma (HCC) cells was associated with increased gene expression of mediators of lipogenesis, transcription factors involved in tumorigenesis, and proinflammatory cytokines, including TNF-α and IL-6. It was also found that, in patients with HCC and tumor-enhanced lipogenesis, increased paracrine production of FNDC5/Ir could compensatively inhibit lipid synthesis. Altay et al. [57] reported a significantly increased FNDC5 expression in WAT and BAT in mice with induced gastric cancer. Cancer development corresponded to an increase in FNDC5 expression in the adipose tissue. In addition, a significant increase in serum Ir levels was demonstrated in unhealthy mice. The increase in serum Ir levels was accompanied by increased levels of TNF-α and IL-6. It was also noted that increased Ir levels in the adipose tissue could result in excess weight loss and cachexia in mice. In their in vitro study, Gannon et al. [30]

demonstrated the inhibitory effect of Ir on the population size and migratory capacity of malignant breast cancer cell lines. Furthermore, they demonstrated that Ir induced apoptosis of malignant cells by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity. This may indicate a potential anti-inflammatory effect of Ir against proinflammatory cytokines (i.e., TNF-α). The inhibitory effect of Ir on cancer cell proliferation has also been demonstrated in other studies. Tekin et al. [58] found the antiproliferative effect of Ir on prostate cancer cells in their in vitro study. Additionally, under cell culture conditions, Shao et al. [59] and Fan et al. [60] demonstrated the antiproliferative effect of Ir on lung cancer cells. Kong et al. [61] reported the inhibitory effect of Ir on the proliferation of osteosarcoma cells, whereas Liu et al. [62] reported this effect on pancreatic cancer cells. In addition to HIF-1α, other proinflammatory factors, transcription factors (NF-κB, STAT3, TNF-α, IL-6), and chemokines promote tumor proliferation by enhancing cancer cell survival, stromal remodeling, angiogenesis, and the metastatic process. The process of cancer transformation also depends on the inhibition of apoptosis, which is manifested by decreased expression of the tumor suppressor gene p53 [53]. Apoptosis is also influenced by Akt, which directly participates in the phosphorylation of proapoptotic proteins or indirectly affects transcription factors such as NFkB39 [53]. Shi et al. [31] showed that Ir stimulated the proliferation of liver cancer cells under in vitro conditions by activating the PI3K/Akt pathway (Figure 2). The above study results are contrary to those obtained by Gannon et al. [30]. In turn, Moon and Mantzoros [63], in their in vitro study, showed no effect of Ir on the proliferation of endometrial, colon, thyroid, or esophageal cancer cells. Many studies support the antiproliferative effect of Ir in an in vitro model. The conflicting findings may be due to tissue and cell specificity of Ir, as reported by Shi et al. [31] (Table 1).

Table 1. Summary of the results of Irisin levels in in vitro model studies.

Research Team	Cancer/Cell Lines	Irisin	Results	Reference Number
Moon et al. [63]	Endometrial (KLE, RL95-2) Colon (HT29, MCA38) Thyroid (SW579, BHP7 Esophageal (OE13, OE33) KLE, RL95-2, HT29, SW579 BHP7, OE13, OE33-American Type Culture Collection (ATCC, Manassas, VA, USA) MCA38, National Cancer Institute, National Institute of Health, Dr. Nicholas Restifo	Human recombinant Ir Aviscera Bioscience (Santa Clara, CA, USA) Phoenix Pharmaceuticals (Burlingame, CA, USA) Ir levels: 5–10 nmol/L (physiological) 50–100 nmol/L (pharmacological)	No impact of Ir on tumor cell proliferation, adhesion, or number compared to controls ($p < 0.05$)	[63]
Gannon et al. [30]	Breast MCF-7 MDA-MB-231 MCF-10a- control (American Type Culture Collection; Manassas, VA, USA)	Human recombinant nonmodified Ir-INM Cayman Chemical (Ann Arbor, MI, USA) Human recombinant modified and active (glycosylated) Ir-IM PlexBio (San Francisco, CA, USA) Ir levels: 0.625–20 nM	Reduced number of cancer cells (INM), and migration (INM, IM) Induction of tumor cell apoptosis (INM) Inhibition of NF-κB activity (INM) Enhancement of the effect of Dox on cancer cells by Ir (INM at all tested concentrations; IM at 1.0 μgM)	[30]
Tekin et al. [58]	Prostate cancer LNCaP DU-145 PC3	Ir (Phoenix peptide, Burlingame, CA, USA) Ir levels: 0.1–100 nM	Antiproliferative effect Decreased survival time of LNCaP cells at higher Ir levels (10–100 nM; $p < 0.05$; $p < 0.01$)	[58]
Shi et al. [31]	Hepatocellular carcinoma HepG2 SMMC7721	Human recombinant modified and active (glycosylated) Ir-IM PlexBio (San Francisco, CA, USA) Human recombinant non-modified Ir-INM CaymanChemical (Ann Arbor, MI, USA) Ir levels: 0.625–20 nM	Ir increased liver cancer cell viability in all cell lines (IM, INM) The Ir-IM-level of 2.5 nM stimulated an increase in migration and invasiveness of HepG2 cells compared to controls. This increase was statistically significant The level of modified Ir-IM of 2.5 nM significantly inhibited the cytotoxicity of Dox	[31]

Table 1. *Cont.*

Research Team	Cancer/Cell Lines	Irisin	Results	Reference Number
Shao et al. [59]	Lung cancer A549 (NSCLC) NCI-H446 (SCLC) Institute of Biochemistry and Cell Biology, Chinese Academy of Science, China	Ir levels: 0–50 nM	Ir at levels of 20–50 nM significantly inhibited A549 cell proliferation Ir at levels >20 nM inhibited migration and invasiveness of A549 cells	[59]
Kong et al. [61]	Osteosarcoma U2OS MG-63 American Type Culture Collection (ATCC, Manassas, VA, USA)	Ir levels: 0–200 ng/mL	Ir inhibited proliferation, migration, and invasiveness of U2OS and MG-63 cells in a dose- and time-dependent manner	[61]
Liu et al. [62]	Pancreatic cancer MIA PaCa-2 Panc03.27 ATCC (Manassas, VA, USA)	Human recombinant glycosylated E-Ir Human nonrecombinant P-Ir Sangon Biotech, Shanghai, China Ir levels: 0–100 nM	Both Ir forms inhibited the growth, migration, and invasiveness of pancreatic cancer cells	[62]

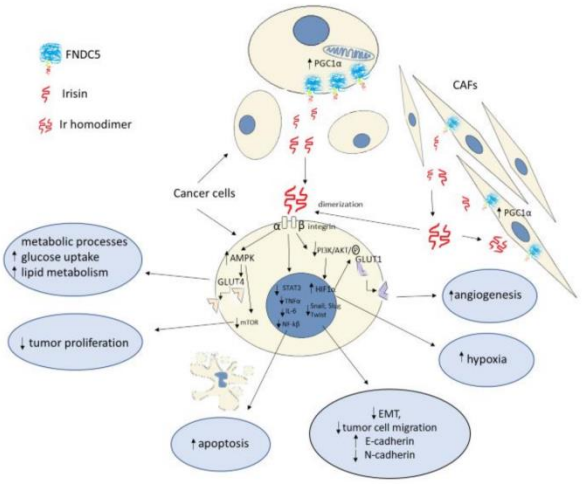

Figure 2. Potential roles of Irisin (Ir) in different signaling pathways and molecular processes involved in cancer progression, proliferation, angiogenesis, apoptosis, hypoxia, metabolic changes, and epithelial–mesenchymal transition (EMT) and migration of cancer cells. Ir has been observed in cancer cells and is also expressed in cancer-associated fibroblasts. This protein is cleaved from FNDC5 prohormone and has the potential to affect the neighboring cells (paracrine) or the cells from which it has been released (autocrine). Ir dimerizes and creates a homodimer with a beta sheet positioned between monomers and binds as a ligand to the integrin receptor. Ir binding to the receptor can affect many signaling pathways. Ir inhibits proliferation via the AMPK–mTOR pathway and increases glucose uptake via GLUT4 incorporation into the cell membrane. Ir affects the STAT3/Snail pathway, inhibits IL-6, and reverses EMT. Ir decreases the expression of N-cadherin and increases the expression of E-cadherin. Snail is downregulated by Ir via decreased phosphorylation of PI3K/Akt.

5. Impact of Irisin on Epithelial–Mesenchymal Transition

5.1. Epithelial–Mesenchymal Transition as the Background of Metastatic Processes

Malignant tumors mainly originate from the epithelial tissue, which is well structured. The cells are located on the basement membrane, form cell–cell junctions (known as

desmosomes or nexus), show the expression of specific markers (i.e., E-cadherin), and are polarized [64]. Tumor cells can spread either locally through a dynamic growth or distantly. Metastasis is a multistep process that requires cancer cells to undergo changes related to motility and the ability to migrate. This process describes the phenomenon of epithelial–mesenchymal transition (EMT). Cell migration is possible due to its polarity and signaling proteins (Rho family GTPases) that influence the remodeling of the cell cytoskeleton via the formation of protrusions and the loosening of cell–cell junctions. [64] Poor oxygen conditions in the tumor increase the expression of HIF-1α, which stimulates VEGF synthesis and angiogenesis, which is possible through the activation of the PI3K/Akt pathway [65]. Cell-secreted proteases (i.e., MMP proteins) digest the extracellular matrix and allow tumor cells to penetrate the circulatory system [66]. A change in the cancer cell phenotype is the result of EMT. These cells start to resemble mesenchymal cells. The changes primarily involve the synthesis of many proteins. Surface proteins (E-cadherin) responsible for forming cell–cell junctions (integrins) are replaced with proteins responsible for migration and the loosening of these junctions. These include mesenchymal cell markers such as *N*-cadherin, vimentin, α- SMA, and the Rho proteins. Altered protein synthesis is determined by altered expression of transcription factors involved in the EMT process (Snail, SLUG, Twist) [67].

5.2. Results of the In Vitro Model Indicating the Impact of Irisin on Epithelial–Mesenchymal Transition

In an in vitro model, Shao et al. [59] showed the inhibitory effect of Ir on lung cancer cell migration. In the same study, Ir inhibited the expression of *N*-cadherin and vimentin and increased the expression of E-cadherin. In addition, the study showed the inhibitory effect of Ir on PI3K/Akt phosphorylation and the transcription factor Snail, which is the major regulator of E-cadherin that is responsible for inhibiting its gene expression. Ir alters EMT markers by inhibiting the PI3K/Akt signaling pathway in lung cancer cells, which, according to the authors, indicates its involvement in the inhibition of migration and metastasis. The inhibitory effect of Ir on EMT markers was reported by Kong et al. [61] In vitro studies showed that Ir inhibited the migration of osteosarcoma cells, thus reducing its metastatic potential. IL-6 inhibits the expression of E-cadherin. In the above study, the authors showed that Ir reversed the effect of IL-6 by increasing E-cadherin expression. However, it has an inhibitory effect on the expressions of E-cadherin, vimentin, and MMP proteins whose expression is stimulated by IL-6. In the same study, the authors also demonstrated an inhibitory effect of Ir on the STAT3 signaling pathway and transcription factor Snail, which are activated by IL-6 and are crucial for EMT of osteosarcoma. Similar results were reported by Liu et al. [62] in an in vitro model using pancreatic cancer cells. As in previous studies, Ir inhibited the migration and metastatic ability of pancreatic cancer cells. In the same study, Ir was found to inhibit the mTOR signaling pathway by activating AMPK, which is involved in the maintenance of cell energy homeostasis and is also necessary for the initiation of EMT. Different experimental results were presented by Shi et al. [31]. Under the influence of Ir, liver cancer cells increased their ability to migrate and metastasize. Moreover, Ir activated the PI3K/Akt signaling pathway. Figure 3 presents a summary of the results of in vitro studies of the influence of Irisin on EMT.

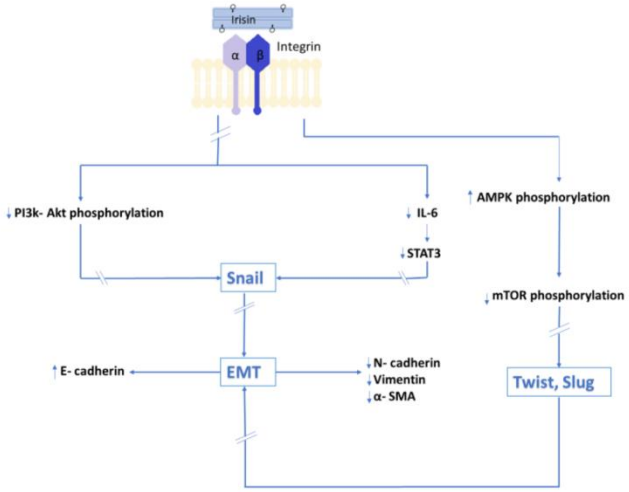

Figure 3. Irisin (Ir) inhibits PI3K/Akt signaling pathway and the transcription factor Snail, which is the major downregulator of E-cadherin gene expression. Ir reverses the effect of IL-6 by increasing E-cadherin expression. Ir has also an inhibitory effect on the STAT3 signaling pathway and transcription factor Snail, which are activated by IL-6 and are crucial for EMT. Ir increases the phosphorylation of AMPK and decreases the phosphorylation of the downstream molecule of AMPK signaling (mTOR pathway), which leads to the inhibition of the expression of transcription factors and the inhibition of EMT.

6. Irisin and Its Potential Role in Cancer Therapy

The effect of Ir on doxorubicin (Dox) therapy was investigated by Gannon et al. [30]. The results of this study showed the inhibitory effects of Ir on the proliferation and migration of breast cancer cells (MDA-MB-231) in an in vitro model. Dox, which can be used in breast cancer therapy, can cause many adverse effects, including cardiotoxicity. In the above study, Ir increased the cytotoxic effect of Dox, while reducing its uptake by tumor cells. Of note, Ir enhanced the cytotoxicity of Dox only in malignant cells (MCF-7) without affecting nonmalignant cells (MCF-10a). Thus, Ir may enhance the efficacy of Dox by affecting the reduction in its toxic effect on healthy cells, thus reducing the number of complications of cancer therapy. Shi et al. [31] also analyzed the effect of Ir on Dox therapy and showed that Ir reduced the cytotoxicity of Dox in liver cancer cells (HepG2). Further studies are warranted to determine whether the inconclusive study results obtained by different research groups are only related to the tissue specificity of Ir. These studies should also determine its potential role in cancer therapy.

Fan et al. [60] investigated the potential effect of Ir on paclitaxel therapy, which is used in many NSCLC treatment regimens. Silencing of FNDC5 decreased the sensitivity of NSCLC cells to paclitaxel. However, cancer cells in patients who were given Ir before treatment showed increased sensitivity to the drug, and they were characterized by higher activity of proapoptotic cells (Bax, p53) and lower levels of antiapoptotic proteins (Bcl-2). Fan et al. [60] concluded that the combined use of Ir and paclitaxel could be beneficial in the treatment of NSCLC. Moreover, it may reduce the frequent phenomenon of increasing resistance to paclitaxel in the late stage of chemotherapy.

7. The Role of Irisin in Selected Cancer Diseases

7.1. Breast Cancer and Reproductive Tract Cancer

7.1.1. Serum Irisin Level in Patients with Breast Cancer

Provatopoulou et al. [13] showed lower serum Ir levels in cancer patients compared to controls (Table 2). Furthermore, it was estimated that a one unit increase in serum Ir levels resulted in the reduction in the risk of breast cancer by almost 90%. It was also shown that Ir could be a breast cancer screening marker. At a cutoff point of 3.21 µg/mL, the sensitivity and specificity were 62.7% and 91.1%, respectively. A positive correlation was found between Ir level and the clinical stage of the tumor (S). No statistically significant correlation was found with respect to tumor size (T), lymph node metastasis (N), and histological malignancy of the tumor (G). Different results were obtained by Panagiotou et al. [68]. After analyzing the results of Provatopoulou et al. [13] Panagiotou et al. [68] paid attention to different ELISA kits used by various investigators. They performed their study using ELISA, which was previously validated using mass spectrometry by Jędrychowski et al. [12]. Panagiotou et al. [68] found elevated serum Ir levels in benign and malignant breast tumors. No differences were found in Ir levels between benign and malignant tumors. However, when Ir was used with omentin-1, which is an adipokine with the properties similar to adiponectin, elevated Ir levels indicated tumor malignancy. Those authors explained different results from Provatopoulou et al. [13] by the fact that a different ELISA kit was used. Panagiotou et al. [68] reported that patients with benign breast lesions were not enrolled in the previous study. The results were explained by unknown pathophysiological phenomena occurring in malignant tumors. In addition, the authors showed a positive correlation between Ir and Ki-67, which is a marker of cell proliferation. Ir levels also increased with the grade of malignancy as described by the Elston–Ellis score and were higher in patients with a positive estrogen receptor (ER^+). The researchers expressed the opinion that their findings indicated the possible involvement of Ir in breast tumor formation from benign lesions to malignant progression, which makes Ir a promising diagnostic and prognostic marker. In turn, Zhang et al. [15] analyzed patients with breast cancer and spinal metastasis. Their results showed that serum Ir levels were higher in patients without metastasis (M0). Furthermore, the presence of serum Ir was shown to be associated with a protective effect against the occurrence of spinal metastasis. Additionally, a positive correlation was found between serum Ir levels and BMI of patients, which could suggest that a higher amount of body fat in female patients was associated with a higher release of Ir into the serum. This suggests that the study group should be matched in terms of BMI to exclude the influence of Ir which is expressed and released from adipocytes. It may explain the opposite results when the immunohistochemical (IHC) method was used, in which Ir expression was analyzed only in the tumor tissue.

Table 2. Summary of the study results of Irisin levels in human plasma detected by ELISA.

Research Team	Study Method	Results	Study Group	Reference Number
Provatopoulou et al. [13]	ELISA (AdipoGen International, Liestal, SW); results expressed as µg/mL	Lower serum levels of Ir in patients compared to the control group (2.47 ± 0.57 (mean ± SD) vs. 3.24 ± 0.66 (mean ± SD)) $p < 0.001$	101 female patients with invasive ductal breast cancer 51 healthy women (the control group)	[13]
Gaggini et al. [56]	ELISA (Adipogen AG, Liestal, Switzerland); results expressed as µg/mL	Plasma Ir levels did not differ between HCC patients and controls (3.56 ± 0.2 (mean ± SEM) vs. 4.4 ± 0.15 (mean ± SEM)) $p = 0.749$	18 patients with HCC 18 deceased donors	[56]
Shi et al. [31]	ELISA (USCN life Science, Wuhan, China); results expressed as µg/mL	Plasma Ir levels were not different between HCC patients and controls	20 patients with HCC	[31]

Table 2. *Cont.*

Research Team	Study Method	Results	Study Group	Reference Number
Altay et al. [14]	ELISA (USCN, Life Science Inc., Catalog No. USCN-E82576Hu, P.R. China); results expressed as pg/mL	Higher FNDC5/Ir levels in renal tumor patients compared to the control group (208 ± 97 (mean ± SD) vs. 110 ± 79 [mean ± SD) $p = 0.0001$	23 patients with renal tumor 25 healthy individuals	[14]
Zhang et al. [15]	ELISA (Aviscera Biosciences, Santa Clara, CA, USA); results expressed as ng/mL	Higher Ir levels in patients without spinal metastases (7.60 ± 3.80 (mean ± SD) vs. 6.10 ± 2.62 (mean ± SD)) $p = 0.012$	148 patients with breast cancer, including 53 subjects with spinal metastasis	[15]
Zhu et al. [69]	ELISA (USCN Life Science Inc., Wuhan, China); results expressed as µg/mL	Lower Ir levels in patients with colorectal cancer and normal weight compared to controls (0.17 ± 0.01 (mean ± SD) vs. 0.22 ± 0.01 (mean ± SD)) $p < 0.05$)	76 patients—38 patients with colon cancer and 38 subjects with rectal cancer 40 healthy controls	[69]
Aslan et al. [70]	ELISA (Yl Biont Biotech Co. Shanghai, China); results expressed as pg/mL	Mean Ir level was lower in prostate cancer patients compared to controls (6.92 ± 2.44 (mean ± SD) and 13.5 ± 6.21 (mean ± SD)) $p < 0.05$	50 patients with primary prostate cancer 30 healthy male subjects	[70]
Esawy and Abel [71]	ELISA (Bio Vendor Laboratory Medicine, Brno, Czech Republic) [Catalog No. RAG018R]; results expressed as µg/mL	Lower Ir levels in patients with bladder cancer compared to controls (1.07 (0.51–1.96) (mean ± SD) vs. 1.8 (0.5–2.44) (mean ± SD)) $p < 0.001$	75 patients with bladder cancer 75 healthy subjects	[71]
Pazgan-Simon et al. [72]	ELISA (Bio Vendor-Laboratorini Medicina a.s. catalog No. RAG018R); results expressed as µg/mL	Lower Ir levels in HCC patients compared to controls (2.52 ± 1.14 (median ± SD) vs. 4.46 ± 1.34 (median ± SD)) $p = 0.02$	69 patients with cirrhosis and hepatocellular carcinoma 24 patients with non-viral cirrhosis 20 healthy volunteers	[72]

SD—standard deviation, SEM—standard error of mean.

7.1.2. Irisin Tissue Expression Levels in Patients with Breast Cancer and Reproductive Tract Cancer

Kuloglu et al. [73] performed IHC reactions which showed no Ir expression in normal breast tissue. However, Ir expression levels were significantly higher in invasive lobular carcinoma, intraductal papillary carcinoma, invasive ductal carcinoma, invasive papillary carcinoma, and mucinous carcinoma compared to healthy breast tissue. As opposed to the mammary gland, normal luteal cells in the ovarian region showed Ir expression. Positive IHC was found for ovarian endometrial cancer. Low Ir expression was found in mucinous ovarian cancer tissues, as well as in atypical endometrial proliferation. High levels of Ir expression were noted in benign endometrial proliferation and in cervical squamous cell carcinoma.

Further studies are warranted to determine the relationship between serum Ir levels and its expression in the tissue, as there is no available research on their relationship. The lack of knowledge about the receptor in the mammary gland tissue does not allow understanding of Ir transport from the serum to the tissue or vice versa. Different results obtained by various research teams may also be due to the lack of validation of the ELISA assays, as well as differences related to the study design and patient selection. However, the above findings indicate that Ir may be a promising biochemical marker that is a complement to screening for breast cancer. The analysis of the above studies showed that decreased serum Ir levels in women could indicate the occurrence of breast cancer and its distant metastasis.

Moreover, determination of Ir expression by IHC in biopsy material may be helpful to determine the occurrence of not only breast cancer, but also cancers of the reproductive tract. However, it is necessary to examine the relationship between Ir expression in the cancer tissue and clinicopathological factors, as was done in the case of its serum levels.

Studies also indicate potential protective effects of Ir and the possibility of its use in targeted therapy.

7.2. Irisin in Prostate, Kidney, and Bladder Cancer

Aslan et al. [70] compared serum Ir levels in prostate cancer patients and healthy male subjects. Prostate-specific antigen levels (PSA) were significantly higher in prostate cancer patients compared to controls. The opposite trend was found in the case of serum Ir levels in cancer patients in whom these levels were decreased compared to healthy male subjects. However, no differences were found in Ir levels in patients from different groups based on the Gleason classification. The results of the above study indicated that Ir could be a useful diagnostic biomarker, which could be used as an adjunct to diagnosis using PSA.

Esawy and Abdel-Samd [71] investigated serum Ir levels in bladder cancer (BC) patients. They showed lower serum Ir levels in patients with BC compared to the control group. At the cutoff level of Ir $\leq$ 1.2 μg/mL, the sensitivity and specificity were 74.7% and 99.7%, respectively. No differences were found in the concentrations of biochemical parameters, i.e., fasting glucose, triglycerides, HDL, and LDL cholesterol in BC patients except for total cholesterol, which was significantly lower in the patient group. Ir levels correlated positively with BMI of patients and negatively with cholesterol levels. The association between Ir levels and grades of histological differentiation (G) was also investigated. Ir level was significantly lower in G3 compared to G1 and it also significantly decreased in the subsequent clinical stages of BC (S). The 1 year mortality rate in patients with high Ir levels was 5% compared to 38.2% in patients with low Ir levels. Patients with high Ir levels had significantly higher overall survival (OS) rates than patients with low Ir levels. The authors of the study concluded that Ir could be both a helpful marker in the diagnosis of BC and could also act as a prognostic factor for the survival of BC patients.

Altay et al. [14] showed higher serum Ir and carcinoembryonic antigen (CEA) levels in renal cancer patients compared to controls. Ir level had higher sensitivity and specificity compared to CEA level, which is a recognized cancer marker of endodermal and ectodermal origin. The authors of the study were cautious in formulating a thesis about the usefulness of Ir in the diagnosis of renal cell carcinoma. The study was conducted on a small patient population (n = 23). This may be the reason why those researchers had different results from those obtained in other urinary tract cancers.

Further studies are warranted on larger patient populations to confirm the results. A study on a much larger patient population was conducted by Kuloglu et al. [74] who analyzed kidney cancer tissues using IHC. Ir was not found in clear cell or papillary renal cell carcinomas. Significantly decreased Ir levels were noted in chromophobe renal cell carcinoma samples. No differences were found in the level of Ir expression in benign oncocytoma than healthy tissues, which indicates that investigation of Ir expression levels may be a useful test for differentiating benign lesions from renal cancer.

The above studies support the thesis about the usefulness of Ir in the diagnosis of urinary tract cancers. Investigation of serum Ir levels may support the diagnosis as an adjunct to the assessment of PSA level in prostate cancer and CEA level in renal cancer. In addition, the study results indicated a protective effect of Ir and higher mortality rates in patients with decreased Ir levels. This molecule can be used as a prognostic factor in BC. Studies on tissues and serum are available only in the case of renal cancer. Studies on material obtained from the same patients are necessary due to the inverse relationship (i.e., elevated serum Ir levels and decreased Ir expression in renal cancer tissue). So far, only the assessment of Ir expression using IHC has seemingly been useful for renal cancer differentiation.

7.3. Irisin in Gastrointestinal Cancers

Zhu et al. [69] compared serum Ir and the activating transcription factor (ATF3) levels in patients with colorectal cancer (CRC) who were overweight, obese, or of normal weight. Patients with normal weight had lower Ir levels than controls. No differences were found

in serum Ir levels or FNDC5 mRNA expression in the adipose tissue of CRC patients with normal or abnormal weight. However, higher serum ATF3 levels were found in patients with normal and abnormal body weight. Moreover, Ir levels were positively correlated with triglyceride levels in CRC patients and controls. After adjusting for age, sex, BMI, and other biochemical parameters, high Ir levels reduced the risk of developing CRC by 78%, whereas high ATF3 levels increased the risk of developing this cancer. At the cutoff value for 0.46 ng/mL for ATF3, the sensitivity and specificity for the discrimination of CRC were 74% and 65%, respectively. At the cutoff value of 0.19 µg/mL for Ir, the sensitivity and specificity for the discrimination of CRC were 63% and 65%, respectively. When ATF3 and Ir were included in the analysis, the sensitivity and specificity were 73% and 80%, respectively. Therefore, combining ATF3 and Ir in the diagnostic process may increase the accuracy of CRC diagnosis.

Pazgan-Simon et al. [72] examined serum Ir and betatrophin levels in patients with cirrhosis and HCC. Ir levels were decreased in patients with HCC, whereas no significant differences were found in patients with cirrhosis compared to controls. However, betatrophin levels were higher in HCC and cirrhosis patients compared to the control group. Moreover, Ir levels were significantly decreased in more advanced stages of HCC (A vs. C according to the Barcelona Clinic Liver Cancer (BCLC)) and more advanced stages of cirrhosis (A vs. B according to the Child–Pugh score (C–P score)). The above studies indicated that Ir could have a protective effect, and its low level promotes faster fibrosis and tumor progression.

In gastrointestinal cancers, Ir was lower in the analyzed papers except for one study. Shahidi et al. [75] reported higher serum Ir levels in gastric cancer (GC) patients compared to controls. According to these authors, Ir could be a valuable biomarker in the early detection of GC. However, the small sample size was the limitation of the study. In turn, the findings of Shahidi et al. [75] are in line with the analysis of Ir levels in tissue material conducted by Aydin et al. [76] The researchers performed Ir detection with IHC using tumor fragments from patients with gastrointestinal cancers which were compared to healthy tissues. Aydin et al. [76] also found positive IHC reactions for Ir detection in healthy gastric, esophageal, colon, hepatic, and pancreatic tissues. They observed higher Ir expression in gastric adenosquamous adenocarcinoma, gastric neuroendocrine carcinoma, and gastric signet-ring cell carcinoma. The expression of Ir in gastric signet-ring cell carcinoma was higher than that in gastric neuroendocrine carcinoma and gastric adenosquamous adenocarcinoma. This cancer is characterized by a significant presence of mucin in the cell cytoplasm [77]. However, Altay et al. [57] did not demonstrate Ir expression in healthy gastric tissues or experimentally induced GC in mice. However, their study showed higher Ir levels in WAT and BAT in GC mice compared to controls and an increase in Ir expression in both adipose tissues with cancer progression.

Furthermore, the increase in Ir levels in the adipose tissue corresponded to the increase in serum Ir levels in diseased mice. Perhaps a similar mechanism also occurs in humans, which could explain the results obtained by Shahidi et al. [75] in patients with GC. The mechanism of the relationship between cancer and the increase in FNDC5 mRNA expression in the adipose tissue and the increase in serum Ir levels is unknown. The authors of this study suggested that the autocrine effect of Ir on the adipose tissue may result in weight loss in diseased mice and underlie cancer cachexia.

Aydin et al. [76] also analyzed other types of gastrointestinal cancers. They found that Ir expression in esophageal epidermoid carcinoma, esophageal adenocarcinoma, and esophageal neuroendocrine carcinoma was higher than in the healthy tissue. The level of Ir was not significantly different in various histological types of esophageal cancer. The researchers also demonstrated increased Ir expression in colon adenocarcinoma and colon mucinous adenocarcinoma. The intensity of the IHC reaction was similar for these two cancers. In intralobular and interlobular ducts of cancerous pancreatic tissue, Ir expression was also higher than in the healthy tissue. However, the results related to HCC are

inconclusive. Although the healthy liver tissue showed the presence of Ir in hepatocytes, the researchers found no difference between Ir expression levels in HCC and healthy tissue.

Different study results were obtained by Zhang et al. [78]. They demonstrated decreased FNDC5 mRNA expression in tissues obtained from HCC patients, as well as decreased serum Ir levels in patients before hepatectomy. However, Gaggini et al. [56] showed overexpression of FNDC5 mRNA in HCC patients. However, no differences were found between serum Ir levels in HCC patients and healthy controls. Similar results were obtained by Shi et al. [31], who found an increase in *FNDC5* gene expression in HCC tissues and no differences between serum Ir levels in HCC patients and healthy controls. According to Gaggini et al. [56], no relationship between *FNDC5* gene expression in the tumor tissue and the protein level is very common and could be related to post-transcriptional and post-translational events (such as protein half-life, protein damage, or degradation). Recent studies have also indicated that the liver and kidney may be involved in the clearance and metabolism of Ir.

The above studies demonstrate a potential role of Ir in the diagnosis of gastrointestinal cancers. Most studies indicate an increased lr level in tumor tissues compared to healthy tissues in patients. However, there have been no studies on its association with clinicopathological factors. Studies on a mouse model indicated no relationship between the expression of Ir protein in the tissue and its serum levels. However, there are no such studies on patient material. Contradictory results are related to hepatic cancers. They may be associated with the participation of the liver in Ir metabolism. Further studies are warranted to clarify these issues. They should consider larger patient populations and critical remarks related to methodology and patient selection for studies. Differences in circulating serum Ir levels may be related to the fact that it is released by many tissues (muscle tissue, adipose tissue), which was confirmed by a study using a mouse model. Moreover, the final Ir level may depend on systemic or local expression. Another reason which has already been indicated in this paper may be the presence of monoclonal antibodies used in ELISA kits, whose sensitivity and specificity have been questioned in many studies [7,11,56].

7.4. Irisin in Lung Cancer

To date, only two studies have been conducted on lung cancer, including one using an in vitro model only. However, Nowinska et al. [51] conducted a study on a significantly larger population (n = 729) than other studies related to different cancer types. The results of their study showed Ir expression in NSCLC cells and stromal cells. Expression of Ir in the stromal cells has not been reported in any other type of cancer. This can be characteristic only of lung cancers. Ir levels were higher in cancer cell cytoplasm and stromal cells in adenocarcinoma compared to squamous cell carcinoma. Higher expression of FNDC5 mRNA in NSCLC tissues was confirmed by molecular studies (RT-PCR). Using laser capture microdissection (LCM), cancer cells and tumor cells were very precisely collected. It was shown that the expression of FNDC5 mRNA from NSCLC tissues in stromal cells was higher than that in cancer cells. No Ir expression was found in normal lung tissue except for lung macrophages. In addition, Nowinska et al. [51] showed an association between Ir expression levels in tumor cells and clinicopathological parameters. In tumor cells, Ir expression levels were decreased with higher grades (G) of malignancy and in larger tumors (T). Changes in Ir expression were also observed in relation to lymph node metastases. The expression level of Ir in tumors with mediastinal lymph node metastasis (N2) was higher than that in the group without lymph node metastasis (N0) and in the group with hilar and mediastinal lymph node metastases (N1). No association was found between Ir expression in tumor cells and overall survival (OS). Those researchers also analyzed the relationship between Ir in tumor stromal cells and clinicopathological parameters. The level of Ir expression increased in advanced pT status. They also showed a positive correlation between Ir expression in stromal cells and the level of Ki-67 antigen in cancer cells. Those authors suggested that Ir expression in stromal fibroblasts could influence NSCLC cell proliferation. This is also supported by shorter survival of patients with higher

Ir expression in NSCLC stromal cells. Higher Ir expression was demonstrated in patients with distant metastases (M1) compared to nonmetastatic patients (M0). Furthermore, the study results confirmed that Ir expression in stromal cells might be an independent prognostic factor.

The role of Ir in lung cancer has not yet been fully understood. Nowinska et al. [51] were the first to describe Ir in the context of lung tissue and lung cancer. An earlier study by Shao et al. [59], who used an in vitro model, demonstrated the inhibitory effect of Ir on lung cancer cell proliferation, migration, and invasion by inhibiting the PI3K/Akt pathway. Furthermore, Ir can reverse the EMT process by inhibiting the expression of the transcription factor Snail. Studies using tissues collected from lung cancer patients also indicated its association with cell proliferation and lymph node and distant metastases. However, the mechanism present in the tissues obtained from patients appears to be more complicated due to a significant impact of Ir expression in the tumor stroma on disease progression. The authors suggested that high Ir levels in cancer cells during the first stage of the disease could be related to changes in their metabolism and mitochondrial biogenesis. However, in later stages of the disease, Ir expression may be inhibited due to the impact of Ir on UCP1 expression and ATP synthesis reduction. A decreased ATP level is associated with activation of AMPK and inhibition of the mTOR pathway. The AMPK–mTOR pathway plays an important role in cell proliferation. However, further studies are warranted to explain the mechanism of how Ir affects cancer cells. Additionally, there have been no studies related to the assessment of serum Ir levels in patients with lung cancer.

7.5. Irisin in Thyroid Cancer

Ugur et al. [79] conducted a study on different histological types of thyroid carcinomas and compared them to healthy tissue. Ir expression was assessed using IHC, and Ir levels were measured using ELISA. Tissue samples were homogenized. Ir expression was slightly increased in patients with papillary thyroid carcinoma (PTC) and significantly increased in oncocytic papillary thyroid carcinoma (OPTC) and anaplastic thyroid carcinoma (ATC). However, no differences were found in follicular thyroid carcinoma (FTC). Ir expression was higher in the tissues of patients with oncocytic follicular thyroid carcinoma (OFTC) than in FTC. No Ir immunoreactivity was found in the tissues of patients with medullary thyroid carcinoma (MTC). The assay using ELISA confirmed the IHC results. Those authors indicated that most oncocytic follicular cells have a structure similar to Hürthle cells (HCs).

On the other hand, HC metaplasia is an important feature of chronic lymphocytic thyroiditis (Hashimoto's thyroiditis; HT) [80]. HCs have many mitochondria and are associated with energy production. Ugur et al. [79] demonstrated increased Ir expression in oncocytic carcinomas and tissues obtained from HT patients. The researchers concluded that the thyroid tissue rich in HC (in other words, rich in mitochondria) produced more heat and caused the death of oncocytic cells, which occurred due to increased Ir levels in oncocytic tumors and increasing concentration of UCP1 in mitochondria. PTCs and FTCs with fewer mitochondria and lower Ir expression generated less heat and were perhaps more clinically aggressive than oncocytic variants. Additionally, Ugur et al. [79] found suppressed Ir expression in MTCs. The more aggressive course of these cancers may be due to decreased Ir synthesis, which may show a protective effect, as reported by those authors. Ir is a promising biomarker which is useful for differentiating oncocytic variants of OPTC and OFTC from non-oncocytic forms of PTC and FTC. Ir may mediate thyroid carcinogenesis and participate in oncocytic cell apoptosis through increased heat production.

7.6. Osteosarcoma

Cheng et al. [81] showed a decreased level of FNDC5/Ir in serum and tissues of osteosarcoma patients. The researchers also performed in vitro studies using osteosarcoma U2OS cell lines and found that Ir inhibited U2OS cell viability in a concentration- and time-dependent manner and could inhibit tumor cell migration and invasion. The microRNA

(miR) 214-3p was also used in this study. MicroRNAs (miRNA/miR) are small noncoding RNAs involved in tumor initiation, growth, and progression. In previous studies, miR-214 was upregulated in osteosarcoma cells and was associated with tumor progression and poor prognosis [82]. Cheng et al. [81] found that miR-214-3p inhibited FNDC5/Ir expression, thus contributing to the activation of migration, invasion, and EMT of osteosarcoma cells (Figure 4). Previous studies showed that Ir activated osteocytes through the αV/β5 integrin receptor [36].

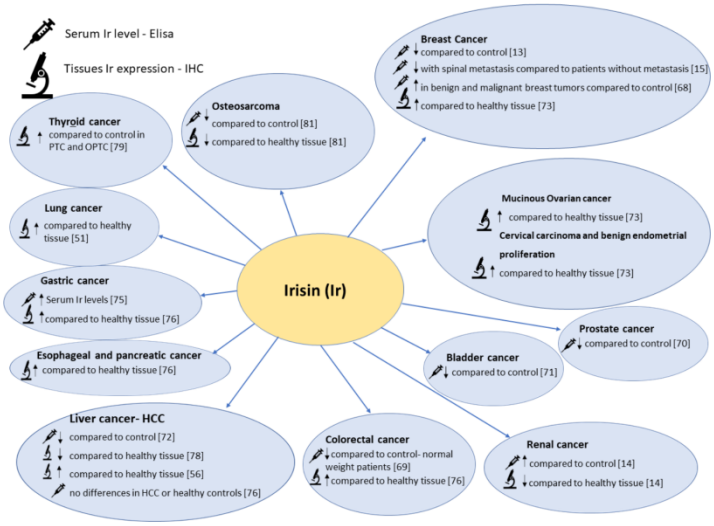

Figure 4. The figure shows the comparison between serum Irisin levels and IHC Irisin expression levels in different types of cancers.

Osteocytes control skeletal remodeling by inducing osteoclastogenesis and inhibiting osteogenesis. Kim et al. [36] showed that deletion of *FNDC5* inhibited bone resorption by blocking the increase in the number of osteoclasts, thus preventing the loss of bone mass. Other findings were obtained by Colaianni et al. [33] who reported that Ir which was given to mice with osteoporosis prevented bone mass loss and induced bone regeneration. Moreover, it was found that Ir protected against the loss of muscle mass in immobilized mice. Kim et al. [36] suggested that Ir could affect bone resorption and remodeling when dosed appropriately. Parathormone (PTH) shows a similar effect. Peritumoral osteolysis is essential in the development of osteosarcoma. Osteoclast activity leads to bone degradation and the release of protumor factors, such as insulin-like growth factor 1 (IGF1) or transforming growth factor-β (TGF-β), which enhance osteosarcoma cell proliferation [83]. Cheng et al. [81] indicated a protective role of Ir in the proliferation and metastasis of osteosarcoma. These authors indicated that Ir could be used for osteosarcoma therapy in the future.

8. Conclusions

Irisin has been described relatively recently in the literature as a myokine released by skeletal muscles under the influence of physical exercise. It soon became evident that Ir expression is also present in other tissues, including normal and abnormal tissues such as cancer tissues. Many studies have been conducted to determine the role of Ir in both physiological and pathological processes.

Studies on animal models and cell lines are underway. They are related to the role of Ir in cancer disease, including many types of malignancies, i.e., breast, lung, gastrointestinal, reproductive tract, and bone cancers. The recent studies mostly showed an inhibitory effect of Ir on the proliferation, migration, and invasiveness of cancer cells. They also indicated the inhibitory effect of Ir on the processes related to EMT, which is crucial for cancer cell metastasis. The conflicting results found in gastrointestinal cancers are probably due to the tissue specificity of Ir, and further studies are warranted in this respect. Additionally, molecular studies which use both tumor tissues and serum of patients are also being conducted. Serum Ir levels are different in cancer patients. They decrease or increase in patients with breast cancer, increase in patients with renal cancer, and remain stable in patients with liver cancer. The observed differences may be due to the release of Ir by different tissues, and its level is the result of local and systemic production. It may also result from the circulation of Ir isoforms and the type of assays used to detect it. Lastly, the most important issue is related to the structure and occurrence of the receptor for Ir. Additionally, cancer stromal cells show the expression of Ir. Their role in promoting tumor proliferation seems particularly interesting. Ir also shows therapeutic potential. However, further studies are warranted to determine its effect on Dox therapy.

To conclude, many studies have been conducted to determine the role Ir plays in the body in health and disease. Small sample sizes, the lack of correlations between the study results and clinicopathological factors, and studies using only animal models or in vitro experiments were some of the identified limitations, as admitted by the researchers themselves. Comprehensive studies defining the role of Ir in pathological processes and in the development of cancer could show its real usefulness in disease prevention, diagnosis, and treatment.

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Abbreviations

Ir	Irisin
FNDC5	fibronectin type III domain-containing protein 5
WAT	white adipose tissue
BAT	brown adipose tissue
UCP	uncoupling protein
UCP1	uncoupling protein 1
PPAR	peroxisome proliferator-activated receptor
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1 alpha
FAK	focal adhesion kinase;
BMP-7	bone morphogenetic protein 7
CAFs	cancer-associated fibroblasts
GLUT	glucose transporters;
HIFs	hypoxia-inducible factors
VEGF	vascular endothelial growth factor
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

Akt	protein kinase B (PKB)
PI3K-3	phosphatidylinositol 3-kinase;
mTOR	mammalian target of rapamycin kinase
AMPK	5′AMP-activated protein kinase
MMP	matrix metalloproteinase
EMT	epithelial–mesenchymal transition
α-SMA	alpha-smooth muscle actin
NSCLC	non-small-cell lung carcinoma
SCLC	small-cell lung carcinoma
DOX	doxorubicin
INM	human recombinant nonmodified irisin
IM	human recombinant modified and active (glycosylated irisin)
HCC	human hepatocellular carcinoma;
DCIS	ductal carcinoma in situ
CEA	carcinoembryonic antigen
RCC	renal cell carcinoma
ccRCC	clear cell renal cell carcinoma
CRC	colorectal cancer
OS	overall survival
PTC	papillary thyroid carcinoma
FTC	follicular thyroid carcinoma
OPTC	oncocytic variant of papillary carcinoma of the thyroid
OFTC	oncocytic variant of follicular carcinoma of the thyroid;
BC	bladder cancer
GC	gastric cancer

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Irisin Association with Ki-67, MCM3 and MT-I/II in Squamous Cell Carcinomas of the Larynx

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Abstract: Background: Current studies indicate irisin role in carcinogenesis. The aim of the study was to investigate the expression of irisin in LSCCs and to determine its association with clinicopathological factors, as well as recognized markers of proliferation, i.e., Ki-67 and MCM3,5,7 and MT-I/II proteins. Material and methods: The research material consisted of 140 cases of LSCCs, 57 cases of laryngeal papillomas (BLs) and 14 controls (benign hypertrophic changes). Tissue microarrays were used to perform IHC. Western blot and immunofluorescence were performed in laryngeal cancer cell lines and normal keratinocytes. Results: Irisin expression levels were significantly increased in LSCC compared to BLs ($p < 0.0001$) and controls ($p = 0.001$). We noted a positive moderate and weak correlation between irisin and Ki-67, MCM3 and MT-I/II. We observed an elevated level of irisin expression with increasing tumor size (T1–2 vs. T3–4; $p = 0.0348$). The levels of irisin were higher in N0 than in N1 and N2–3 ($p = 0.0031$ and $p = 0.0457$, respectively). Our in vitro study revealed a higher level of irisin in Larynx Epidermoid Carcinoma 2 (HEp-2) cells compared to the control Normal Human Keratinocyte (HaCat) cell line. Conclusions: Increased irisin expression levels in LSCC and its correlation with clinicopathological and proliferation factors may indicate the potential role of irisin as a biomarker in the diagnostic process of LSCC.

Keywords: irisin; Ki-67; MCM3; MCM5; MCM7; MT-I/II; LSCC

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1. Introduction

The larynx is the most common localization of head and neck cancers, and the most prevalent type is squamous cell carcinoma (SCC), which is diagnosed in 95% of cases [1]. The risk of laryngeal cancer is eight times more frequent in men. In the male population, laryngeal cancer is the seventh most common cancer [2]. Total laryngectomy still remains the gold standard of therapeutic management due to the lack of specific methods for the diagnosis of laryngeal cancer [3,4]. Radical surgery is associated with the loss of function of the larynx, depriving patients of the possibility of communication, which has a very negative impact on their functioning and recovery [5,6]. Due to the difficulties associated with the diagnosis of laryngeal cancer, there is a necessity to search for specific markers related to carcinogenesis in this area. Irisin could be one such marker. The increased level of irisin expression has been reported in many cancers. Its elevated level of expression has been observed, for example, in thyroid and lung cancer [7–9]. The anatomic proximity of the thyroid and larynx, as well as the location of the larynx on the border between the upper and lower respiratory tract raise the question about the potential role of irisin in the carcinogenesis of laryngeal cancer. The expression of irisin in laryngeal carcinomas has not been studied yet.

Irisin was described as a myokine released by skeletal muscle in response to physical exercise in 2012. Bostrom et al. [10] observed that irisin was released as a result of physical exercise and affected white adipose tissue (WAT) cells, which turn into beige. Phenotypically and functionally, they resemble brown adipose tissue (BAT). Irisin is released as a result of post-translational modifications of the FNDC5 prohormone, encoded by the FNDC5 gene. The peptide composed of 112 amino acids is the result of cleavage and glycosylation of the extracellular domain of the prohormone [11,12]. FNDC5 is an N-glycan with two potential glycosylation sites (Asn36 and Asn81) [13]. Glycosylation is one of the most important post-translational modifications affecting the physicochemical properties of protein [14]. Inhibition of glycosylation decreases irisin secretion [13] and also increases its molecular mass from 13 kD to 20 kD [11]. The increase in FNDC5 expression under the influence of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) elevates the expression of the BAT uncoupling protein 1 (UCP1) marker. The effect of thermogenin activity is energy dissipation, which has a positive effect on metabolism, and may prevent obesity and related diseases, including type II diabetes [15,16]. In addition to muscle and adipose tissue, many studies have shown the presence of irisin in other tissues and organs, such as the brain, testes, epididymis, heart muscle cells, skin, liver, pancreas, and stomach [17]. Moreover, the study showed the impact of irisin on tissues as a ligand of integrin receptor [18–20].

Irisin expression was also observed in many types of cancer, including breast, prostate, gastrointestinal tract, bone, and lung cancer as well as gynecological carcinomas [21–24]. In the case of non-small-cell lung carcinoma (NSCLC), the presence of irisin has been noted not only in cancer cells but also in cancer-associated fibroblasts (CAFs). The elevated level of irisin expression in CAFs was associated with a worse prognosis in patients and the proliferation of NSCLC cells [8]. Moreover, CAFs participate in carcinogenesis by stimulating neoplastic angiogenesis and the epithelial–mesenchymal transition (EMT) [25]. Other studies have revealed that irisin may be involved in limiting neoplastic expansion. Irisin was shown to inhibit EMT by blocking PI3K/Akt/Snail signaling, affecting the inhibition of migration and invasion of lung cancer cells in an in vitro model [9]. In addition, many studies on the in vitro model revealed that irisin also inhibited cell proliferation, for example, in breast, lung, bone, prostate, and pancreatic gland cancer [9,22,24,26,27]. Their findings are contrary to the studies that showed that irisin did not affect the proliferation, adhesion, or colony formation of malignant cells of the colon, endometrium, thyroid, or esophagus [28].

The influence of irisin on the proliferative activity of cancer cells seems to be of significant importance for its potential role in the diagnostic process. Therefore, in our study, we estimated the correlation with recognized markers of proliferation, such as Ki-67 antigen and minichromosome maintenance proteins (MCM3, MCM5, and MCM7) [29]. Ki-67 is a non-histone protein in the cell nucleus that is responsible for cell proliferation. In LSCC cancers, a significant correlation was demonstrated between the tumor grade (G) and the Ki-67 proliferation index value [30,31]. However, the MCM family includes proteins from MCM1 to MCM10. The basic function of MCM proteins is participation in the mechanism of initiation of replication and regulation of DNA synthesis. MCM proteins play an important role in maintaining genome integrity by preventing DNA from another replication in the same cell cycle. MCM proteins are not expressed in the resting phase (G0). However, they are detected during all other phases (G1, S, G2 and M) and can, therefore, serve as potential markers of cell proliferation and can also be useful to assess the degree of tumor cell proliferation [32]. Studies showed an increased expression of MCM5 and a positive correlation with Ki-67 [33], as well as an increased expression of MCM2, MCM3, and MCM7 in Larynx Epidermoid Carcinoma 2 (HEp-2) cell lines and positive correlations with Ki-67 in LSCC [34]. In addition, we decided to investigate the correlation of irisin with metallothioneins I and II (MT-I/II). MT-I/II are low-molecular-weight proteins that are characteristic of most tissues. They participate in the neutralization of free oxygen radicals and the homeostasis of many chemical elements, and play an important role in the

detoxification of heavy metals [35]. They are also involved in carcinogenesis and stimulate cell proliferation and angiogenesis. The increase in MT concentration in the cell correlates with the tumor grade. MTs are involved in the immune response, wound healing, and the development of multidrug resistance (MDR) [36]. The importance of MT expression as a tumor biomarker is mainly observed in squamous neoplasms of the larynx [34], lung [37], stomach, intestine, pancreas, uterus, and breast [38].

Studies on irisin expression in neoplastic tissues have demonstrated its potential as an independent diagnostic and prognostic factor. The expression of irisin in laryngeal cancer has not been studied yet. Therefore, the aim of the study was to determine the localization and the level of irisin expression in LSCC, as well as to investigate its relationship with such markers as Ki-67, MCM3,5,7, or MT-I/II and with clinicopathological factors in LSCC.

2. Materials and Methods

2.1. Patients Cohort

The study was conducted on material consisting of 140 cases of laryngeal squamous cell carcinomas (LSCCs) and 57 cases of laryngeal papillomas (BLs), which were collected between 1997 and 2003. All patients were diagnosed and treated at the Department of Pathomorphology of the J. Babinski Regional Hospital of Wroclaw and the Department and Clinic of Otolaryngology, Head and Neck Surgery, Wroclaw Medical University. Fourteen blocks with the sections of vocal cord nodules and Reinke's edema were used as the control. Patients gave their written informed consent, and the study was approved by the Bioethical Committee of Wroclaw Medical University (ID No. KB-355/2019). The mean age of the patients during the treatment period was 59 years (41–79 years). The treatment and follow-up included 20 women and 120 men based on the TNM classification of the International Union Against Cancer (UICC) [39], the grade of malignancy (G), and the clinical stage of LSCC were determined.

2.2. Cell Line Culture

Protein expression studies (Western blot and immunofluorescence [IF]) were conducted using the reference Larynx Epidermoid Carcinoma 2 (HEp-2) (collection of cell lines of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland) adherent laryngeal cancer cell line, and the Normal Human Keratinocyte cell line (HaCaT) (The American Type Culture Collection, Manassas, VA, USA), an adult normal immortalized human keratinocyte cell line, was used as the control. HEp-2 cells were cultured in EMEM medium (Lonza, Basel, Switzerland). HaCaT cells were cultured in DMEM medium (Lonza). Both media were supplemented with 10% fetal bovine serum (FBS) (Merck, Darmstadt, Germany), 1% penicillin/streptomycin (Merck), and L-glutamine (Merck). The HERA cell incubator (Heraeus, Hanau, Germany) was used to maintain constant conditions of cell cultures, i.e., a temperature of 37 °C, 5% CO_2 concentration, and a 95% humidity level.

2.3. Tissues Microarray (TMA) Preparation

Tissue sections were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Tissue microarrays (TMA) were performed from paraffin blocks—1 TMA containing the material with vocal cord nodules, 5 TMAs containing LSCC tumors, and 2 TMAs with BLs. Routinely, paraffin blocks were cut and hematoxylin and eosin (HE) staining was performed. The HE stained slides were examined under light microscopy (BX-42; Olympus, Tokyo, Japan) by two independent pathologists. Subsequently, the HE slides were scanned using the Pannoramic Midi II histological scanner (3DHistech, Budapest, Hungary). Using the Pannoramic Viewer Program (3DHistech), representative cancers sites with a core size of 1.5 mm were selected, followed by their transfer to the tissue recipient arrays using the TMA Grand Master (3DHistech Ltd., Budapest, Hungary).

2.4. Immunohistochemistry (IHC)

TMAs were cut into 4-µm-thick paraffin sections and mounted on Superfrost Plus slides (Menzel Gläser, Braunschweig, Germany). Deparaffinization, hydration, and thermal demasking of epitopes were performed using the Pre Treatment Link Station (Dako, Glostrup, Denmark). The slides were incubated at 97 °C for 20 min with the Target Retrieval Solution in high-pH buffer (Agilent Technologies, Santa Clara, CA, USA). IHC reactions were performed using specific polyclonal anti-irisin rabbit antibodies (dilution 1:50; code no. NBP2-14024; Novus Biologicals, Littleton, CO, USA), mouse monoclonal antibodies: anti-MCM5 (clone E-10, dilution 1:100, Santa Cruz Biotechnology, Dallas, TX, USA), anti-Ki-67 (clone MIB1, ready to use; Agilent Technologies), anti-MT-I/II (clone E9, dilution 1:100; Agilent Technologies), anti-MCM2 (clone CRCT2.1, dilution 1:15; Leica Biosystems, Nussloch, Germany), anti-MCM3 (clone 101, dilution 1:50; Agilent Technologies), and anti-MCM7 (clone DCS-141.1, dilution 1:50; Leica Biosystems). The antibody diluent (Agilent Technologies), with a background reducing component, was used to dilute the primary antibodies. IHC reactions were performed using the Autostainer Link (Agilent Technologies) and the EnVision™ FLEX high-pH Link visualization system (Agilent Technologies). Control of the immunohistochemical reaction was performed without the addition of the primary antibody.

2.5. Evaluation of Immunohistochemistry (IHC)

A positive IHC reaction of irisin, Ki-67, MCM3,5,7, and MT-I/II in tissue specimens in LSCCs, BLs, and control was observed using the BX41 light microscope (Olympus, Tokyo, Japan) coupled with the Cell D program (Olympus). The analysis of the expression of the proteins was performed using ×200 magnification. The intensity of the cytoplasmic reaction detecting the presence of irisin and of the MT-I/II semiquantitative Immunoreactive Score (IRS) method, according to Remmele and Stegner [40], were used.

The evaluation of the IHC reaction was conducted by two independent pathologists. The intensity of the color reaction in the cytoplasm of cells (from 0 to 3 points) and the percentage of neoplastic cells with a positive reaction (from 0 to 4 points) were estimated. The score is the result of the multiplication of color intensity and percentage of cells with positive reactions. The result can take values from 0 to 12 points. Details of the IRS scale are presented in Table 1.

Table 1. The semiquantitative scale assessing the irisin and MT-I/II expression in control tissues of the larynx, BLs, and LSCC according to Remmele and Stegner (IRS) [40]. Modified table according to Nowinska [34].

Points	Percentage of Cells with Positive Reaction	Points	Color Intensity of Positive Reaction in Cells
0	0%	0	lack
1	1–10%	1	weak
2	11–50%	2	moderate
3	51–80%	3	strong
4	>80%		

The percentage of cells with a positive reaction was calculated compared to the total number of tumor cells. The level of Ki-67, MCM3, MCM5, and MCM7 expression was assessed with the use of a five-point scale (0 point—>0%; 1 point—>0–10%; 2 points—>10–25%; 3 points—>25–50%; 4 points—>50–100% of expression) [41,42].

2.6. Immunofluorescence (IF)

To perform IF, 24-h microculture cells were placed on slides (Millicell EZ 8-well glass slides; Merck). For microculture, 600 µL of 20 × 10^4 cells/mL suspension was instilled into each well on the slides. Microcultures with cells were placed in an incubator at 37 °C for 24 h. After the incubation, the cells were fixed with the use of 4% formaldehyde and further

incubation was conducted with the specific polyclonal rabbit anti-irisin/FNDC5 antibody (dilution 1:50; code no. NBP2-14024; Novus Biologicals) at 4 °C overnight. After rinsing, the slides were incubated for 1 h with polyclonal donkey anti-rabbit secondary AlexaFluor 568 conjugated antibody (dilution 1:2000; code no. A10042; Invitrogen, Carlsbad, CA, USA) in the reagent with a background-reducing component (Agilent Technologies). The slides were mounted using the Prolong DAPI Mounting Medium (Invitrogen). The observations were made at x600 magnification with the use of Fluoview FV3000 confocal microscopy (Olympus) coupled with CellSense software (Olympus, RRID:SCR_016238).

2.7. Western Blot Analysis

For each analysis, 3×10^6 HEp-2 and HaCat cells were taken. The extraction of whole cell proteins was made using the RIPA buffer (50 mM Tris HCl; 150 mM NaCl; 0.1% SDS; 1% Igepal (CA-630, Merck, Darmstadt, Germany); 0.5% sodium deoxycholate; protease inhibitor cocktail (Merck); 0.5 mM PMSF). The concentration of proteins was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The proteins were denatured in sample loading buffer (250 mM Tris-HCl, 40% glycerol, 20% β-mercaptoethanol, 8% SDS and bromophenol blue), transferred to a PSQ membrane (Millipore, Burlington, MA, USA), and blocked with 2% non-fat milk (Bio-Rad, Marnes-la-Coquette, France) in 0.1% TBST for 1 h at room temperature. Next, the membrane was incubated with rabbit polyclonal anti-irisin/FNDC5 antibody diluted in 0.5% milk in 0.1% TBST (1:200, code no. NBP2-14024, Novus Biologicals) overnight at 4 °C with gentle shaking. Incubation with the secondary horseradish peroxidase, conjugated with donkey anti-rabbit antibody and diluted in 0.5% milk in 0.1% TBST (1:3000, code no. 711-035-052; Jackson ImmunoResearch, Cambridgeshire, UK), was performed for 1 h at room temperature. The proteins were visualized using the Luminata Classico Western HRP Substrate (Millipore). The membrane was stripped and incubated again with monoclonal mouse anti-βactin antibody diluted in 1% milk in 0.1% TBST (dilution 1:5000, clone 2D4H5, code no. 66009-1-Ig, Proteintech, Rosemont, IL, USA), and this was used as the control for the amount of protein loading. The data were collected in the Chemi-Doc XRS Molecular Imager apparatus (Bio-Rad). The optical density of the protein band was measured with the use of the Image Lab (Bio-Rad) software. The experiment was repeated three times.

2.8. Statistical Analysis

The Kolmogorov–Smirnov test was used to check the normality of the distribution. The differences in the expression of irisin in BLs and LSCCs, as well as their relationship with clinicopathological factors, were examined using the Kruskal–Wallis test or the Mann–Whitney U test. Associations between irisin and the MCM2, MCM3, and MCM7 proteins, Ki-67 antigen, and MT-I/II were assessed by the Spearman rank correlation test. The Kaplan–Meier analysis and the Cox regression method were used to check the relationship between the intensity of irisin expression and overall survival. The unpaired t-test was used to assess the differences between the level of irisin in cell lines. The statistical analysis was performed using Prism 5.0 (GraphPad, La Jolla, CA, USA) software and p values < 0.05 were considered statistically significant.

3. Results

3.1. Immunohistochemical (IHC) Detection of Irisin Expression in Tissue Microarrays (TMAs)

Our study showed the expression of irisin in controls, BLs, and LSCCs (Figure 1). In LSCC, irisin expression was found in the cytoplasm of cancer cells. No significant difference of irisin expression was found between the control group and BLs. However, irisin expression levels were significantly increased in LSCCs compared to BLs (Mann–Whitney U test, $p < 0.0001$), and the control group (Mann–Whitney U test, $p = 0.001$) (Figure 2A). The associations between low and high expression and clinicopathological characteristics of patients are shown in Table 2.

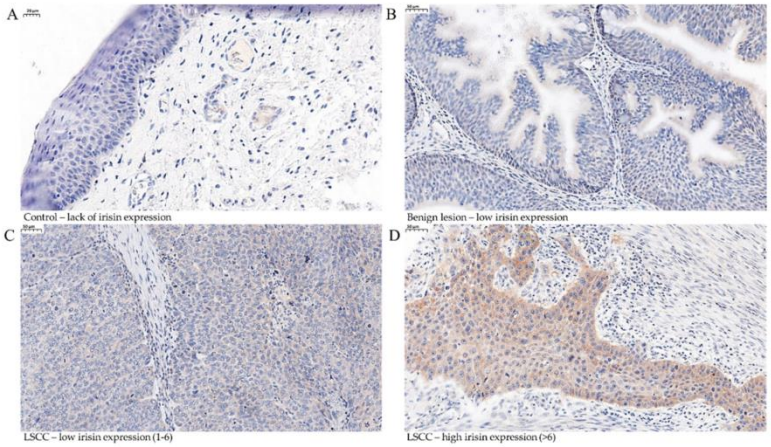

Figure 1. Immunohistochemical (IHC) reactions (brown) indicating irisin expression in the cytoplasm performed on control tissue (**A**) (magnification ×400, scale bar 20 µm, benign lesions (BLs)) and (**B**) in laryngeal squamous cell cancer (LSCC) (low (1–6) (**C**) and high (>6) (**D**)) (magnification ×200).

Table 2. Clinicopathological characteristics of LSCC patients related to irisin expression.

Clinicopathological Parameter		n 140 (%)	Irisin Expression in LSCC Cancer Cells		
			Low 1–6	High >6	Chi^2 Test *p* Value
Age *	≤60	76 (56,3)	66 (47,10)	10 (7,1)	0.9457
	>60	59 (43,7)	51 (36,4)	8 (5,7)	
Sex	Male	120 (85,7)	49 (35,0)	71 (50,7)	0.7262
	Female	20 (14,3)	9 (6,4)	11 (7,8)	
Tumor size (T)	T1–T2	54 (38,6)	50 (35,7)	4 (2,8)	0.0834
	T3–4	86 (61,4)	70 (50,0)	16 (11,4)	
Lymph nodes * (N)	N0	98 (72,6)	86 (61,4)	12 (8,6)	0.8475
	N1	15 (11,1)	13 (9,3)	2 (1,4)	
	N2–N3	24 (17,7)	20 (14,3)	4 (2,8)	
Stage *	I	7 (5,1)	6 (4,3)	1 (0,7)	0.0216
	II	37 (27,2)	37 (26,4)	0 (0,0)	
	III–IV	94 (69,1)	77 (55,0)	17 (12,1)	
Grade of malignance (G)	G1	30 (21,5)	27 (19,3)	3 (2,1)	0.3803
	G2	92 (65,7)	79 (56,4)	11 (7,8)	
	G3	18 (12,8)	13 (9,3)	4 (2,8)	

Abbreviations: LSCC—laryngeal squamous cell carcinoma; significance in bold; * missing data: Age—5 cases; Lymph nodes—3 cases; Stage—2 cases.

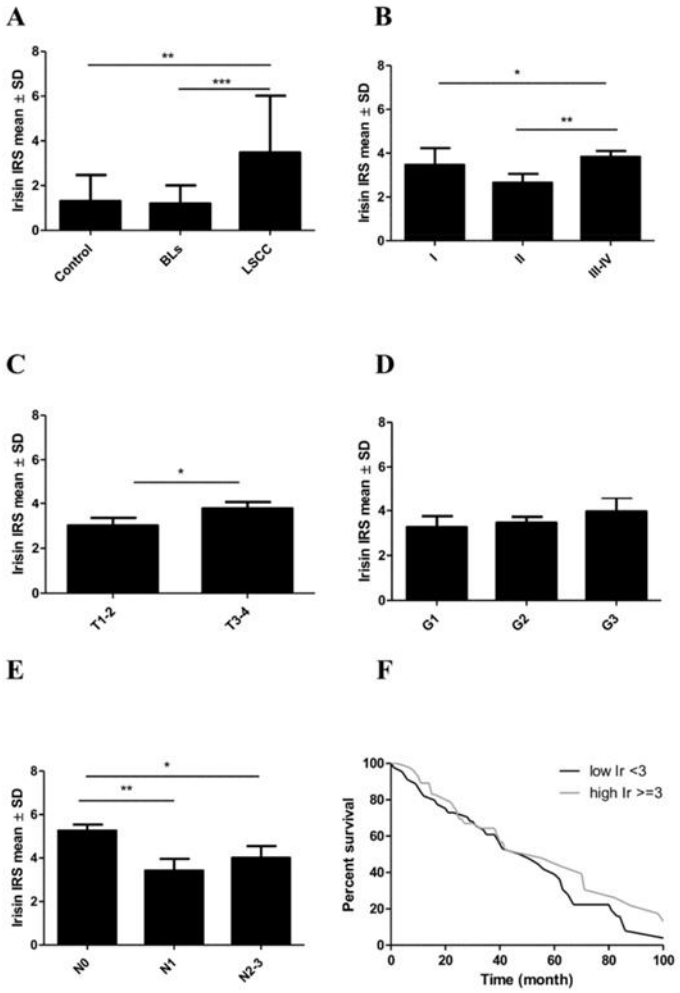

Figure 2. Comparison of irisin expression levels detected by immunohistochemistry (IHC) in control tissues, benign lesions (BLs), and laryngeal squamous cell cancer (LSCC) (**A**). Comparison of irisin expression levels in LSCC cells according to the tumor stage (**B**), tumor size (**C**), the grade of malignancy (**D**), and the lymph node status (**E**). Kaplan–Meier survival curves show the prognostic impact of irisin expression levels on overall survival (OS) of patients with LSCC. Patients were grouped according to the median value of expression levels (**F**), * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.001$.

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

The relationships between the level of irisin expression in cancer cells and the clinical and pathological parameters in LSCC are given in Table 3. The mean level of irisin expression for stage I was 3.46 ± SD 1.86, then it decreased to 2.65 (±SD 2.52) in stage II and increased again in stages III and IV (mean 3.86 ± SD 2.55). We observed a statistically

significant difference between stage II and stages III–IV (Mann–Whitney U test, $p = 0.0083$) (Figure 2B).

Table 3. Associations of irisin expression level with clinicopathological characteristics in patients with LSCC.

LSCC	*p* Value (Mann–Whitney U Test)		Mean Value ± SD
Lymph nodes		Lymph nodes	
N0 vs. N1	**0.0031**	N0	5.25 ± 2.02
N0 vs. N2–3	**0.0457**	N1	3.41 ± 2.10
N1 vs. N2–3	0.5101	N2–3	4.00 ± 2.68
Tumor size		Tumor size	
T1–2 vs. T3–4	**0.0348**	T1–2	3.00 ± 2.45
		T3–4	3.78 ± 2.58
Stage		Stage	
I vs. II	0.2785	I	3.46 ± 1.86
I vs. III–IV	0.7991	II	2.65 ± 2.52
II vs. III–IV	**0.0083**	III–IV	3.83 ± 2.55
Grade of malignancy	0.5354	Grade of malignancy	3.26 ± 2.70
G1 vs. G2	0.2857	G1	3.45 ± 2.47
G1 vs. G3	0.4415	G2	3.95 ± 2.64
G2 vs. G3		G3	

Abbreviations: LSCC—laryngeal squamous cell cancer; significance in bold.

We also observed an elevated level of irisin expression with increasing tumor size (T). The differences between T1–2 and T3–4 were statistically significant (Mann–Whitney U test, $p = 0.0348$). The highest mean level of irisin was observed in T3–4 (mean 3.78 ± SD 2.58) compared to T1–2 (mean 3.00 ± SD 2.45) (Figure 2C). The level of irisin expression in LSCC increased slightly with the increase in tumor malignancy (G). The differences between the individual groups were not statistically significant (Figure 2D).

We also noticed an association between irisin expression levels and lymph node metastasis. The highest mean value was observed for N0 (5.25 ± SD 2.02) and the lowest for N1 (mean 3.41 ± SD 2.10). Finally, the mean value (4.00 ± SD 2.68) increased again with lymph node metastasis (N2–3). The differences between the levels of irisin in N0 and N1 and in N0 and N2–3 were statistically significant (Mann–Whitney U test, $p = 0.0031$ and $p = 0.0457$, respectively) (Figure 2E). We did not observe any association between overall survival (OS) and irisin, MCM3,5,7, and Ki-67 in patients with LSCC (Table 4, Figure 2F).

Table 4. Univariate and multivariate Cox proportional hazards analyses in 140 patients with LSCC.

Clinicopathological Parameter	Univariate Analysis HR (95% CI) *p*
pT	0.98 (0.60–1.62)
T1–T2 vs. T3–T4	0.9787
pN	0.76 (0.43–1.35)
N0 vs. N+	0.3705
Grade	0.90 (0.50–1.60)
G1 vs. G2–G3	0.7320
Stage	0.80 (0.48–1.33)
I–II vs. III–IV	0.3883

Table 4. *Cont.*

Clinicopathological Parameter	Univariate Analysis HR (95% CI) *p*
Irisin <25% vs. ≥25%	1.46 (0.90–2.40) 0.1183
Ki-67 <25% vs. ≥25%	1.16 (0.68–1.98) 0.5779
MT- I/II <25% vs. ≥25%	1.18 (0.72–1.88) 0.5000
MCM3 <25% vs. ≥25%	1.37 (0.82–2.24) 0.2153
MCM5 <25% vs. ≥25 %	1.42 (0.89–2.27) 0.1442
MCM7 <25% vs. ≥25%	3.32 (0.60–18.33) 0.1665

Abbreviations: HR—hazard ratio; CI—confidence interval; LSCC—laryngeal squamous cell cancer.

3.3. Associations between Irisin and Cancer Cell Proliferation

The expression of other proteins (Ki-67, MCM3,5,7) that are associated with proliferation was observed in nuclei, and the expression of MT-I/II was found in the cytoplasm (Figure 3A–F).

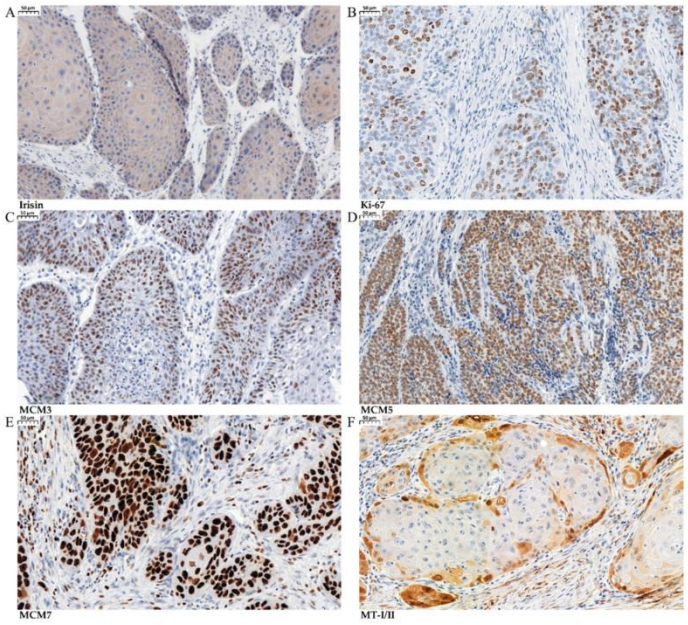

Figure 3. Immunohistochemical (IHC) reactions (brown) indicating irisin expression in the cytoplasm in laryngeal squamous cell cancer (LSCC) (**A**) with antigen Ki-67 (**B**), MCM3 (**C**), MCM5 (**D**), MCM7 (**E**), and MT-I/II (**F**) in LSCC (magnification ×200).

We found a positive moderate correlation between irisin and the Ki-67 antigen expression level in the nuclei of LSCC cells (r = 0.36; $p < 0.0001$) (Figure 4A). In addition, we identified a weak positive relationship between irisin and MCM3 expression levels (r = 0.25; $p = 0.0033$) (Figure 4B). However, we did not observe any significant correlation between irisin and MCM5 expression (r = 0.12, $p = 0.1512$), or between irisin and MCM7 (r = −0.17, $p = 0.790$). A moderate positive correlation was also demonstrated by comparing the level of irisin expression in LSCC and MT-I/II (r = 0.35, $p < 0.0001$) (Figure 4C).

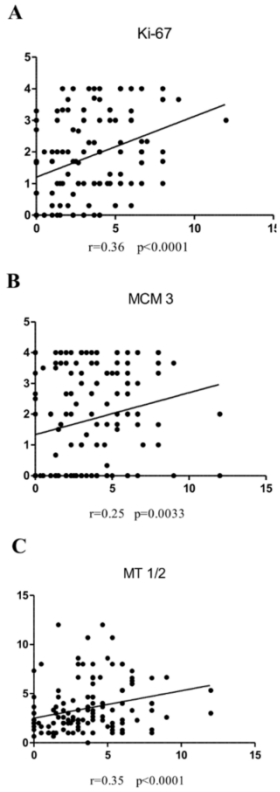

Figure 4. Correlations of irisin expression levels with diagnostic markers are moderate positive Ki-67 (**A**), weak positive MCM3 (**B**), and moderate positive MT-I/II (**C**) in LSCC cells.

3.4. Irisin Expression Levels in Cancer Cell Lines

Our in vitro study indicated the presence of irisin expression in both cell lines. However, a higher level of irisin was observed in HEp-2 laryngeal cancer cells compared to its level in control HaCat cells. The difference was not statistically significant. The results obtained using Western blot (Figure 5) were confirmed by confocal microscopy (Figure 6).

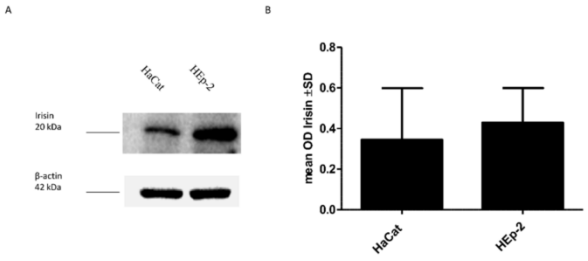

Figure 5. Comparison of irisin expression levels detected by Western blot in Normal Human Keratinocytes (HaCat) and Larynx Epidermoid Carcinoma 2 (HEp-2) (**A**). The mean value of the densitometric optical analysis of the 3 repeats of Western blot detecting irisin expression levels (**B**).

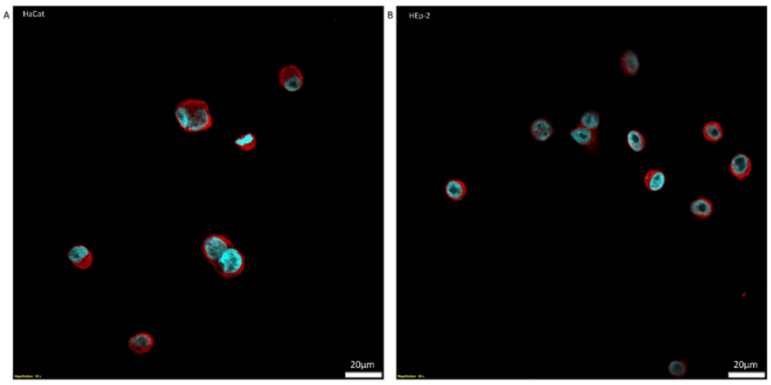

Figure 6. Localization of irisin (red color) around the nucleus (blue color) expression in cells' cytoplasm detected by immunofluorescence reaction (IF) in Normal Human Keratinocytes (HaCat) (**A**) and Larynx Epidermoid Carcinoma 2 (HEp-2) (**B**) (magnifications ×600, scale bar-20 µm (lower right corner)).

4. Discussion

Our study is the first to assess irisin expression in LSCC tissues. The investigation showed the expression of irisin in both non-cancerous and LSCC cells. A significantly higher concentration of irisin was observed in LSCC with regard to BLs and controls. Irisin was expressed primarily in the cytoplasm of laryngeal cancer cells. Previous studies were focused on the distal part of the respiratory tract. It was also the first study that used the tissue obtained from NSCLC tumors and the results were correlated with the clinicopathological data of patients [8]. Former studies confirming the expression of irisin in cancer cells of the respiratory system were carried out in an in vitro model using lung cancer cell lines [9]. An increased irisin expression in cancer cells of the head and neck area was observed in the tissues of thyroid tumors [7]. Ugur et al. [7] also showed that the increased concentration of irisin could be used in the differential diagnosis between malignant tumors and benign hyperplastic changes in the thyroid gland. Among other tumor tissues, increased irisin expressions were observed in cancers of the breast, ovary, cervix [21], and gastrointestinal tract [23], with the exception of hepatocellular carcinoma. In contrast, decreased irisin levels were observed in renal cancer tissues [43].

Most studies on irisin levels in cancers were based on its detection in the serum of patients. It was not possible to determine whether local or systemic production was respon-

sible for the increase in irisin concentration in tumor cells. The results of the tests using ELISA determining the serum concentration of irisin were inconclusive compared to the control group. Irisin levels were higher in patients with renal cancer [44], lower in patients with breast cancer [45], and unchanged in subjects with hepatocellular carcinoma [46]. A high expression of irisin was observed in skeletal muscles, whereas a moderate expression was found in adipose tissue [47]. However, the probability of irisin production by skeletal muscles and adipose tissue in cancer patients was reduced by their physical condition, and often by progressive cachexia. A decreased level of irisin in circulating blood could be the result of increased local production and a signal for the systemic regulation [45].

Moreover, in our studies, irisin expression levels were compared with clinicopathological parameters. We observed a higher irisin expression in larger tumors (T3–4) and tumors with a high grade of malignancy (G3). However, in the case of malignancy grade, we did not observe statistical significance. However, Panagiotou et al. [48] observed an increase in serum irisin levels in higher grades (Elston-Ellis) and the differences were significant, which confirmed our observation. Tumor growth requires a lot of energy and glucose, which are crucial for tumor proliferation. The consumption of glucose by cancer cells through aerobic respiration increases [8] and the overexpression of membrane glucose transporters (GLUTs) occurs. Overexpression and translocation to the membrane of GLUTs have been observed in many cancers, including breast, large intestine, salivary gland, and stomach cancer [49]. There is evidence that irisin affects GLUT4 translocation in the skeletal muscle of diabetic mice. Studies showed that irisin increased glucose tolerance and uptake. Irisin also improved glucose metabolism by increasing the phosphorylation of 5′AMP-activated protein kinase (AMPK) in myocytes and hepatocytes in in vitro and in vivo studies [50]. Irisin may change the metabolism of cancer cells, which could explain its relationship with the increase in its proliferation. However, further studies are warranted to explain this mechanism.

Most studies performed on the in vitro model revealed the inhibitory effect of irisin on the proliferation of cancer cells [9,27,28]. However, there are no studies that determined the existence of such a relationship in neoplastic tissues. Considering the results of these studies and the lack of knowledge in this area, we decided to investigate the correlation of irisin expression in LSCC with the standard markers of cancer cell proliferation. We obtained a positive moderate and weak correlation with Ki-67, MCM3, and MT-I/II antigens, which could indicate the potential role of irisin in the proliferation of LSCC cells. Positive correlations of irisin expression with cellular proliferation markers were also reflected and confirmed by association with clinicopathological factors (G, pT, pN, and Stage) in LSCC. Panagiotou et al. [48] also observed a positive correlation of serum irisin levels with the Ki-67 antigen in patients with benign and malignant breast cancer. Moreover, Shi et al. [46] reported that in human hepatocellular carcinoma (HHC), irisin could enhance the proliferation of neoplastic cells via the PI3K/AKT pathway. Contrary to our results, Shao et al. [9] indicated that a higher level of expression could lead to the inhibition of proliferation, migration, and the epithelial–mesenchymal transition (EMT). However, these studies used an in vitro model. In our previous study, we observed associations between irisin and cancer cell proliferation in NSCLC cells. Irisin expression in cancer cells correlated negatively with the expression of the Ki-67 antigen in NSCLC cells [8]. The differences may be due to tissue specificity and, hence, further research is warranted.

The phenomenon of EMT is the basis of metastasis [51]. Previous studies showed that irisin could be involved in cancer metastasis by inhibiting the EMT [24,27]. In our study, we observed the highest expression of irisin in the group of patients without lymph node metastasis (N0). This could demonstrate the protective effect of irisin in the pre-metastatic tumor. A similar protective effect of high levels of irisin (serum) was observed in breast cancer. The study on patients with breast cancer revealed that serum irisin level was higher in the group without spinal metastasis. The patients with higher serum levels of irisin had a 20% reduction in the possibility of metastasis [47]. In our opinion, the increased level of irisin in the N0 group of patients is associated with the inhibition of EMT and the

prevention of metastasis in LSCC. So far, only a few studies have analyzed the possible relationship between irisin expression and EMT. The results of these studies suggest that irisin may reverse the EMT process of cells. Kong et al. [24] observed that irisin reverses EMT depending on the IL-6 pathway in osteosarcoma. They suggest that irisin regulates Snail expression via STAT3. In our study, the level of irisin decreased in patients who had metastasis to a single lymph node on the same side of the neck as the tumor (N1). The level of irisin increased again if cancer cells spread to more lymph nodes (N2–3). Despite the re-increase in irisin levels in N2–3, it did not reach a level as high as that of N0. The explanation of the re-increase in irisin expression in the N2–3 group requires further research in order to enable full understanding of this phenomenon.

Our IHC study revealed a higher irisin expression in LSCC cells compared to control tissues. The levels of irisin expression determined by Western blot and IF were the same as expected. In the cell lines, irisin expression was significantly higher in the laryngeal cancer cell line (HEp-2) compared to the control cell line (HaCat). IHC confirmed the results obtained in the in vitro model. Our previous study on lung cancer cells also indicated the higher expression of irisin in cancer cells compared to control cells [8]. Shi et al. [46] showed increased proliferation, migration, and invasiveness of hepatocellular carcinoma cells caused by activation of the PI3K/Akt pathway after irisin treatment. On the other hand, Moon et al. [28] showed the inhibiting effect of irisin treatment on the proliferation of endometrial (KLE and RL95-2), colon (HT29 and MCA38), thyroid (SW579 and BHP7), and esophageal (OE13 and OE33) cancer cell lines. The ambiguous results of the studies may arise from the tissue and cell specificity in irisin expression [46] or may be associated with the form of irisin used (glycosylated or non-glycosylated) [9,24,26–28,46]. The studies in the in vitro model investigating the effects of irisin on cancer cells and the expression levels in cancer cells showed unclear results. More research is warranted to determine the effects of irisin on cancer cells.

5. Conclusions

In conclusion, our study was the first to show irisin expression in LSCC. We observed a higher irisin expression for LSCC compared to control tissues and its association with tumor growth and lymph node metastasis. Moreover, we analyzed irisin expression with clinicopathological factors and established cancer proliferation factors, indicating the potential role of irisin as a biomarker in the diagnostic process of LSCC.

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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 on reasonable request. To access protocols or datasets, contact katarzyna.nowinska@umw.edu.pl.

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7. Podsumowania i wnioski

Przeprowadzone w ramach pracy doktorskiej badania wskazały na potencjalną rolę Ir w procesach kancerogenezy raka płaskonabłonkowego krtani (LSCC). Obserwowane dodatnie średnie korelacje ekspresji Ir z uznanymi markerami proliferacji komórek nowotworowych: Ki-67, MCM3 i MT-I/II mogą sugerować związek ekspresji Ir z procesem proliferacji komórek LSCC. Na podstawie uzyskanych wyników dowiedziono, że komórki raka płaskonabłonkowego krtani wykazują wyższy poziom ekspresji Ir niż w tkankach kontrolnych (łagodne zmiany krtani). Ponadto wzrastający poziom ekspresji Ir obserwowano w guzach większych oraz w wyższych stadiach zaawansowania klinicznego. Może to wskazywać na potencjalną użyteczność Ir w diagnostyce raków płaskonabłonkowych krtani o większym potencjale proliferacyjnym.

Bardzo interesujące wydają się być wyniki wskazujące na zmiany poziomu ekspresji Ir w przebiegu powstawania przerzutów do kolejnych grup węzłów chłonnych. Najwyższy poziom ekspresji Ir obserwowano u pacjentów bez przerzutów do węzłów chłonnych (N0). Mogłoby to sugerować supresyjną rolę Ir w procesie przerzutowania nowotworowego. Wraz ze wzrostem masy guza i pojawieniem się przerzutów do regionalnych węzłów chłonnych (N1) poziom Ir spada. W czasie przerzutowania (N2-3) ponownie wzrasta, nie osiągając poziomu ze stadium N0. Rosnący ponownie poziom ekspresji Ir w stadiach N2-3 może być związany z dalszym niekontrolowanym już wzrostem masy guza. Może wynikać również z uwalniania Ir poza samym guzem np. z komórek podścieliska nowotworu. Otwarte pozostaje nadal pytanie, czy spadek ekspresji Ir jest przyczyną powstania przerzutów, czy zjawiskiem wtórnym.

Konieczne są dalsze badania, które ocenią znaczenie zmian poziomu Ir wraz z objęciem kolejnych okolicznych węzłów chłonnych. Istotne wydaje się także ustalenie, czy zmiany są wynikiem specyfiki tkankowej raka krtani. Jeśli tak, ocena poziomu ekspresji Ir mogłaby być także przydatna do prognozowania powstawania przerzutów. Stąd konieczne są dalsze badania nad rolą Ir w procesach kancerogenezy oraz progresji raka płaskonabłonkowego krtani i potencjalnej użyteczności tego białka w diagnostyce LSCC.

Wnioski:

1. Podwyższony poziom Ir w rakach płaskonabłonkowych krtani (LSCC), w porównaniu do zmian łagodnych wskazuje, że może być on użyteczny jako potencjalny marker różnicujący zmiany łagodne i LSCC.

2. Obserwowana korelacja Ir z markerami proliferacji komórek nowotworowych: Ki-67, MCM3 and MT-I/II może wskazywać na jej rolę w proliferacji komórek raków płaskonabłonkowych krtani i jej użyteczność w ocenie rokowniczej tych nowotworów.

3. Obniżenie poziomu Ir w komórkach raka krtani może wskazywać na możliwość pojawienia się przerzutów odległych tego nowotworu.

8. Załączniki

a. Oświadczenia współautorów

b. Opinia Komisji Bioetycznej

c. Dorobek Naukowy

Lek. Agnieszka Pinkowska
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Wrocław, dn. 07.02.2023 r.

OŚWIADCZENIE

Oświadczam, że w pracy

Agnieszka Pinkowska, Marzenna Podhorska- Okołów, Piotr Dzięgiel, Katarzyna Nowińska. The Role of Irisin in Cancer Disease. Cells 2021, 10, 1479. https://doi.org/10.3390/cells1006147
IF: 7.666; Pkt. MNiE: 140.000

mój udział polegał na opracowaniu koncepcji pracy, zebraniu niezbędnego piśmiennictwa, napisaniu manuskryptu i sporządzeniu rycin.

Podpis

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Wrocław, dn. 07.02.2023 r.

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Agnieszka Pinkowska, Marzenna Podhorska- Okołów, Piotr Dzięgiel, Katarzyna Nowińska. The Role of Irisin in Cancer Disease. Cells 2021, 10, 1479. https://doi.org/10.3390/cells1006147
IF: 7.666; Pkt. MNiE: 140.000

mój udział polegał na nadzorze merytorycznym przygotowywanej pracy poglądowej, a także recenzowaniu i korekcie wstępnej wersji manuskryptu.

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Wrocław, dn. 07.02.2023 r.

OŚWIADCZENIE

Oświadczam, że w pracy

Agnieszka Pinkowska, Marzenna Podhorska- Okołów, Piotr Dzięgiel, Katarzyna Nowińska. The Role of Irisin in Cancer Disease. Cells 2021, 10, 1479. https://doi.org/10.3390/cells10061479 IF: 7.666; Pkt. MNiE: 140.000

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Wrocław, dn. 07.02.2023 r.

OŚWIADCZENIE

Oświadczam, że w pracy

Agnieszka Pinkowska, Marzenna Podhorska- Okołów, Piotr Dzięgiel, Katarzyna Nowińska. The Role of Irisin in Cancer Disease. Cells 2021, 10, 1479. https://doi.org/10.3390/cells10061479 IF: 7.666; Pkt. MNiE: 140.000

mój udział polegał na pomocy w zebraniu i analizie niezbędnej do napisania pracy literatury, pomocy w przygotowaniu tekstu manuskryptu.

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Wrocław, dn. 07.02.2023 r.

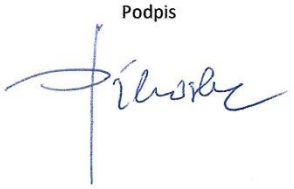
OŚWIADCZENIE

Oświadczam, że w pracy

Agnieszka Pinkowska, Katarzyna Nowińska, Urszula Ciesielska, Marzenna Podhorska- Okołów. Irisin Association with Ki-67, MCM3 and MT-I/II in Squamous Cell Carcinomas of the Larynx. Biomolecules 2022, 12, 52. https://doi.org/10.3390/biom12010052, IF: 6.064; Pkt. MNiE: 100,00

mój udział polegał na opracowaniu koncepcji pracy, przeglądzie i zebraniu piśmiennictwa, pomocy w części eksperymentalnej badań, analizie uzyskanych wyników i opracowaniu manuskryptu.

Podpis



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mój udział polegał na pomocy w części eksperymentalnej badań opisanych w tej pracy, analizie statystycznej wyników eksperymentów i pomocy w przygotowaniu tekstu manuskryptu.

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mój udział polegał na pomocy w przygotowaniu i tłumaczeniu na język angielski tekstu manuskryptu.

Podpis

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Wrocław, dn. 07.02.2023 r.

OŚWIADCZENIE

Oświadczam, że w pracy

Agnieszka Pinkowska, Katarzyna Nowińska, Urszula Ciesielska, Marzenna Podhorska- Okołów. Irisin Association with Ki-67, MCM3 and MT-I/II in Squamous Cell Carcinomas of the Larynx. Biomolecules 2022, 12, 52. https://doi.org/10.3390/biom12010052, IF: 6.064; Pkt. MNiE: 100,00

mój udział polegał na nadzorze merytorycznym całego projektu naukowego obejmującego badania opisane w tej pracy, a także recenzowaniu i korekcie wstępnej wersji manuskryptu.

Podpis

M. Podhorska-Okołów

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

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 355/2019

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

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

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

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

„Ekspresja iryzyny w rakach płaskonabłonkowych krtani"

zgłoszonym przez **lek. Agnieszkę Pinkowską** zatrudnioną w Zakładzie Anatomii Prawidłowej Katedry Morfologii i Embriologii Człowieka Uniwersytetu Medycznego we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła **wyrazić zgodę** na przeprowadzenie badania w Klinice Otolaryngologii, Chirurgii Głowy i Szyi USK oraz Zakładzie Anatomii Prawidłowej Katedry Morfologii i Embriologii Człowieka UMW pod nadzorem prof. dr hab. Marzenny Podhorskiej - Okołów **pod warunkiem zachowania anonimowości uzyskanych danych**.

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

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

Opinia powyższa dotyczy projektu badawczego będącego podstawą rozprawy doktorskiej.

Wrocław, dnia 1 kwietnia 2019 r.

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

Dorobek naukowy

1. Publikacje w czasopismach naukowych

1.1 Publikacje w czasopiśmie z IF

Lp	Opis bibliograficzny	IF	Punkty
1	**Pinkowska Agnieszka**, Podhorska-Okołów Marzenna, Dzięgiel Piotr, Nowińska Katarzyna: The role of irisin in cancer disease, Cells, 2021, vol. 10, nr 6, art.1479 [22 s.], DOI:10.3390/cells10061479	7,666	140
2	**Pinkowska Agnieszka**, Nowińska Katarzyna, Ciesielska Urszula, Podhorska-Okołów Marzenna: Irisin association with Ki-67, MCM3 and MT-I/II in squamous cell carcinomas of the larynx, Biomolecules, 2022, vol. 12, nr 1, art.52 [15 s.], DOI:10.3390/biom12010052	6,064*	100
	Podsumowanie	13,730	240

IF 2021

1.2 Publikacje w czasopiśmie bez IF

Lp	Opis bibliograficzny	Punkty
1	Mastalerz-Migas Agnieszka, Muszyńska Agnieszka, Pokorna-Kałwak Dagmara, Pawłowska-**Pinkowska Agnieszka**, Czajczyńska Anna, Wabik Aleksandra, Drobnik Jarosław, Steciwko Andrzej: Czy należy mierzyć ciśnienie dzieciom w wieku przedszkolnym?, Family Medicine & Primary Care Review, 2007, vol. 9, nr 3, s. 528-531	3
2	Muszyńska Agnieszka, Pawłowska-**Pinkowska Agnieszka**, Mastalerz-Migas Agnieszka, Pokorna-Kałwak Dagmara, Wychowaniec Katarzyna, Steciwko Andrzej: Trudności diagnostyczne w skąpoobjawowych, pozaszpitalnych zapaleniach płuc - opis przypadku, Family Medicine & Primary Care Review, 2007, vol. 9, nr 3, s. 916-918	3
3	Muszyńska Agnieszka, Pawłowska-**Pinkowska Agnieszka**, Pokorna-Kałwak Dagmara, Mastalerz-Migas Agnieszka, Roth Hanna, Steciwko Andrzej: Ocena przydatności szybkich testów CRP w ostrych infekcjach u dzieci, Family Medicine & Primary Care Review, 2008, vol. 10, nr 3, s. 535-538	6
4	Pokorna-Kałwak Dagmara, Pawłowska-**Pinkowska Agnieszka**, Sapilak Bartosz J., Mastalerz-Migas Agnieszka, Muszyńska Agnieszka, Steciwko Andrzej: Wyszczepialność szczepionkami skojarzonymi w Praktyce Lekarza Rodzinnego we Wrocławiu, Family Medicine & Primary Care Review, 2008, vol. 10, nr 3, s. 612-614	6

5	Pawłowska-**Pinkowska Agnieszka**, Pokorna-Kałwak Dagmara, Roemer-Ślimak Roma, Muszyńska Agnieszka: Najczęstsze problemy zdrowotne u 2- i 4-latków w praktyce lekarza rodzinnego, Family Medicine & Primary Care Review, 2010, vol. 12, nr 3, s. 778-780	6
6	Pokorna-Kałwak Dagmara, Roemer-Ślimak Roma, Muszyńska Agnieszka, Pawłowska-**Pinkowska Agnieszka**, Steciwko Andrzej: Zachorowalność na ospę wietrzną w populacji dzieci szczepionych jednorazową dawką szczepionki Varilrix w praktyce lekarza rodzinnego, Family Medicine & Primary Care Review, 2010, vol. 12, nr 3, s. 791-793	6
7	Pokorna-Kałwak Dagmara, Roemer-Ślimak Roma, Pawłowska-**Pinkowska Agnieszka**, Muszyńska Agnieszka, Steciwko Andrzej: Wyszczepialność przeciwko kleszczowemu zapaleniu mózgu w populacji dzieci od 1. do 18. roku życia w praktyce lekarza rodzinnego, Family Medicine & Primary Care Review, 2010, vol. 12, nr 3, s. 794-796	6
8	Pokorna-Kałwak Dagmara, **Pinkowska Agnieszka**, Muszyńska Agnieszka: Szczepienia przeciw wirusowi HPV w praktyce lekarza rodzinnego, Family Medicine & Primary Care Review, 2011, vol. 13, nr 3, s. 497-500	6
9	Domagała Zygmunt, **Pinkowska Agnieszka**, Piotrowska Aleksandra, Domański Jurand, Tarkowski Victoria, Zimmer-Stelmach Aleksandra, Śliwa Jakub: Utility of the Movat pentachrome stain technique in the microanatomical analysis of the human placenta, Italian Journal of Anatomy and Embryology, 2022, vol. 126, nr 2, s. 25-32, DOI:10.36253/ijae-13882	40
	Podsumowanie	82

1.3 Publikacje w czasopiśmie - prace kontrybutorskie -

2. Monografie naukowe

2.1 Książka autorska -
2.2 Książka redagowana -

2.3 Rozdziały

Lp	Opis bibliograficzny	Punkty
1	Pawłowska-**Pinkowska Agnieszka**, Mastalerz-Migas Agnieszka, Steciwko Andrzej: Badania hematologiczne, układu krzepnięcia, moczu, kału, badania mikrobiologiczne, W: Vademecum umiejętności praktycznych lekarza rodzinnego, (red.) Andrzej Steciwko, Wrocław 2007, Akad. Med., s. 49-62, ISBN 978-83-7055-127-8	6
2	Pawłowska-**Pinkowska Agnieszka**, Mastalerz-Migas Agnieszka, Steciwko Andrzej: Badania biochemiczne i immunochemiczne w surowicy krwi, W: Vademecum umiejętności praktycznych lekarza rodzinnego, (red.) Andrzej Steciwko, Wrocław 2007, Akad. Med., s. 63-78, ISBN 978-83-7055-127-8	6

3	Pawłowska-**Pinkowska Agnieszka**, Muszyńska Agnieszka, Pokorna-Kałwak Dagmara, Siejka Dominika: Zapalenia opon mózgowo-rdzeniowych w praktyce lekarza rodzinnego, W: Wybrane zagadnienia z praktyki lekarza rodzinnego. T.13: Kardiologia, nefrologia, psychologia, pediatria, geriatria, (red.) Andrzej Steciwko, Agnieszka Mastalerz-Migas, Wrocław 2008, Wydawnictwo Continuo, s. 133-142, ISBN 978-83-89629-93-7	3
4	**Pinkowska Agnieszka**, Rohan-Fugiel Anna, Gworys Bohdan: Knowledge of cervix cancer and human papilloma infection in 13-year-old females in relation to conducted protective vaccination. A preliminary study, W: Wellness and age, (red.) Ryszard Asienkiewicz, Małgorzata Biskup, Lublin 2016, NeuroCentrum, s. 121-135, ISBN 978-83-61495-91-8	5
5	Rohan Anna, Fugiel Jarosław, Buraczyńska Sandra, Kowalski Henryk, **Pinkowska Agnieszka**, Gworys Bohdan: Fitness effects of resistance tube exercise in women over 60 years of age, W: Wellness and age, (red.) Ryszard Asienkiewicz, Małgorzata Biskup, Lublin 2016, NeuroCentrum, s. 137-146, ISBN 978-83-61495-91-8	5
6	**Pinkowska Agnieszka**, Dąbrowski Paweł, Domagała Zygmunt, Woźniak Sławomir, Rohan-Fugiel Anna, Karykowska Aleksandra: Secondary prevention and oncological screening methods of breast cancer and cervical cancer: recommendations in Poland and abroad, W: Health and its determinants, (red.) Wiesław Kurlej, Halina Król, Lublin 2017, NeuroCentrum, s. 159-172, ISBN 978-83-61495-67-3, [Publikacja w wydawnictwie spoza listy MNiSW]	5
7	Rohan Anna, Fugiel J., Nyc M., **Pinkowska Agnieszka**, Dąbrowski Paweł, Domagała Zygmunt, Kacała Ryszard, Woźniak Sławomir: Physical activity and the quality of life in men over the age of 60, W: Health and its determinants, (red.) Wiesław Kurlej, Halina Król, Lublin 2017, NeuroCentrum, s. 173-181, ISBN 978-83-61495-67-3, [Publikacja w wydawnictwie spoza listy MNiSW]	5
8	Rohan Anna, Chromik K., Jakubiec D., **Pinkowska Agnieszka**, Sobiech K.A.: Selected lifestyle factors in first-year male university students, W: Prevention and health education, (red.) Henryk Duda, Małgorzata Biskup, Tomasz Wójcik, Lublin 2017, NeuroCentrum, s. 157-166, ISBN 978-83-61495-55-0, [Publikacja w wydawnictwie spoza listy MNiSW]	5
9	Dąbrowski Paweł, Grzelak Joanna, Kotylak Aleksandra, Olchowy Cyprian, Kulus Michał, Kurc-Darak Bożena, Domagała Zygmunt, Jura Maksym, Woźniak Sławomir, Rohan-Fugiel Anna, **Pinkowska Agnieszka**: Wstępna ocena stanu zdrowia i kondycji biologicznej ludności pochowanej na cmentarzu miejskim w Raciborzu (XIX/XX w.), W: Zagrożenie życia i zdrowia człowieka, (red.) Józef Tatarczuk, Bożena Zboina, Paweł Dąbrowski, Lublin 2017, NeuroCentrum, s. 39-62, ISBN 978-83-61495-71-0, [Publikacja w wydawnictwie spoza listy MNiSW]	5

10	**Pinkowska Agnieszka**: Opieka nad pacjentem z rakiem krtani po laryngektomii, W: Kompleksowa opieka nad pacjentem w chorobach cywilizacyjnych, (red.) Marzenna Podhorska-Okołów, Adam Matkowski, Izabella Uchmanowicz, Wrocław 2020, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, s. 175-179, ISBN 978-83-7055-636-5	20
	Podsumowanie	65

3. Varia

3.1 Komentarz -

3.2 Inne

Lp	Opis bibliograficzny
1	Muszyńska Agnieszka, Pawłowska-**Pinkowska Agnieszka**: Sprawozdanie z I Kongresu Top Medical Trends 2007. Polska, Poznań, 16-18 marca 2007 r., Family Medicine & Primary Care Review, 2007, vol. 9, nr 2, s. 321-324
2	Pawłowska-**Pinkowska Agnieszka**, Muszyńska Agnieszka: Sprawozdanie z II Kongresu Top Medical Trends 2008, Poznań 7-9 marca 2008 r., Family Medicine & Primary Care Review, 2008, vol. 10, nr 2, s. 281-284
3	Muszyńska Agnieszka, Pawłowska-**Pinkowska Agnieszka**: II Kongres Top Medical Trends 2008 [Poznań, 7-9 marca 2008 r.], Medium. Gazeta Dolnośląskiej Izby Lekarskiej, 2008, nr 7-8, s. 23

4. Abstrakty

Lp	Opis bibliograficzny
1	Domagała Zygmunt, **Pinkowska Agnieszka**, Piotrowska Aleksandra, Kobierzycki Christopher, Gomułkiewicz Agnieszka, Kurc-Darak Bożena, Dzięgiel Piotr: Ekspresja nestyny w prawidłowym dojrzałym łożysku ludzkim - doniesienie wstępne, W: 52. Zjazd Naukowy Polskiego Towarzystwa Histochemików i Cytochemików "Immunohistochemia i biologia molekularna w morfologii". Białystok, 13-16 września 2018. Streszczenia prezentacji ustnych oraz plakatowych 2018, 4 poz.U3
2	**Pinkowska Agnieszka**, Zimmer Aleksandra, Śliwa Jakub, Tarczyńska Anna, Piotrowska Aleksandra, Gomułkiewicz Agnieszka, Kurc-Darak Bożena, Dzięgiel Piotr, Domagała Zygmunt: Ekspresja wybranych białek w łożysku ludzkim, W: 52. Zjazd Naukowy Polskiego Towarzystwa Histochemików i Cytochemików "Immunohistochemia i biologia molekularna w morfologii". Białystok, 13-16 września 2018. Streszczenia prezentacji ustnych oraz plakatowych 2018, 5 poz.U4

3	Domagała Zygmunt, **Pinkowska Agnieszka**, Zimmer Aleksandra, Śliwa Jakub, Piotrowska Aleksandra, Woźniak Sławomir, Dzięgiel Piotr: Are any lymphatics vessels in normal human placenta - podoplanin, LYVE-1 and VGFR3 immunohistochemistry expression - the preliminary study, W: 114th Annual Meeting - 33. Arbeitstagung der Anatomischen Gesellschaft. Würzburg, 25-27 September 2019. Posterabstracts [online] 2019, poz.158, [[Dostęp 13.11.2019]. Dostępny w: https://anatomische-gesellschaft.de/data/uploads/content/abstract-archiv/AGM-2019-114-Poster.pdf]
4	Kurc-Darak Bożena, Kornafel Danuta, **Pinkowska Agnieszka**, Woźniak Sławomir, Domagała Zygmunt: Kondycja biologiczna chorych na zwyrodnienie stawu kolanowego, W: XLVII Ogólnopolska Konferencja Naukowa Polskiego Towarzystwa Antropologicznego "Antropos - między naturą a kulturą". Kraków, 11-13 września 2019 r. Program oraz streszczenia referatów i prezentacji plakatowych 2019, 36a

Sumaryczny impact factor: 13,730

	Punktacja MNiSW
do roku 2018	**87,0**
od roku 2019	**300,0**
Razem:	**387,0**