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PRACA DOKTORSKA

Aleksandra Izabela Partyńska

Ekspresja zyksyny w niedrobnokomórkowych rakach płuc

Promotor Pracy: prof. dr hab. Piotr Dzięgiel

Promotor pomocniczy: dr Agnieszka Gomułkiewicz

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Spis treści

Wykaz publikacji stanowiących podstawę Pracy Doktorskiej:

1. **Partyńska Aleksandra**, Gomułkiewicz Agnieszka, Dzięgiel Piotr, Podhorska-Okołów Marzenna. *The role of zyxin in carcinogenesis*. Anticancer Research, 2020, Vol. 40, no. 11, s. 5981-5988, DOI:10.21873/anticanres.14618

   IF=2,480 Pkt MEiN=70,00

2. **Partyńska Aleksandra**, Gomułkiewicz Agnieszka, Piotrowska Aleksandra, Grzegrzółka Jędrzej, Rzechonek Adam, Ratajczak-Wielgomas Katarzyna, Podhorska-Okołów Marzenna, Dzięgiel Piotr. *Expression of zyxin in non-small cell lung cancer – a preliminary study*. Biomolecules, 2022, Vol. 12, no. 6, art. 827, DOI:10.3390/biom12060827

   IF (2021)=6,064 Pkt MEiN=100,00

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Streszczenie

Rak płuc jest jednym z najczęstszych nowotworów złośliwych, charakteryzującym się wysoką zapadalnością i śmiertelnością. Pod względem histologicznym dzieli się na niedrobnokomórkowego raka płuc (ang. *non-small cell lung cancer*, NSCLC), stanowiącego ok. 85% wszystkich przypadków, oraz drobnokomórkowego raka płuc (ang. *small cell lung cancer*, SCLC). W niedrobnokomórkowym raku płuc wyróżnia się raka płaskonabłonkowego (ang. *squamous cell carcinoma*, SCC), gruczolakoraka (ang. *adenocarcinoma*, AC) oraz raka wielkokomórkowego (ang. *large cell carcinoma*, LCC). Ze względu na wysoką śmiertelność spowodowaną tą chorobą, badania nad poszukiwaniem markerów prognostycznych tego nowotworu wydają się nadal uzasadnione.

Zyksyna (ZYX) jest białkiem należącym do rodziny białek z domeną LIM (ang. *LIM domain proteins*), powszechnie znanym jako komponent ognisk adhezyjnych. Bierze ona udział w organizacji i remodelingu cytoszkieletu. Cząsteczka zyksyny składa się z miejsca wiążącego α-aktyninę, powtórzeń bogatych w prolinę ActA, sekwencji eksportu jądrowego (ang. *nuclear export sequence*, NES) oraz z trzech domen LIM. Wykazano, że białko to może ulegać przemieszczeniu z cytoplazmy do jądra komórkowego, co sugeruje jego udział w przekazywaniu sygnału z zewnątrz do wnętrza komórki oraz udział w regulacji ekspresji genów. Eksperymenty wykazały, że ZYX może uczestniczyć w takich procesach jak apoptoza czy przejście epitelialno-mezenchymalne.

Przeprowadzone dotychczas badania sugerują udział zyksyny w onkogenezie. Może ona pełnić zarówno funkcję promującą, jak i supresorową w procesie nowotworzenia. Onkogenną rolę ZYX wykazano między innymi w raku gruczołu piersiowego oraz jelita grubego. Natomiast supresorową rolę tego białka postuluje się w raku prostaty i pęcherza moczowego. Istnieje jednak niewielka liczba publikacji weryfikujących udział ZYX w powstawaniu niedrobnokomórkowego raka płuc. Badania pokazują zmniejszoną ekspresję ZYX w linii komórkowej niedrobnokomórkowego raka płuc. Inne eksperymenty demonstrują z kolei rolę ZYX w migracji i adhezji komórkowej. Wykazano również zmniejszony poziom ZYX w guzach nowotworowych w mysim modelu raka płuc. Dlatego też, celem naszych badań było określenie poziomu ekspresji ZYX w przypadkach niedrobnokomórkowego raka płuc, pozyskanych od pacjentów leczonych operacyjnie i skorelowanie otrzymanych wyników z danymi kliniczno-patologicznymi. Tym samym podjęto próbę odpowiedzi na pytanie, czy poziom ekspresji tego białka może mieć związek z rozwojem NSCLC?

Pierwsza praca zawarta w cyklu publikacji ma charakter przeglądowy. Jej celem było usystematyzowanie wiedzy na temat udziału zyksyny w rozwoju różnych typów nowotworów (*Partyńska Aleksandra, Gomułkiewicz Agnieszka, Dzięgiel Piotr, Podhorska-Okołów Marzenna. The role of zyxin in carcinogenesis. Anticancer Res., 2020, Vol. 40, no. 11, s. 5981-5988, DOI:10.21873/anticanres.14618)*. We wstępie pracy opisano budowę oraz funkcje ZYX. Dalsza część pracy skupia się na roli tego białka w rozwoju i progresji różnego typu nowotworów.

Druga publikacja jest pracą oryginalną opisującą ekspresję ZYX, zarówno na poziomie mRNA, jak i białka, w materiale klinicznym niedrobnokomórkowego raka płuc oraz w wybranych liniach komórkowych stanowiących model *in vitro* tego nowotworu (*Partyńska Aleksandra, Gomułkiewicz Agnieszka, Piotrowska Aleksandra, Grzegrzółka Jędrzej, Rzechonek Adam, Ratajczak-Wielgomas Katarzyna, Podhorska-Okołów Marzenna, Dzięgiel Piotr. Expression of zyxin in non-small cell lung cancer − a preliminary study. Biomolecules, 2022, Vol. 12, no. 6, art.827, DOI:10.3390/biom12060827*). Badania na materiale klinicznym zostały wykonane wykorzystując następujące metody badawcze: immunohistochemię (IHC), Western Blot, real-time PCR oraz RT-qPCR wykonany na mRNA pochodzącym z komórek wyizolowanych za pomocą mikrodysekcji laserowej. W badaniach IHC wykorzystano 399 przypadków NSCLC oraz 85 wycinków tkanki płuc niezmienionej nowotworowo, utrwalonych w postaci bloczków parafinowych oraz przygotowanych jako mikromacierze tkankowe. Wykonane reakcje IHC wykazały obecność ZYX w cytoplazmie, błonie komórkowej oraz jądrze komórkowym komórek nowotworowych oraz komórek prawidłowej tkanki płuc. Analiza statystyczna wykazała istotnie niższy poziom cytoplazmatycznej ZYX w komórkach NSCLC niż w tkance prawidłowej. Z kolei poziom jądrowej ZYX był podwyższony w komórkach NSCLC w stosunku do prawidłowych komórek płuc. Dalsze analizy pokazały wyższy poziom jądrowej ZYX w komórkach SCC niż w AC. Badania zademonstrowały również istotnie niższy poziom cytoplazmatycznej ZYX w komórkach NSCLC i SCC, w guzach pT3-4 niż w pT1. Zauważono także istotnie niższy poziom cytoplazmatycznej ZYX w komórkach NSCLC i SCC w stadium zaawansowania klinicznego III-IV niż w stadium I. Analizy wykazały, że ZYX nie może być traktowana jako niezależny czynnik prognostyczny w NSCLC. Metoda Western Blot pokazała niższy poziom białka ZYX w guzach NSCLC i SCC niż w tkance kontrolnej. Badania real-time PCR zademonstrowały niższy poziom mRNA *ZYX* w guzach NSCLC i AC niż w prawidłowej tkance płuc. Z kolei RT-qPCR wykonany na mRNA pochodzącym z komórek wyizolowanych za pomocą mikrodysekcji laserowej również wykazał niższy poziom mRNA *ZYX* w komórkach

NSCLC, SCC i AC niż w prawidłowych komórkach płuc. Poziom ZYX został zbadany także w liniach NSCLC (linia płaskonabłonkowego raka płuc − NCI-H1703, linia gruczolakoraka płuc − NCI-H522) oraz w linii kontrolnej prawidłowych fibroblastów płucnych IMR-90. Wykorzystane techniki badawcze obejmowały: immunofluorescencję, immunocytochemię, Western Blot oraz real-time PCR. Reakcje immunocytochemiczne wykazały obecność ZYX w cytoplazmie. Dodatkowo reakcje immunofluorescencyjne ujawniły obecność ZYX w jądrze komórkowym badanych komórek. Eksperymenty wykonane na liniach komórkowych zademonstrowały niższy poziom białka ZYX w liniach NSCLC niż w linii kontrolnej IMR-90. Z kolei analiza metodą real-time PCR wykazała wyższy poziom mRNA *ZYX* w linii komórkowej NCI-H1703 niż w linii prawidłowej IMR-90. Niższy poziom mRNA *ZYX* w stosunku do linii kontrolnej IMR-90 występował w linii NCI-H522.

Podsumowując, obniżony poziom ZYX w komórkach NSCLC w porównaniu do tkanki kontrolnej sugeruje, że ZYX może pełnić rolę białka supresorowego w rozwoju NSCLC. Jednakże uzyskane wyniki wskazują, że ZYX nie ma potencjału prognostycznego w tym typie nowotworu.

Summary

Lung cancer is one of the most frequent cancers, described with high incidence and mortality. Based on histological classification, non-small cell lung cancer (NSCLC), accounting for nearly 85% of all lung cancer cases, and small cell lung cancer (SCLC) are distinguished. Non-small cell lung cancer is subdivided into squamous cell carcinoma (SCC), adenocarcinoma (AC), and large cell carcinoma (LCC). As this disease is characterized by high mortality, research concerning searching for prognostic factors is still justified.

Zyxin (ZYX) is a protein owned by *LIM domain proteins* family. ZYX is commonly acknowledged as a component of focal adhesions. It is responsible for organization and remodeling of cytoskeleton. The molecule of zyxin is made up of the following sequences: α-actinin binding site, proline-rich ActA repeats, nuclear export sequences (NES), and three LIM domains. The studies demonstrated this protein could translocate from cytoplasm into nucleus. It advocates the contribution of ZYX to extra-to-intracellular signaling and gene expression regulation. The experiments showed ZYX could take part in processes such as apoptosis or epithelial-mesenchymal transition.

Studies so far undertaken have suggested the role of ZYX in oncogenesis. This protein might promote as well as suppress carcinogenesis. The oncogenic function of ZYX has been shown, inter alia, in breast cancer and colorectal cancer. However, the suppressive role of this protein has been postulated in prostate cancer and bladder cancer. In the literature, there are not enough publications verifying the contribution of zyxin to non-small cell lung cancer development. Downregulation of ZYX expression was described in a NSCLC cell line. Other experiments demonstrated the position of ZYX in migration and cell adhesion. Decreased level of zyxin was shown in tumours of lung cancer murine model. Therefore, the aim of our study was to evaluate the expression of ZYX in NSCLC cases, collected from patients treated surgically, and to correlate the obtained results with the clinicopathological data. These measures let answer the question of whether the expression level of zyxin could be associated with NSCLC development?

Summary

The first paper included in a publication series is a review paper. Its purpose was to systemize the knowledge about the role of zyxin in cancer development (*Partyńska Aleksandra, Gomułkiewicz Agnieszka, Dzięgiel Piotr, Podhorska-Okołów Marzenna. The role of zyxin in carcinogenesis. Anticancer Res., 2020, Vol. 40, no. 11, s. 5981-5988, DOI: 10.21873/anticanres.14618)*. In the introduction section, the structure and function of ZYX are described. The further part of the publication concerns the role of this protein in the development and progression of different cancer types.

The second publication is classified as an original research paper describing the mRNA and protein ZYX levels in NSCLC clinical specimens as well as in chosen cell lines which constitute the *in vitro* model of this cancer (*Partyńska Aleksandra, Gomułkiewicz Agnieszka, Piotrowska Aleksandra, Grzegrzółka Jędrzej, Rzechonek Adam, Ratajczak-Wielgomas Katarzyna, Podhorska-Okołów Marzenna, Dzięgiel Piotr. Expression of zyxin in non-small cell lung cancer − a preliminary study. Biomolecules, 2022, Vol. 12, no. 6, art. 827, DOI: 10.3390/biom12060827*). Clinical sample studies were executed using the following methods: immunohistochemistry (IHC), Western Blot (WB), real-time PCR, and RT-qPCR performed on mRNA stemmed from cells isolated with laser microdissection. IHC technique was carried out on 399 NSCLC cases and 85 non-malignant lung tissue specimens, fixed in parraffin blocks and prepared as tissue microarrays. IHC reactions demonstrated the presence of ZYX in the cytoplasm, cell membrane, and nucleus of cancer cells and normal lung tissue cells. The statistical analysis showed significantly decreased level of cytoplasmic ZYX in NSCLC cells compared to normal tissue. However, the nuclear ZYX level was elevated in NSCLC cells in relation to normal lung cells. Further analyses showed higher nuclear ZYX level in SCC cells than in AC cells. The study also presented statistically decreased cytoplasmic ZYX levels in NSCLC cells and SCC cells of pT3-4 tumours in relation to pT1 tumours. Statistically lower cytoplasmic ZYX levels in NSCLC cells and SCC cells of stage III-IV compared to stage I were noticed. The results showed ZYX could not be considered an independent prognostic factor in NSCLC. Western Blot method demonstrated decreased ZYX protein level in NSCLC and SCC tumours compared to the control tissue. Real-time PCR studies presented lower *ZYX* mRNA levels in NSCLC and AC lesions than in normal lung tissue. In addition, RT-qPCR carried out on mRNA derived from cells isolated with laser microdissection showed decreased *ZYX* mRNA expression in NSCLC, SCC, and AC cells in comparison to normal lung cells.

The expression of ZYX was also investigated in NSCLC cell lines (lung squamous cell carcinoma cell line − NCI-H1703, lung adenocarcinoma cell line − NCI-H522) and in the

control cell line comprising normal lung fibroblast cell line IMR-90. The applied research methods included immunofluorescence, immunocytochemistry, Western Blot, and real-time PCR. Immunocytochemical reactions revealed the presence of ZYX in the cytoplasm. Moreover, immunofluorescence reactions demonstrated the residence of ZYX in the nucleus of the examined cells. The experiments performed on the cell lines showed decreased ZYX protein levels in NSCLC cell lines in relation to the control cell line IMR-90. In turn, real-time PCR analysis presented higher *ZYX* mRNA expression in the cell line NCI-H1703 than in the normal cell line IMR-90. Lower *ZYX* mRNA level was detected in the cell line NCI-H522 than in the control cell line IMR-90.

In conclusion, decreased ZYX expression in NSCLC cells in comparison to the control tissue suggests that ZYX might function as a suppressor protein in NSCLC development. Nevertheless, the obtained outcomes point out that ZYX does not exhibit the prognostic potential in this cancer type.

Wstęp

Rak płuc jest jednym z najczęściej diagnozowanych nowotworów złośliwych na świecie, charakteryzującym się wysoką zapadalnością i śmiertelnością [1]. W związku z tym, zalicza się do najważniejszych celów terapeutycznych. Czynnikami predysponującymi do zachorowania na raka płuc są: długoczasowe palenie papierosów, czynniki genetyczne oraz ekspozycja na metale ciężkie [2,3]. Rak płuc dzieli się na niedrobnokomórkowego raka płuc (ang. *non-small cell lung cancer*, NSCLC) oraz drobnokomórkowego raka płuc (ang. *small cell lung cancer,* SCLC) [4]. Szacuje się, że NSCLC stanowi około 85% nowych przypadków raka płuc [3,4]. NSCLC można podzielić na raka płaskonabłonkowego (ang. *squamous cell carcinoma*, SCC), gruczolakoraka (ang. *adenocarcinoma*, AC) i wielkokomórkowego raka płuc (ang. *large cell carcinoma,* LCC) [3,4].

Zyksyna (ZYX) jest białkiem należącym do rodziny białek z domeną LIM. Białka z domeną LIM biorą udział w szeregu procesów, takich jak różnicowanie komórek, regulacja transkrypcji czy organizacja cytoszkieletu [5]. ZYX jest składnikiem ognisk adhezyjnych oraz włókien naprężeniowych [6]. Bierze ona udział między innymi w remodelowaniu oraz naprawie włókien naprężeniowych [6], poprzez swoją rolę w polimeryzacji aktyny [7]. W strukturze ZYX wyróżnia się miejsce wiążące α-aktyninę, powtórzenia ActA (bogate w prolinę) oraz dwie sekwencje eksportu jądrowego (ang. *nuclear export sequence* − NES) [6]. Na C-końcu białka znajdują się trzy domeny LIM [6]. Wykazano, że zyksyna może ulegać przemieszczeniu z ognisk adhezyjnych/cytoplazmy do jądra komórkowego pod wpływem różnych czynników [8–11]. Niektóre badania sugerują jej udział w regulacji ekspresji genów [8,11]. Należy nadmienić, że ZYX może pełnić rolę w apoptozie oraz przejściu epitelialno-mezenchymalnym [9,12]. Badania wykazały, że ZYX może również brać udział w procesie gojenia się ran [5]. Sugeruje się, że lokalizacja ZYX w komórce może mieć wpływ na rozwój nowotworów [13,14].

Przeprowadzone dotychczas badania wykazały zwiększoną ekspresję ZYX w komórkach raka jelita grubego, gruczołu piersiowego oraz raka wątrobowokomórkowego [15–17]. Supresorową rolę ZYX w procesie nowotworzenia sugeruje się natomiast w raku prostaty czy pęcherza moczowego [18–20]. Zyksyna może odgrywać rolę także w rozwoju czerniaka, ze względu na jej podwyższony poziom w komórkach tego nowotworu w porównaniu do prawidłowych melanocytów [21]. Istnieje jednak niewiele doniesień na temat ekspresji oraz znaczenia ZYX w raku płuc. Badania Mise et al. sprawdzały rolę ZYX w migracji i adhezji komórek niedrobnokomórkowego raka płuc [22]. Komórki A549 pozbawione ekspresji ZYX

charakteryzowały się zwiększoną szybkością przemieszczania się [22]. Dalsze badania wykonane na myszach zademonstrowały niższy poziom ZYX w guzach raka płuc [22]. Z kolei Cadinu et al. wykazali niższą ekspresję ZYX w linii komórkowej HCC4017 (NSCLC) [23]. Niższą ekspresją charakteryzowały się także inne białka związane z cytoszkieletem [23]. Przeprowadzono również badania weryfikujące poziom zyksyny w osoczu oraz egzosomach surowicy pacjentów z NSCLC [24,25].

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Założenia i cel pracy

Celem niniejszej pracy było określenie lokalizacji i poziomu ekspresji ZYX w guzach niedrobnokomórkowego raka płuc i tkance płuc niezmienionej nowotworowo. Do określenia poziomu ZYX w NSCLC wykorzystano również komercyjne linie komórkowe ww. nowotworu. Ocena lokalizacji i poziomu białka ZYX w materiale klinicznym odbywała się za pomocą metody immunohistochemicznej oraz Western Blot. Poziom mRNA *ZYX* w guzach NSCLC został określony metodą real-time PCR. RT-qPCR posłużył również do określenia poziomu mRNA *ZYX* w wyizolowanych metodą mikrodysekcji laserowej komórkach nowotworowych jak i komórkach płucnych niezmienionych nowotworowo. Wyniki otrzymane metodą immunohistochemiczną skorelowano następnie z danymi kliniczno-patologicznymi. Uzyskane dane pozwoliły na zweryfikowanie, czy poziom ZYX ulega zmianie podczas rozwoju NSCLC oraz czy ZYX posiada potencjał prognostyczny w przypadku tego rodzaju nowotworu. Materiał badany stanowiły również dwie ludzkie linie NSCLC (NCI-H1703 − SCC płuc i NCI-H522 − AC płuc) oraz kontrolna linia prawidłowych ludzkich fibroblastów płucnych IMR-90. Wymienione linie komórkowe posłużyły do badań metodami Western Blot, real-time PCR, immunofluorescencji i immunocytochemii.

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Review

The Role of Zyxin in Carcinogenesis

ALEKSANDRA PARTYNSKA[1], AGNIESZKA GOMULKIEWICZ[1], PIOTR DZIEGIEL[1,2] and MARZENNA PODHORSKA-OKOLOW[3]

[1]*Department of Histology and Embryology, Department of Human Morphology and Embryology, Faculty of Medicine, Wroclaw Medical University, Wroclaw, Poland;*
[2]*Human Biology Unit, Faculty of Physiotherapy, University School of Physical Education in Wroclaw, Wroclaw, Poland;*
[3]*Department of Ultrastructural Research, Faculty of Medicine, Wroclaw Medical University, Wroclaw, Poland*

Abstract. *Zyxin (ZYX) is a LIM domain protein whose presence has been detected in the cytoplasm and nucleus. ZYX can translocate between these two compartments and therefore, can take part in the regulation of various cellular processes. VASP and α-actinin are examples of proteins that interact with ZYX. As ZYX is present in focal adhesions (FAs), an immense part of research is focused on the role of this protein in the organisation and function of the cytoskeleton. Other studies aim to explain the impact of zyxin on other intracellular processes. Zyxin has been shown to take part in apoptosis, as well as in wound healing. Additionally, zyxin contribution to cancer development is gaining growing interest. This paper aims to systematise the knowledge on zyxin and its role in carcinogenesis.*

Zyxin (ZYX) is classified as a LIM domain protein (1). LIM domain contains two cysteine and histidine-rich zinc finger motifs (sequence C-X_2-C-X_{17-19}-H-X_2-C-X_2-C-X_2-C-X_{15-19}-C) (2, 3). ZYX can interact through these domains with other molecules and thus, regulate various intracellular processes (1). The name "LIM domain" comes from the proteins in which it was discovered for the first time: Lin-11, Isl1, and Mec-3 (2). Paxilin, TRIP6, testin, and LASP belong to the same large family of proteins (1). LIM domain proteins are involved in many intracellular processes, such as cytoskeleton organisation, transcription regulation, cell differentiation, and oncogenesis (Figure 1) (2).

Gene encoding human ZYX is located on chromosome 7 (4, 5). Zyxin is a phosphorylated protein with a molecular mass of 82-84 kDa (6). This protein can be present not only in the cytoplasm, but also in the nucleus (2) (Figure 2), and the translocation between these two compartments can occur under various conditions (7), such as mechanical forces or EGF (epidermal growth factor) (7, 8). ZYX is a component of focal adhesions (FAs) responsible for the interaction between a cell and the extracellular matrix, and also takes part in the organisation and regeneration of the cytoskeleton (1). This protein is a pivotal component of stress fibres (SFs), *i.e.* contractile actomyosin fibres determining cell migration (1, 9). Zyxin is known as a mechanosensor – under mechanical forces it i) translocates into the nucleus where it can regulate gene expression, or ii) localises along stress fibres to take part in actin polymerisation (10-12). The structure of ZYX consists of sequences that enable the protein to perform its functions. These are: α-actinin [*i.e.* protein crosslinking actin filaments (13)] binding site, 4 proline-rich repeats (so-called ActA repeats), and 2 leucine-rich nuclear export sequences (NES) (Figure 3) (1). ActA repeats, identified for the first time in ActA protein of *Listeria monocytogenes* bacterium (14, 15), are responsible for interaction with VASP (Vasodilator-stimulated phosphoprotein), which plays a role in actin polymerisation. In addition, three LIM domains (LIM1, LIM2, and LIM3) essential for ZYX targeting to focal adhesions and the cytoskeleton are located at the C-terminus (1). LIM domains allow zyxin to interact with other molecules, such as cell cycle and apoptosis regulator protein-1 (CARP-1) (16) or transcription factor ZNF384, involved in osteoblast differentiation (7).

Activity of ZYX might be regulated, *inter alia*, through its head-tail interactions (17, 18). These interactions are based on LIM domain binding to the proximity of proline-rich regions (ActA repeats) and the release of zyxin from

Correspondence to: Aleksandra Partynska, Department of Histology and Embryology, Department of Human Morphology and Embryology, Wroclaw Medical University, T. Chalubinskiego 6a, 50-368 Wroclaw, Poland. Tel: +48 717841354, e-mail: ola.partynska@onet.pl

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binding with proteins (*i.e.* so-called closed conformation) (17, 18). Serine phosphorylation at position 142 (S142) was shown to be indispensable for the elimination of the ZYX head-tail interaction that recovers the ability to bind other proteins (17, 18).

The study of Guo and Wang (19), performed on a murine embryonic fibroblast cell line (NIH3T3), revealed that zyxin is transported retrogradely in the form of so-called "tails" from focal adhesions to the cell interior. This phenomenon was dependent on the stiffness of the substrate, on which cells were grown. In addition, the number of "tails" was negatively correlated with the speed of cell migration (19). Another study showed that mechanical stress resulted in zyxin translocation from focal adhesions to actin filaments (20). What is more, it turned out that zyxin was essential for VASP localisation along stress fibres in cells subjected to mechanical stress (20).

The contribution of zyxin to cellular processes has also been described in apoptosis. Experiments carried out on a murine embryonic fibroblast cell line (MEF) treated with UV-C radiation showed that zyxin promoted cell death (21). Translocation of zyxin from the cytoplasm into nucleus, as well as an increase in both caspase-3 activity and the percentage of apoptotic cells (detected with TUNEL method) were detectable under UV-C radiation (21).

The study of Han *et al.* suggests that ZYX may play a role in wound healing (22). Zyxin was shown to influence von Willebrand factor secretion from murine endothelial cells (22). Knockout of Zyx in mice caused longer bleeding time after epinephrine stimulation, in comparison to wild type (WT) mice (22). Moreover, Zyx knockout negatively affected thrombus formation (22).

Numerous studies are focused on the role of ZYX as a component of focal adhesions and the cytoskeleton, and as a mechanosensor. It is commonly known that changes in the expression of cytoskeleton-organising proteins have an impact on cell migration, which is important in metastasis (9, 23). Not only for this reason, but also for its above-mentioned features, the role of ZYX in carcinogenesis is getting much attention from researchers.

The Role of Zyxin in Carcinogenesis

The role of ZYX in oncogenesis is not clearly defined. The research carried out in recent years has confirmed the contribution of ZYX to the development of various cancer types, including melanoma, ovarian, breast, glioma, lung, and oral squamous cell carcinoma (6, 24-28). Recent observations have revealed that ZYX can act as an oncoprotein or a tumour suppressor, depending on the cancer type (16).

Breast cancer. Many studies have shown that ZYX can promote breast carcinogenesis. Increased level of ZYX was detected in radiotherapy-resistant (RR) breast cancer cell lines MCF-7RR and MDA-MB-231RR, in comparison to the sensitive ones (29). However, the studies of Hodgkinson *et al.* presented decreased ZYX expression in chemotherapy-resistant breast tumours when compared to cancer cells that are sensitive to chemotherapy (30). These results suggest, that zyxin expression level may determine the sensitivity of cancer cells to a particular kind of anticancer therapy and may be helpful in predicting its effectiveness.

Ma *et al.* noticed that ZYX might act as an oncogene in breast cancer cells. It was shown that ZYX-silenced MDA-MB-231 cells were characterised by decreased migration. Such cells injected into mice, formed tumours of smaller mass than controls (25). Furthermore, based on the results of immunohistochemical reactions (IHC) performed on breast tumours resected from patients, a positive correlation between zyxin level and cancer stage was demonstrated (25).

During further experiments explaining the mechanism of ZYX interactions in cancer cells, Ma *et al.* observed that this protein takes part in the regulation of Hippo signalling pathway (25). Activation of Hippo signalling pathway leads to cell growth and proliferation inhibition (31, 32). The direct reason for this phenomenon is Yes-associated protein (YAP) and Transcriptional co-activator with PDZ-binding motif (TAZ) phosphorylation by Large Tumour Suppressor 1/2 (LATS 1/2) factor (31, 32). Such phosphorylation leads to protein accumulation and degradation in the cytoplasm, resulting in cell proliferation inhibition (31). The discussed studies showed that under hypoxic conditions and after TGFβ stimulation, ZYX forms a complex with LATS2 and Siah2 (E3 ubiquitin ligase, involved in LATS2 degradation), which leads to Hippo pathway deactivation (25) and cell proliferation. It is worth mentioning that YAP and TAZ proteins have an impact on the activity of TEAD and SMAD transcription factors, which are responsible, *inter alia*, for cell survival and growth (31). The results of these studies suggest that ZYX may play a role in the development and progression of breast cancer.

The contribution of ZYX to the regulation of Hippo signalling pathway is confirmed by Diepenbruck *et al.* (32). Their studies showed that zyxin expression in breast cancer cells is controlled by Tead2 (transcription factor), and more specifically by Tead2-Taz complex (32). Tead2 over-expression in murine breast cancer cells resulted in increased cell invasion (32). Further analyses showed that cells characterised by Tead2 over-expression but with Zyx silencing, demonstrated significantly decreased invasion (32). According to earlier studies, YAP and TAZ proteins take part in the regulation of epithelial-mesenchymal transition (EMT) (33-35). It can thus be suggested that ZYX, together with these proteins, can also impact the EMT process and thus, influence breast cancer cell invasion. The study of Mori *et al.* seems to confirm this hypothesis, as they

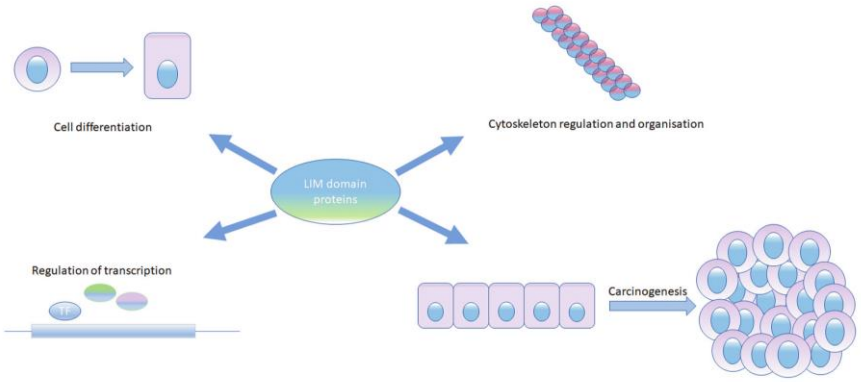

Figure 1. *Exemplary functions of LIM domain proteins [based on (2)].*

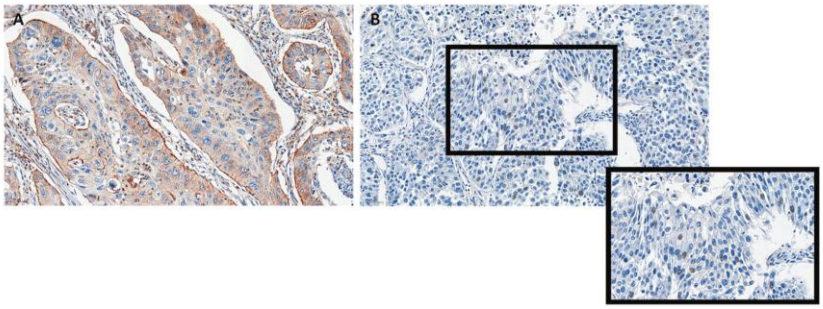

Figure 2. *Cytoplasmic (A) and nuclear (B) localisation of zyxin in non-small cell lung cancer (property of the Department of Histology and Embryology, Wroclaw Medical University).*

Figure 3. *Schematic structure of zyxin [based on (1, 17)].*

showed the role of Zyx in migration of normal murine mammary gland epithelial cells NMuMG (36). It was observed that under TGFβ treatment, significant increase in zyxin expression and translocation to stress fibres appeared, initiating the migration of the examined cells. It was also noticeable that an increase in zyxin expression under TGFβ stimulation is controlled by Twist1 (transcription factor) with an established role in EMT (36).

Other studies carried out on BT-20 breast cancer cell line presented that ZYX localisation in a cell can be probably related to breast cancer development (37). Lim and SH3 domain protein (LASP-1) is responsible for ZYX cellular

distribution and plays a role in ZYX localisation to focal adhesions (37, 38). Studies performed on this cell line revealed that LASP-1 silencing leads to changes in ZYX localisation, inhibits proliferation, and decreases cell migration (37).

Lung cancer. Some research focused on the effect of ZYX in lung cancer pathogenesis suggests its suppressive role in carcinogenesis. The study of Hodgkinson *et al*. revealed that an increase in ZYX expression occurred after subjecting A549 lung cancer cell line to cyclooxygenase-2 (COX-2) specific inhibitor (29). COX-2 is an enzyme that catalyses the conversion of arachidonic acid to prostaglandins in response to inflammation (39, 40). It was shown that increased expression of COX-2 in non-small cell lung cancer (NSCLC) plays a significant role in lung cancer development through its effect on angiogenesis and immune response suppression in tumour cells (40, 41). The literature data show that carcinogenesis can be decelerated by the use of COX-2 inhibitors not only as single agents, but also in combined therapy (40-42). Therefore, an increased level of ZYX in response to the application of COX-2 inhibitor suggests the ability of ZYX to inhibit oncogenesis.

Stimulation of A549 cells with TGFβ, a commonly recognized EMT inducer, resulted in increased ZYX expression (28). It was demonstrated that ZYX expression is dependent on SMAD3 (28), the effector molecule activated upon TGFβ treatment that takes part in gene expression regulation (43, 44). Furthermore, upon TGFβ stimulation, ZYX-silenced A549 cells exhibit increased expression of the integrin α5, *i.e*. a molecule responsible for cell adhesion and migration (28). An increased velocity of ZYX-deficient cells *versus* controls with unchanged ZYX expression was also noticed (28). In addition, significantly decreased expression of ZYX was detected in the K-ras^{LA2} lung cancer mouse model (28). The above results suggest that ZYX might decelerate cancer progression through inhibition of cell migration.

The studies of Cadinu *et al*. confirm the suppressive function of ZYX in lung cancer development. Decreased ZYX expression was noticed in HCC4017 cells (NSCLC) in comparison to normal HBEC30KT cells (23). Also, lowered expression of other cytoskeleton-organising proteins was observed (23). Based on these results, a conclusion can be drawn that a change in cytoskeletal protein expression (including ZYX) may be cancer transformation-related.

Incubation of H1299 cells (NSCLC) with retinoic acid (RA) resulted in ZYX translocation from the cytoplasm into the nucleus (45). The use of retinoic acid in anticancer therapy is a very promising approach (46). After translocation into the nucleus, retinoic acid interacts with its receptor Retinoic Acid Receptor (RAR) that forms a complex with Retinoid X Receptor (RXR) (47). As a result, interaction with co-activators and transcription initiation can occur (47). It was demonstrated that after translocation into the nucleus, ZYX forms a complex with prostate tumour overexpressed 1 domain [PTOV1 domain, present in MED25 protein (48)] and CREB binding protein [CBP, transcription co-activator in RA signalling pathway (49)] (45). It inhibits CBP from interacting with RAR, thus resulting in a poor cytotoxic RA effect (45). This study presents partially the potential mechanism of the regulation of cellular response to ZYX.

Moreover, it was noticed that ZYX plasma concentration is significantly increased in patients diagnosed with NSCLC in comparison to healthy controls (50). Elevated ZYX levels were already detected in early stages of NSCLC, thus classifying it as a potential plasma marker of non-small cell lung cancer (50). In another study, the distribution of ZYX in serum and saliva exosomes was examined (51). Exosomes are exocrine vesicles that transport proteins, nucleic acids or lipids. It is believed that these vesicles take part in the communication between cells (52, 53). The content of ZYX in serum exosomes of patients with NSCLC was significantly lowered when compared to healthy controls (51). However, at present, the reasons for the observed discrepancies in ZYX levels in plasma and exosomes are difficult to be explained.

Melanoma. Elevated levels of ZYX were observed in melanoma cell lines (PM-WK, RPM-EP, RMP-MC, MM-AN) in comparison to normal melanocytes (6). In addition to this, melanoma cells showed shorter doubling time than normal cells (6). The study was extended to the incubation of cells with protein kinase C-activator – 12-O-tetradecanoylphorbol-13-acetate (TPA) (6). Incubation of melanoma cells with TPA resulted in cell proliferation inhibition and in a decrease in ZYX expression (6). These results suggest that ZYX might affect cell proliferation and therefore, takes part in cancer progression (6).

This hypothesis was also confirmed by the study performed on A375 melanoma cell line (54), where it was shown that silencing Wilms' Tumour 1 (WT1, transcription factor) resulted in proliferation inhibition and decreased expression of ZYX and nestin (54). WT1 is a well-known regulator of cell growth and proliferation (55). The effect of WT1 on ZYX levels suggests that ZYX may participate in the regulation of proliferation through interacting with other factors (54). Similar observations were carried out by Michiels *et al*. (56) who examined the effect of Peroxisome Proliferator-Activated Receptor β (PPARβ, transcription factor) on cancer cell proliferation. They showed that PPARβ activation leads to a decrease in the expression of WT1, zyxin, and nestin, and to inhibition of cancer cell proliferation (56).

Colorectal cancer. The role of ZYX in the development and progression of colorectal cancer is still elusive, but existing studies show that this protein is an unfavourable prognostic factor.

The study of Fukumoto *et al*. suggests that the formation of immature focal adhesions may be one of the mechanisms responsible for an increased invasion in DLD-1 colorectal cancer cell line. The lack of FA maturation results from the presence of alpha-actinin 4 inside these structures and the disturbed ZYX localisation (57). Alpha-actinin is a component of focal adhesions responsible for actin filament crosslinking (57). Four isoforms of alpha-actinin have been distinguished: 1, 2, 3, and 4 (57). The existing studies demonstrated that alpha-actinin 4 is related to cancer invasion (57). Disrupted binding of ZYX to alpha-actinin 4 was noticed, that may explain decreased stability of adhesions between cells and the extracellular matrix, and therefore increased cancer cell invasion (57). For comparison, the interaction of ZYX with alpha-actinin 1 was not disrupted, so the mature focal adhesions could appear (57).

Zhong *et al*. showed that ZYX expression was significantly higher in colorectal cancer lesions in comparison to normal tissues (58). The patients with tumours characterised by high ZYX expression had a shorter recurrence-free survival (58). In vitro experiments demonstrated that ZYX silencing in HCT116 colorectal cancer cell line caused decreased cell migration and invasion (58).

Moreover, ZYX fragments were detected in the serum of colorectal cancer patients. It suggests that zyxin can be a potential marker of colorectal cancer (59).

The Role of Zyxin in the Development of Other Cancer Types

The potential participation of ZYX in carcinogenesis has also been examined in other cancer types. The team of Wu *et al*. revealed the regulation of ZYX expression by miRNA-16 in Hep-2 laryngeal cancer cell line (60). The authors postulate that lowering ZYX expression with miRNA-16 increases the ability of Hep-2 cells to migrate (60). The function of miRNA-16-1 and its influence on ZYX expression was also examined in glioma (61). The levels of miRNA-16-1 in U251 and U87 glioma cell lines were significantly lower when compared to control brain tissue (61). It was observed that transfection of the glioma cell line with miRNA-16-1 leads to lower ZYX mRNA levels, decreased cell migration and invasion (61). The results of these experiments suggest that ZYX can have an impact on cancer cell invasion and therefore on the course of cancer.

The studies carried out on K562 chronic myeloid leukaemia cell line showed that silencing of ZYX with the use of specific shRNA resulted in lower levels of Bcl-2 and Bcl-XL, which are classified as antiapoptotic proteins (62). In addition, treatment of ZYX-silenced cells with Imatinib (Gleevec, BCR-ABL kinase inhibitor) led to an increase in apoptosis and to a decrease in cell growth in comparison to the control (62). These outcomes suggest that ZYX may exhibit an antiapoptotic function and thus, may induce cancer cell survival.

On the other hand, the suppressive role of zyxin was noticed in Ewing sarcoma (63). In the case of this cancer, Cerisano *et al*. demonstrated that ZYX takes part in CD99-induced apoptosis (64). CD99 is a transmembrane protein, and its stimulation leads to an activation of caspase-independent apoptosis (64). Treatment of Ewing sarcoma cells with anti-CD99 antibody (CD99 agonist) induced the expression of ZYX at mRNA and protein levels (64). In the following experiments, where ZYX was silenced with antisense oligonucleotides, partial inhibition of CD99-induced apoptosis was found (64).

It is also supposed that ZYX takes part in cervical cancer development (65, 66). This hypothesis was proposed when the interaction of ZYX with E6 protein of human papilloma virus 6 (HPV6) was found (65). As it is commonly known, HPV is responsible, *inter alia*, for the genital warts and leads to cancer development (16, 65). It was demonstrated that the interaction of ZYX with a viral molecule causes its translocation into the nucleus. As a result, ZYX can influence transcription processes (65). These findings suggest that ZYX might promote cervical cancer progression through regulating expression of certain genes.

The contribution of ZYX to cervical cancer was also shown when the impact of thymosin β4 on SiHa cancer cell line was studied (66). Thymosin β4 is responsible for actin depolymerisation and its significant role in processes such as angiogenesis or metastasis was demonstrated (66). Over-expression of thymosin β4 in SiHa cells turned out to increase ZYX expression (66). Similar effect was observed with cells incubated with exogenous thymosin β4 (66). Moreover, ZYX expression was noticed to increase together with its translocation into the nucleus in the first hours of incubation. Afterwards, the protein returns to the cytoplasm (66). Based on these results, the authors suggest that ZYX and thymosin β4 regulate the migratory properties of cells by coordinating actin polymerisation and depolymerisation, respectively (66). In addition, the authors suppose that ZYX might function as a transport molecule allowing thymosin β4 to translocate into the nucleus (66). This could explain why ZYX transports into the nucleus and, after some time, returns to the cytoplasm and focal adhesions (66).

The oncogenic role of ZYX in hepatocellular carcinoma has also been examined. Sy *et al*. showed that ZYX expression is elevated in 33% of cancer cases, in comparison to control tissues (67). *In vitro* experiments demonstrated that ZYX silencing in Hep3B cancer cell line caused decreased cell migration and invasion (67).

Other studies have presented that lowered ZYX expression affects the level of the p53 protein and caspase activation

(68). It was observed that UV radiation of ZYX-silenced HepG2 hepatocellular cancer cell line caused lower phosphorylation of p53 protein (specifically, serine at position 46) that resulted in inhibition of apoptosis and lack of caspase activation (68). Furthermore, elevated levels of ZYX fragments were detected in the sera of hepatocellular cancer patients (69). It suggests that ZYX may also be a potential marker of this cancer.

Summary

Based on the already published papers, the functions of ZYX in a cell are not only restricted to its presence in focal adhesions. As a result of translocation into the nucleus and the regulation of expression of certain genes, zyxin can take part in various cellular processes that occur in normal and cancer cells. The results of the presented studies suggest that ZYX may have a double effect on cancer progression, *i.e.* may act as an oncogene or tumour suppressor, depending on the cancer type. These discrepancies encourage researchers to further evaluate the role of zyxin in carcinogenesis.

Conflicts of Interest

The Authors declare that there are no conflicts of interest in relation to this study.

Authors' Contributions

Writing of the article and review of the literature were performed by AP. Review was performed by AG. Final review and final approval were performed by PD and MPO.

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Expression of Zyxin in Non-Small Cell Lung Cancer—A Preliminary Study

Aleksandra Partynska [1,*], Agnieszka Gomulkiewicz [1], Aleksandra Piotrowska [1], Jedrzej Grzegrzolka [1], Adam Rzechonek [2], Katarzyna Ratajczak-Wielgomas [1], Marzenna Podhorska-Okolow [3] and Piotr Dziegiel [1,4]

[1] Division of Histology and Embryology, Department of Human Morphology and Embryology, Faculty of Medicine, Wroclaw Medical University, 50-368 Wroclaw, Poland; agnieszka.gomulkiewicz@umw.edu.pl (A.G.); aleksandra.piotrowska@umw.edu.pl (A.P.); jedrzej.grzegrzolka@umw.edu.pl (J.G.); katarzyna.ratajczak-wielgomas@umw.edu.pl (K.R.-W.); piotr.dziegiel@umw.edu.pl (P.D.)
[2] Department of Thoracic Surgery, Wroclaw Medical University, 53-439 Wroclaw, Poland; adam.rzechonek@umw.edu.pl
[3] Division of Ultrastructural Research, Faculty of Medicine, Wroclaw Medical University, 50-368 Wroclaw, Poland; marzenna.podhorska-okolow@umw.edu.pl
[4] Division of Human Biology, Faculty of Physiotherapy, University School of Physical Education in Wroclaw, 51-612 Wroclaw, Poland
* Correspondence: aleksandra.partynska@umw.edu.pl

Abstract: Background: The potential involvement of zyxin (ZYX) in carcinogenesis has been investigated in many cancer types. However, there are a limited number of studies on the role of ZYX in the progression of non-small cell lung cancer (NSCLC). Since lung cancer is one of the most frequently diagnosed carcinomas, the aim of our study was to determine the localization and expression levels of ZYX in NSCLC and to correlate the results with the clinicopathological data. Materials and Methods: The expression of ZYX was assessed in NSCLC cases and in cell lines representing this tumor type. Levels of ZYX were determined in the clinical material using immunohistochemistry (IHC) and Western Blot. Real-time PCR was used to assess *ZYX* mRNA levels. The expression of ZYX was also checked in NSCLC cell lines using real-time PCR, Western Blot, and immunofluorescence/immunocytochemistry. Results: The results showed lower levels of ZYX in NSCLC cells compared with control tissues. This trend was observed at the protein and mRNA levels. The assays on the NSCLC model also demonstrated lower levels of ZYX in cancer cells compared with control cells. Conclusions: The decreased expression of ZYX in NSCLC may indicate a suppressor role of this protein in NSCLC.

Keywords: zyxin; non-small cell lung cancer; tumor cells

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1. Introduction

Lung cancer is one of the most commonly diagnosed malignancies worldwide [1] with a high incidence and high mortality rates [1,2]. Therefore, it is one of the most important therapeutic targets. Lung cancer is divided into two subtypes, i.e., non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) [2]. NSCLC accounts for approximately 85% of all new cases of lung cancer [2,3]. NSCLC includes adenocarcinomas (ACs), squamous cell carcinomas (SCCs), and large cell carcinomas (LCCs) [2], of which ACs and SCCs are the most prevalent [3]. Risk factors for lung cancer include long-term smoking, exposure to trace metals, asbestos, and genetic predispositions [3,4].

There is a growing need to search for factors that may be crucial in the development of lung cancer. One of the factors under consideration is zyxin (ZYX), a LIM domain protein commonly known as a component of focal adhesions and stress fibers [5]. ZYX is involved in actin polymerization induced by mechanical stress in structures such as focal adhesions and stress fibers [6], thus enabling remodeling and repair of stress fibers [5]. It

has been shown that ZYX undergoes translocation from focal adhesions/cytoplasm to the cell nucleus under the influence of various factors (e.g., mechanical forces, UV, epidermal growth factor, retinoic acid) [7–10]. Therefore, its role in regulating gene expression has also been postulated [7,10]. Studies have shown that ZYX can interact with many proteins, such as transcription factor ZNF384 (involved in bone metabolism) [11], transcription factor Hepatocyte Nuclear Factor-1β (HNF-1β) [10], and Cell Cycle and Apoptosis Regulator Protein-1 (CARP-1) [8]. Furthermore, ZYX may play a role in von Willebrand factor (vWF) secretion, apoptosis, and epithelial–mesenchymal transition (EMT) [8,12,13].

Many studies have been conducted to determine the role of ZYX in oncogenesis. Overexpression of ZYX has been demonstrated in breast, colorectal, and hepatocellular carcinomas [14–16]. It has also been suggested that ZYX may act as a tumor suppressor protein in prostate and bladder cancers [17]. Moreover, this protein is probably involved in the progression of chronic myeloid leukemia and glioma [18,19]. However, there are only a few studies on the involvement of ZYX in lung cancer progression. Cadinu et al. demonstrated a lower expression of ZYX in the HCC4017 (NSCLC) cell line [20]. Mise et al. described the role of ZYX in migration and adhesion of lung cancer cells [21]. The same authors also presented decreased ZYX levels in a mouse model of lung cancer [21]. Other studies aimed to verify ZYX levels in serum exosomes [22] and in plasma [23] of NSCLC patients.

Considering the above, the aim of this study was to verify the location and intensity of ZYX expression in NSCLC and to compare the results with the clinicopathological data.

2. Materials and Methods

2.1. Patients and Tissue Material

The tissue material was obtained during pulmonary parenchymal resection or lobectomy in patients with NSCLC at the Department of Thoracic Surgery, Wroclaw Medical University between 2007 and 2017. Written informed consent was obtained for the use of clinical material for research. The clinicopathological characteristics of all patients are shown in Table 1. Immunohistochemistry (IHC) reactions were performed on 399 formalin-fixed and paraffin-embedded NSCLC sections (including 169 lung SCCs, 168 lung ACs, and 31 lung LCCs) and 85 non-malignant lung tissue (NMLT) sections. The histological grade (G) of NSCLC cases was determined according to the WHO criteria [24]. TNM Classification of Malignant Tumors eighth edition was used to determine lung cancer stage [25]. Twenty-three frozen NSCLC sections and the corresponding NMLT samples were used for the Western Blot analysis. Real-time PCR was performed on 63 NSCLC specimens and 58 NMLT cases previously fixed in RNAlater solution. Laser microdissection was performed on frozen material including 10 NSCLC sections (5 SCCs and 5 ACs) and 6 NMLTs. The experiments were performed after obtaining the approval of the Bioethics Committee at the Wroclaw Medical University (consent no. KB-483/2018, 6 September 2018 and KB-504/2018, 11 September 2018).

Table 1. Clinicopathological characteristics of patients with non-small cell lung cancer (NSCLC).

Clinical Feature	NSCLC		SCC		AC		LCC	
	***n* = 399**	**%**	***n* = 169**	**%**	***n* = 168**	**%**	***n* = 31**	**%**
Age								
≤62	212	53.13%	≤64 89	52.66%	≤61 90	53.57%	≤62 16	51.61%
>62	187	46.87%	>64 80	47.34%	>61 78	46.43%	>62 15	48.39%
Sex								
Female	116	29.07%	32	18.93%	65	38.69%	6	19.35%
Male	283	70.93%	137	81.07%	103	61.31%	25	80.65%

Table 1. *Cont.*

Clinical Feature	NSCLC		SCC		AC		LCC	
	***n* = 399**	**%**	***n* = 169**	**%**	***n* = 168**	**%**	***n* = 31**	**%**
Histological grade								
G1	24	6.02%	4	2.37%	19	11.31%	1	3.23%
G2	293	73.43%	143	84.62%	109	64.88%	24	77.42%
G3	66	16.54%	22	13.02%	38	22.62%	6	19.35%
No data	16	4.01%	0	0.00%	2	1.19%	0	0.00%
Tumor size								
T1	88	22.06%	39	23.08%	33	19.64%	8	25.81%
T2	186	46.62%	76	44.97%	86	51.19%	13	41.94%
T3	79	19.80%	37	21.89%	31	18.45%	6	19.35%
T4	46	11.53%	17	10.06%	18	10.71%	4	12.90%
Lymph node metastases								
N0	260	65.16%	110	65.09%	108	64.29%	19	61.29%
N1	70	17.54%	40	23.67%	22	13.10%	4	12.90%
N2	69	17.29%	19	11.24%	38	22.62%	8	25.81%
Distant metastases								
M0	395	99.00%	169	100.00%	165	98.21%	30	96.77%
M1	4	1.00%	0	0.00%	3	1.79%	1	3.23%
Clinical stage								
I	145	36.34%	64	37.87%	62	36.90%	7	22.58%
II	130	32.58%	64	37.87%	46	27.38%	12	38.71%
III	120	30.08%	41	24.26%	57	33.93%	11	35.48%
IV	4	1.00%	0	0.00%	3	1.79%	1	3.23%
Smoking								
Smokers	336	84.21%	157	92.90%	128	76.19%	29	93.55%
Non-smokers	63	15.79%	12	7.10%	40	23.81%	2	6.45%
Living in urban areas								
Yes	31	7.77%	16	9.47%	10	5.95%	1	3.23%
No	368	92.23%	153	90.53%	158	94.05%	30	96.77%
Death								
Yes	244	61.15%	92	54.44%	108	64.29%	24	77.42%
No	147	36.84%	74	43.79%	59	35.12%	6	19.35%
No data	8	2.01%	3	1.78%	1	0.60%	1	3.23%

2.2. Preparation of Tissue Microarrays (TMAs)

Lung cancer tissue microarrays were prepared from archival formalin-fixed and paraffin-embedded NSCLC and NMLT tissues. Hematoxylin and eosin-stained sections were used to select representative tissue sites using a Pannoramic Midi II Histology scanner (3D Histech, Budapest, Hungary) and the Pannoramic Viewer software version 1.15.4 (3D Histech, Budapest, Hungary). Next, the selected representative cores of 1.5 mm diameter were transferred from the donor block to the target block using the TMA Grand Master instrument (3DHistech, Budapest, Hungary). These TMAs were used for further immunohistochemical reactions.

2.3. Immunohistochemistry (IHC)

IHC reactions were performed on 4 µm thick TMA sections placed on Superfrost Plus slides (Menzel Gläser, Braunschweig, Germany). The reactions were performed using the EnVision Flex System (Dako, Glostrup, Denmark). Deparaffinization, rehydration, and antigen retrieval (97 °C, 20 min) were performed in low-pH EnVision FLEX Target Antigen Retrieval Solution (pH = 6) using the PT Link (Dako, Glostrup, Denmark). Dako

Autostainer Link48 was used for performing IHC reactions (Dako, Glostrup, Denmark). Endogenous peroxidase activity was blocked by a 5 min incubation with EnVision FLEX Peroxidase-Blocking Reagent (Dako, Glostrup, Denmark). The sections were incubated for 20 min with anti-ZYX monoclonal antibody (1:50, 2D1 clone, catalogue no. sc-293448, Santa Cruz Biotechnology, Dallas, TX, USA) followed by EnVision FLEX + MOUSE LINKER for 15 min. Next, the sections were incubated for 20 min with EnVision FLEX/HRP secondary antibody (Dako, Glostrup, Denmark). DAB+ Chromogen (Dako, Glostrup, Denmark) was used to visualize the reaction. Hematoxylin was used to visualize cell nuclei according to the manufacturer's instructions (Dako, Glostrup, Denmark).

2.4. Assessment of IHC Reactions

IHC reactions were assessed using an Olympus BX41 microscope (Olympus Corporation, Tokyo, Japan). The Remmele and Stegner scoring system (Immunoreactive score—IRS) was used to evaluate the cytoplasmic reaction in cancer cells [26]. This scale is related to reaction intensity (0 points—no reaction; 1 point—weak reaction; 2 points—moderate intensity; 3 points—intense reaction) and the percentage of positive cells (0 points—no positive cells, 1 point—≤10% of positive cells; 2 points—11–50% positive cells; 3 points—51–80% of positive cells; 4 points—>80% of positive cells) [26]. The value of the multiplied components represents the number of points, ranging from 0 to 12. The nuclear reaction was assessed using the scale given in Table 2. The scale includes the percentage of tumor cells in which the nuclear reaction occurred [27].

Table 2. Scoring system for nuclear reaction intensity (modified according to [27]).

Points	Percentage of Cells with Positive Nuclear Reaction
0	0%
1	≤10%
2	11–25%
3	26–50%
4	>50%

2.5. Cell Lines

Two human NSCLC cell lines, i.e., NCI-H1703 (lung SCC) and NCI-H522 (lung AC) were used (ATCC, Manassas, VA, USA). The IMR-90 normal human lung fibroblast cell line (ATCC, Manassas, VA, USA) was used as the control. NCI-H1703 and NCI-H522 cell lines were cultured in RPMI-1640 medium (Gibco, Grand Island, New York, NY, USA) enriched with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine and antibiotics (Gibco, Grand Island, New York, NY, USA). EMEM medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 1xNEAA (Sigma-Aldrich, St. Louis, MO, USA), sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), and 2 mM L-glutamine and antibiotics (Sigma-Aldrich, St. Louis, MO, USA) was used to grow IMR-90 cells. Cells were grown in an incubator at 37 °C with 5% CO_2.

2.6. Immunocytochemistry (ICC)

After 24 h growth, cells were fixed in 4% formaldehyde for 12 min at room temperature (RT) and permeabilized in 0.2% Triton X-100 in PBS for 10 min. The slides were incubated for 5 min with EnVision FLEX Peroxidase-Blocking Reagent (Dako, Glostrup, Denmark). After an hour incubation with anti-ZYX antibody (1:100, 2D1 clone, catalogue no. sc-293448, Santa Cruz Biotechnology, Dallas, TX, USA), the slides were incubated with EnVision FLEX/HRP secondary antibody. DAB+ Chromogen was used for reaction visualization (Dako, Glostrup, Denmark). Hematoxylin was used to stain cell nuclei according to the manufacturer's instructions (Dako, Glostrup, Denmark).

2.7. Immunofluorescence (IF)

Cells were seeded into 8-well Millicell EZ slides (Merck Millipore, Kenilworth, NJ, USA). These cells were left to grow for 24 h and fixed with 4% formaldehyde for 12 min at RT. Permeabilization was performed with 0.2% Triton X-100 in PBS for 10 min. After blocking with 3% BSA in PBST for 45 min, cells were incubated with anti-ZYX antibody (1:100, 2D1 clone, catalogue no. sc-293448, Santa Cruz Biotechnology, Dallas, USA) for 1 h at RT. A secondary anti-mouse antibody conjugated to Alexa-Fluor 488 (1:1000, catalogue no. ab15013, Abcam, Cambridge, UK) was added for 1 h at RT. This was followed by incubation with DAPI (Thermo Fisher Scientific, Waltham, MA, USA) to stain cell nuclei. After washing, the cells were embedded in ProLong Diamond Antifade Reagent (Life Technologies, Carlsbad, CA, USA). Detection and assessment of ZYX expression levels were performed using an Olympus FV3000 confocal microscope (Olympus Corporation, Tokyo, Japan) and CellSense software version 3.2 (Olympus Corporation, Tokyo, Japan).

2.8. Western Blot

To isolate proteins from the tissue material, lysis was performed in T-PER Tissue Protein Extraction Reagent, Halt Protease Inhibitor Cocktail (catalogue no. 78510 and 78430, respectively, Thermo Fisher Scientific, Waltham, MA, USA) and 0.66 mM PMSF using TissueLyser LT (Qiagen, Hilden, Germany). The samples were incubated for 30 min at 4 °C and centrifuged (12,000× *g*, 15 min, 4 °C) to collect the supernatant. The cells were lysed using the RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Igepal CA-630, 0.5% sodium deoxycholate) supplemented with 0.5 mM PMSF and the Halt Protease Inhibitor Cocktail (catalogue no. 78430, Thermo Fisher Scientific, Waltham, USA). After 20-min incubation on ice, the samples were centrifuged (12,000× *g*, 10 min, 4 °C) to collect the supernatant. Protein concentration was measured using the Pierce BCA Protein Assay Kit (catalogue no. 23227, Thermo Fisher Scientific, Waltham, MA, USA). Identical amounts of protein (30 µg per lane) were resuspended in 4× loading buffer (250 mM Tris pH = 6.8, 40% glycerol, 20% (*v*/*v*) β-mercaptoethanol, 0.33 mg/mL bromophenol blue, 8% SDS), denatured for 10 min at 96 °C, and subjected to SDS-PAGE. The proteins were transferred to a PVDF membrane (Immobilon-P; catalogue no. IPVH00005, Merck Millipore, Kenilworth, NJ, USA). The membrane was blocked with 5% skimmed milk (catalogue no. 170-6404, Bio-Rad, Marnes-la-Coquette, France) in 0.1% TBST for 1 h at RT. The membrane was incubated overnight at 4 °C with anti-ZYX antibody (1:200, 2D1 clone, catalogue no. sc-293448, Santa Cruz Biotechnology, Dallas, TX, USA) diluted in 1% milk in 0.1% TBST. The membrane was washed three times and incubated with the HRP-conjugated anti-mouse secondary antibody (AffiniPure Donkey Anti-Mouse IgG (H + L), catalogue no. 715-035-150, Jackson ImmunoResearch, Ely, Cambridgeshire, UK) at a dilution of 1:3000 for 1 h at RT.

After washing, detection was performed with the Immobilon Classico Western HRP Substrate (catalogue no. WBLUC0500, Merck Millipore, Kenilworth, NJ, USA). Densitometric measurements were performed and analyzed using the ChemiDoc MP System instrument and Image Lab Software version 5.0 (Bio-Rad, Marnes-la-Coquette, France). β-actin was used as the reference protein.

2.9. RNA Isolation, Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA from NSCLC, NMLT, and cell line sections was isolated using the RNeasy Mini Kit (catalogue no. 74104, Qiagen, Hilden, Germany) according to the manufacturer's instructions. To remove genomic DNA, the samples were digested using the RNase-Free DNase Set (catalogue no. 79254, Qiagen, Hilden, Germany). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription kit with the RNase inhibitor (catalogue no. 4374966, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The qPCR was performed using the 7500 Real-Time PCR System instrument and 7500 software v2.0.6 (Applied Biosystems, Foster City, CA, USA). The following Taqman probe and primer sets were used in the reactions: *ZYX* (Hs00170299_m1, Applied Biosystems, Foster City, CA, USA) and *ACTB* (Hs99999903_m1,

Applied Biosystems, Foster City, CA, USA). Real-time PCR reaction conditions were as follows: polymerase activation at 50 °C for 2 min, initial denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 sec, annealing and extension at 60 °C for 1 min for 45 cycles. β-actin (ACTB) was used as the reference gene. Changes in gene expression were determined using the ΔΔCt method [28]. Reactions were performed in triplicate.

2.10. Laser Capture Microdissection (LCM) and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Laser capture microdissection (LCM) was performed on NSCLC (SCC and AC) and NMLT specimens. Tumor cells and non-malignant lung cell (NMLC) samples were harvested separately to compare *ZYX* mRNA expression. A Leica CM1950 cryostat (Leica Microsystems, Wetzlar, Germany) was used to slice 10 µm-thick frozen tissue sections that were placed on a polyethylene-terephthalate membrane (catalogue no. 50102, MMI, Glattbrugg, Switzerland). LCM was performed using the MMI CellCut Plus System (MMI, Glattbrugg, Switzerland). RNeasy Micro Kit (catalogue no. 74004, Qiagen, Hilden, Germany) was used to isolate total RNA. The synthesis of cDNA was performed using the QuantiTect Reverse Transcription Kit (catalogue no. 205311, Qiagen, Hilden, Germany). Real-time PCR reactions were performed as described in the section *RNA isolation, reverse transcription–quantitative polymerase chain reaction (RT-qPCR)*.

2.11. Statistical Analysis

The results were statistically analyzed using Prism 5.0 (GraphPad, San Diego, CA, USA) and Statistica 13.1 (StatSoft, Krakow, Poland) software. ANOVA with post-hoc Bonferroni's multiple comparisons test were used to compare ZYX expression in cell lines. The analysis of the results obtained from LCM sections was performed by unpaired t test. Mann–Whitney test or Kruskal–Wallis test with Dunn's post-hoc multiple comparisons test was used to compare ZYX expression in the groups without Gaussian distribution. The paired t-test or unpaired t-test was used to perform statistical analysis of ZYX protein levels in the tissue material as determined by the Western Blot analysis. The survival analysis was performed using the Kaplan–Meier method and the Mantel–Cox test. Univariate and multivariate analyses were performed with the Cox proportional hazards model. The survival analysis was performed only in cases with the complete clinicopathological data. The results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Expression of Zyxin in NSCLC Cell Lines and Normal Lung Fibroblasts

Using ICC and IF, ZYX was detected in the following cell lines: NCI-H1703 (SCC), NCI-H522 (AC), and IMR-90 (normal lung fibroblasts) (Figures 1 and 2). Cytoplasmic and nuclear localization of this protein was observed in all cell lines. Western Blot analysis showed lower levels of ZYX protein in NCI-H1703 and NCI-H522 cell lines compared with control IMR-90 (Figure 3A,B). *ZYX* mRNA expression in NCI-H522 cells was also lower compared with IMR-90 cells (*** $p < 0.001$), while NCI-H1703 cells showed higher *ZYX* mRNA expression compared with control cells (* $p < 0.05$) (Figure 3C). In addition, ZYX expression at mRNA and protein levels was significantly higher in the NCI-H1703 SCC cell line compared with the NCI-H522 AC cell line (*** $p < 0.001$ and * $p < 0.05$; respectively) (Figure 3B,C). Fluorescence intensity measurements showed decreased levels of ZYX in NSCLC cell lines compared with the control line (IMR-90) (Figure 3D).

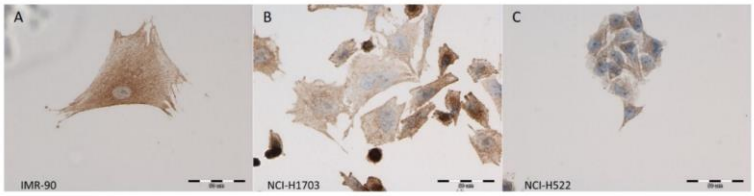

Figure 1. Immunocytochemical (ICC) reactions demonstrating the cytoplasmic localization of ZYX (brown) in the IMR-90 normal lung fibroblast cell line (**A**), NCI-H1703 lung squamous cell carcinoma (SCC) cell line (**B**), and NCI-H522 lung adenocarcinoma (AC) cell line (**C**). Nuclei were counterstained with hematoxylin (blue). Magnification ×400.

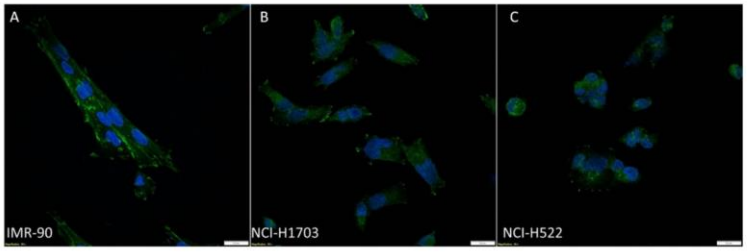

Figure 2. Immunofluorescence (IF) images taken by confocal microscopy showing ZYX expression (green) in the IMR-90 normal lung fibroblast cell line (**A**), NCI-H1703 lung SCC (**B**) and NCI-H522 lung AC (**C**). Nuclei were counterstained with DAPI (blue). Magnification ×600.

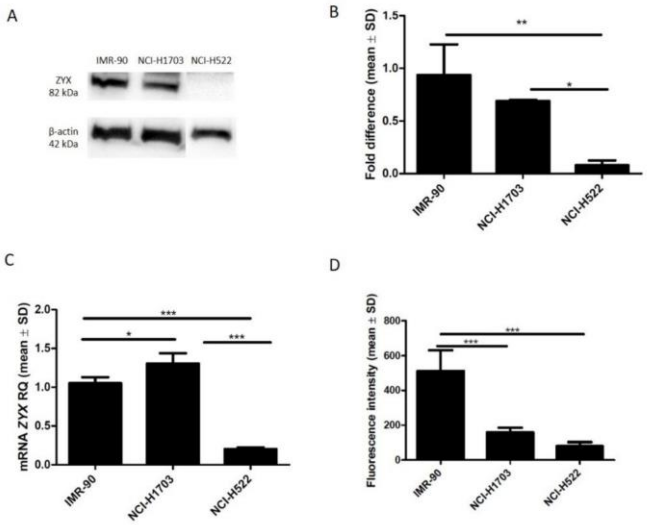

Figure 3. Expression of ZYX in the IMR-90 normal lung fibroblast cell line and in NSCLC cell lines: NCI-H1703 (lung squamous cell carcinoma) and NCI-H522 (lung adenocarcinoma). Western Blot (**A**) and densitometric analysis (**B**) of ZYX protein levels. *ZYX* mRNA expression in the cell lines (**C**). ZYX expression levels determined by measuring fluorescence intensity in the cell lines (**D**). Bonferroni multiple comparisons test (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

3.2. ZYX Expression in Patients with NSCLC

IHC reactions showed the presence of ZYX in the cytoplasm, cell membrane, and in the cell nucleus of tumor cells and NMLT cells. A positive reaction was also found in macrophages in the lung tissue (Figure 4). The representative images of ZYX expression in different lung AC subtypes were also included (Figure S1). The intensity of ZYX expression which was less than or equal to the median in NSCLC cells (IRS $\leq$ 0.33; nuclear score $\leq$ 0.5) was defined as "low expression", while the value of ZYX expression above the median was defined as "high expression". Cytoplasmic expression of ZYX in tumor cells was found in 207 (51.88%) NSCLC cases. The mean value of cytoplasmic ZYX expression in NSCLC cells was 1.354 $\pm$ 1.616 (mean $\pm$ SD). There were 201 (50.38%) cases with low cytoplasmic expression of ZYX in NSCLC cells, and 198 (49.62%) cases with high cytoplasmic expression of ZYX. Nuclear expression of ZYX in NSCLC cells was found in 282 (70.68%) cases. The mean value of nuclear expression of ZYX in NSCLC cells was 0.5242 $\pm$ 0.4662 (mean $\pm$ SD). Low expression of nuclear ZYX in NSCLC cells was present in 236 cases, while high expression was noted in 163 cases.

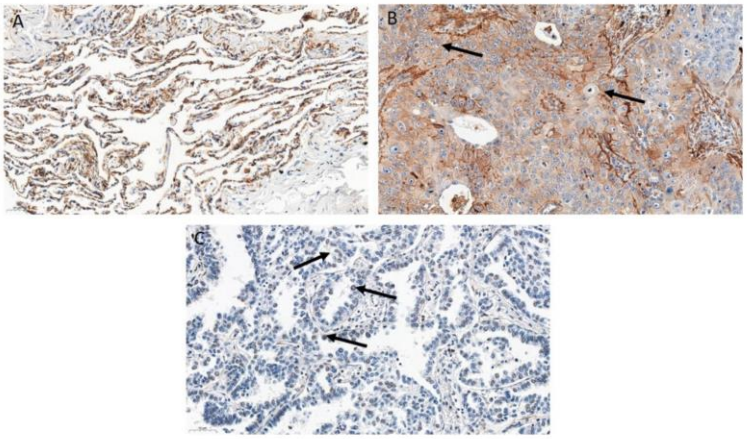

Figure 4. Immunohistochemical (IHC) reactions detecting ZYX protein (brown) performed on non-malignant lung tissue (NMLT) (**A**), lung squamous cell carcinoma (SCC) (**B**), and lung adenocarcinoma (AC) (**C**). Arrows in (**B**) indicate cytoplasmic/membranous localization of ZYX, while arrows in (**C**) show nuclear one. Magnification ×200.

Statistical analysis of IHC reactions results showed significantly lower levels of cytoplasmic ZYX in NSCLC cells compared with control tissue (NMLT) ($p < 0.0001$). However, nuclear ZYX levels were increased in tumor cells compared with control tissue ($p < 0.0001$). These findings were observed not only when the total NSCLC group was analyzed, but also when the SCC and AC subtypes were selected (Figure 5).

The analysis using Western Blot showed significantly reduced expression of ZYX in NSCLC compared with NMLT samples ($p = 0.0021$). Compared with control tissue, reduced levels of ZYX were also found in lung SCCs ($p = 0.0075$) (Figure 6A,B). Lower levels of ZYX were detected in lung ACs compared with control tissue. However, no statistical significance was reported ($p = 0.1254$) (Figure 6C). *ZYX* mRNA levels were significantly decreased in NSCLC and in lung ACs compared to non-malignant lung tissue ($p = 0.0005$; $p = 0.0001$; respectively) (Figure 7A,C). Real-time PCR performed on laser capture microdissected sections showed lower *ZYX* mRNA expression in tumor cells of the whole NSCLC group ($p < 0.0001$), of the SCC subtype ($p < 0.0001$), and of the AC subtype ($p < 0.0001$) compared with non-malignant lung cells (NMLC) (Figure 7D–F).

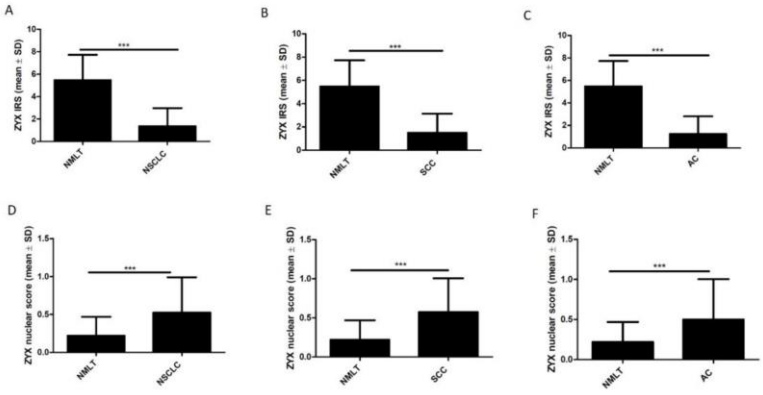

Figure 5. Immunohistochemical (IHC) analysis of ZYX expression in NSCLC cells and in NMLT cells. The graphs showing the intensity of cytoplasmic (IRS) and nuclear (nuclear score) expression of ZYX for the whole NSCLC group (**A**,**D**, respectively), levels of cytoplasmic and nuclear ZYX in lung SCCs (**B**,**E**, respectively), levels of cytoplasmic and nuclear ZYX in lung ACs (**C**,**F**, respectively) compared with NMLT. Mann–Whitney test (*** $p < 0.001$).

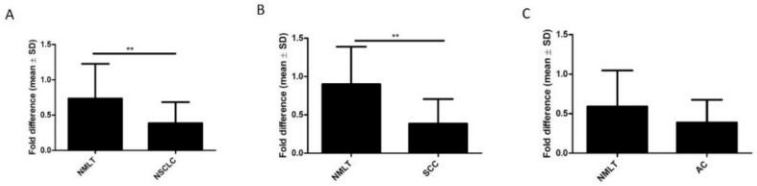

Figure 6. Expression of zyxin in NSCLC (**A**), in the SCC subtype (**B**), and in the AC subtype (**C**) compared with control tissue; Western Blot. Paired t-test (** $p < 0.01$).

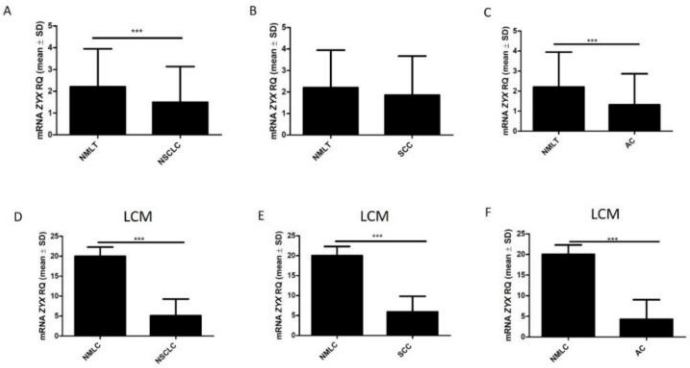

Figure 7. *ZYX* mRNA levels in NSCLC (**A**), lung SCCs (**B**), and lung ACs (**C**) compared with control tissue as determined by real-time PCR. Mann–Whitney test (*** $p < 0.001$). *ZYX* mRNA levels were determined by real-time PCR reactions performed on isolated NSCLC cells (**D**), lung SCC cells (**E**), lung AC cells (**F**), and non-malignant lung cells (NMLCs). Cell isolation was performed by laser capture microdissection (LCM). Unpaired t test (*** $p < 0.001$).

IHC reactions showed higher cytoplasmic and nuclear levels of zyxin in SCC cells than in AC cells (Figure 8A,B). However, statistical significance was observed only in the case of nuclear localization ($p = 0.0292$). Real-time PCR showed higher *ZYX* mRNA levels in the SCC subtype than in the AC subtype (Figure 8D). However, statistical significance was not observed ($p = 0.2604$). Statistical significance was not found when cancer cell isolation was performed using LCM ($p = 0.5758$) (Figure 8E). Western Blot results showed no significant differences in ZYX levels between lung SCCs and lung ACs ($p = 1.000$) (Figure 8C). IHC analysis demonstrated no difference in cytoplasmic ZYX expression between lung SCCs and lung LCCs ($p = 0.6746$) (Figure 8F). Interestingly, higher nuclear ZYX level was detected in SCC than in LCC cells ($p = 0.0404$) (Figure 8G). Higher cytoplasmic ZYX expression in LCC than in AC cells was shown however statistical significance was not observed ($p = 0.9551$) (Figure 8H). Nuclear ZYX level was higher in AC cells than in LCC cells but no statistical difference was noticed ($p = 0.5319$) (Figure 8I).

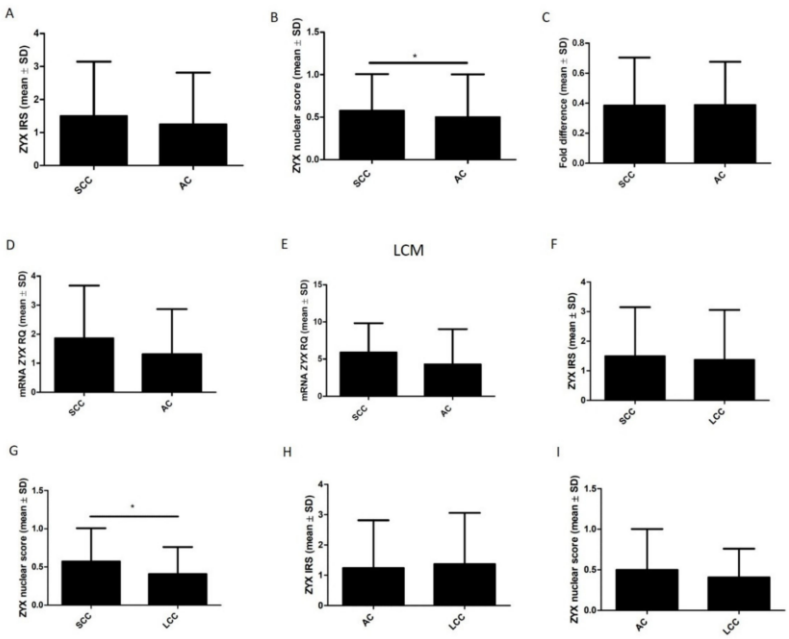

Figure 8. Comparison of zyxin expression in lung SCCs and lung ACs using the following methods: immunohistochemistry (**A**,**B**); Western Blot (**C**); real-time PCR (**D**); and real-time PCR performed on tumor cells isolated using laser capture microdissection (LCM) (**E**). Mann–Whitney test (IHC, RT-qPCR) and unpaired t-test (Western Blot, LCM real-time PCR) (* $p < 0.05$). Comparison of zyxin expression in lung LCCs and other NSCLC subtypes (SCC, AC) with the use of IHC (**F–I**). Mann–Whitney test (* $p < 0.05$).

The levels of both cytoplasmic and nuclear ZYX in NSCLC, SCC, and AC cells were not significantly different according to the histological grade (G) (Figure 9). Further analyses showed that the levels of cytoplasmic ZYX in NSCLC cells decreased with increasing tumor size (pT) (Figure 10A). The same relationships were observed when tumor size (pT) was compared with the levels of cytoplasmic ZYX in lung SCC and lung AC cells (Figure 10B,C). Significantly lower levels of cytoplasmic ZYX in NSCLC and SCC cells were demonstrated for T3-4 compared with T1 (* $p < 0.05$, for both). The levels of nuclear expression of ZYX in NSCLC, SCC, and AC cells showed no relationships (Figure 10D–F). The analysis of the

relationship between the intensity of ZYX expression and the clinical stage showed that the levels of cytoplasmic ZYX in NSCLC cells decreased with the increasing stage (Figure 11A). The same relationship was also present for cytoplasmic ZYX in SCC cells. Significantly lower levels of cytoplasmic ZYX in NSCLC and SCC cells were demonstrated in Stage III–IV compared with Stage I (* $p < 0.05$, for both) (Figure 11A,B). The levels of cytoplasmic ZYX in AC cells showed no relationship with the clinical stage. For nuclear ZYX expression, no correlation was shown between the clinical stage and its levels in NSCLC, SCC, and AC cells (Figure 11D–F).

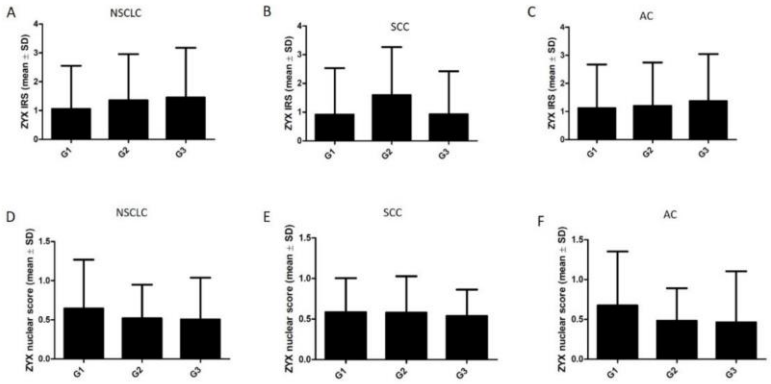

Figure 9. Immunohistochemistry (IRS, nuclear score) of ZYX expression in NSCLC cells in relation to the histological grade. The analysis for NSCLC (**A**,**D**), lung SCC (**B**,**E**), and lung AC (**C**,**F**). Dunn's multiple comparisons test.

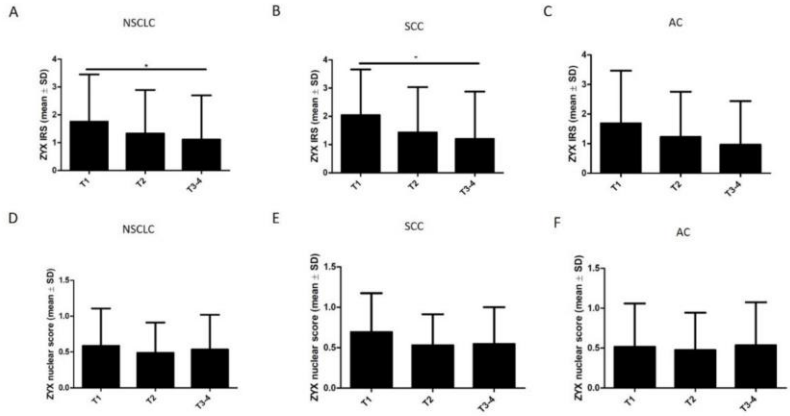

Figure 10. Immunohistochemistry (IRS, nuclear score) of ZYX expression in NSCLC cells in relation to tumor size (pT). Results for NSCLC (**A**,**D**), SCC (**B**,**E**), and AC (**C**,**F**) subtypes. Dunn's multiple comparisons test (* $p < 0.05$).

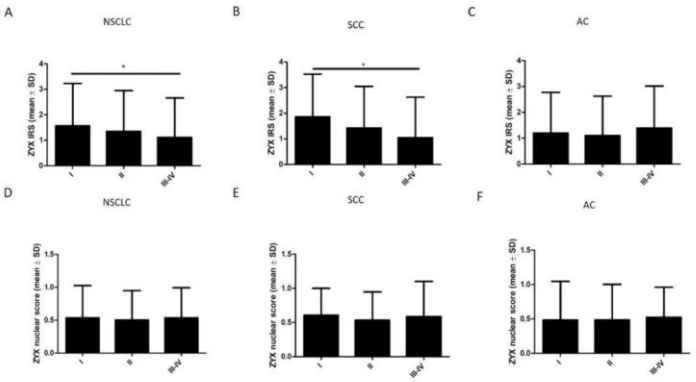

Figure 11. Immunohistochemistry (IRS, nuclear score) of ZYX expression at different clinical stages of cancer. The analysis for the whole NSCLC group (**A**,**D**), SCC (**B**,**E**), and AC (**C**,**F**) subtypes. Dunn's multiple comparisons test (* $p < 0.05$).

3.3. Survival Analysis

Based on IHC, Mantel–Cox tests showed that higher levels of cytoplasmic ZYX in tumor cells were associated with longer overall survival (OS). However, no statistical significance was observed (Figure 12A–D). In terms of nuclear localization, patients with higher ZYX levels had shorter OS except for SCC patients. However, the results were not statistically significant (Figure 12E–H).

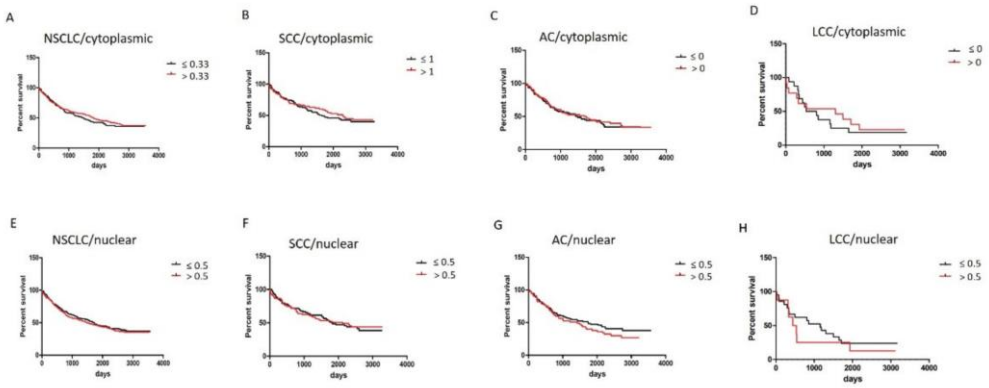

Figure 12. Survival analyses of NSCLC patients in relation to cytoplasmic/nuclear ZYX expression in the cells of NSCLC (**A**,**E**, respectively), SCC (**B**,**F**, respectively), AC (**C**,**G**, respectively), and LCC subtypes (**D**,**H**, respectively). The cut-off point was established in relation to the median value.

Analyses of different ZYX expression groups (low/high level) in relation to survivals of patients demonstrated that overall survivals of NSCLC (in total), SCC, and AC patients were similar in each group (Figure 13A–D). The differences were observed in the case of LCC patients who had shorter OS in comparison with NSCLC, SCC, and AC patients.

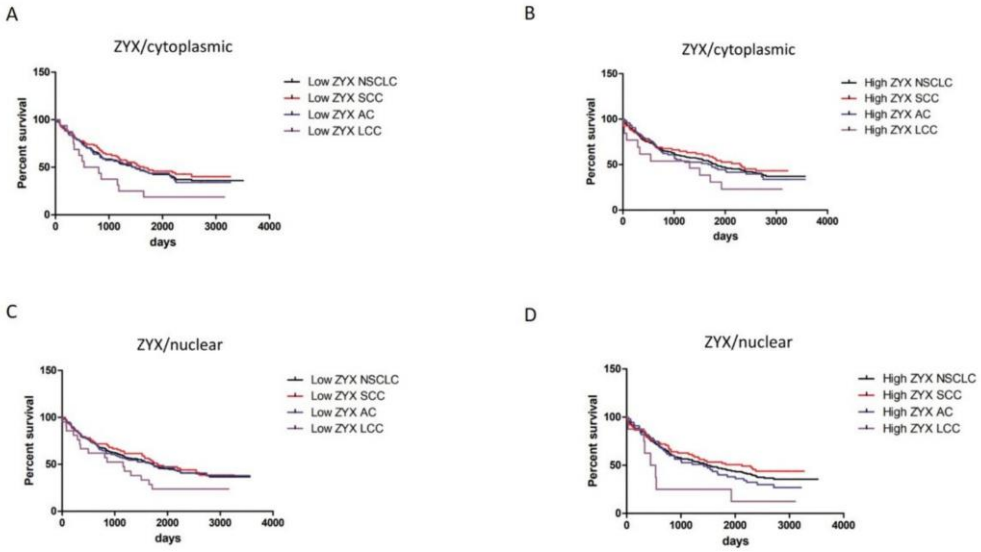

Figure 13. Different cytoplasmic (**A**,**B**) and nuclear (**C**,**D**) ZYX expression statuses in relation to survival of NSCLC (total), SCC, AC, and LCC patients. The cut-off points were set on median values of ZYX expression in NSCLC, SCC, AC, and LCC (IHC).

Univariate analysis showed that OS of NSCLC patients was associated with the clinicopathological factors such as age over 62 years, male sex, higher histological grade (G), larger tumor size (pT), presence of lymph node metastases (pN), and higher clinical tumor stage (Table 3). It was shown that cytoplasmic and nuclear expression of ZYX in NSCLC could not be considered a factor affecting patient survival. For patients with SCC, higher clinical stage, higher histological grade (G) and higher pT affected OS. ZYX expression in SCC did not affect OS (Table 3). Univariate survival analysis of AC patients showed that the male sex, living in urban areas, higher clinical stage, higher pT and the presence of lymph node metastases (pN) were negative factors for survival. Cytoplasmic/nuclear expression of ZYX in AC was not a factor affecting OS (Table 3).

Multivariate survival analysis was performed for all factors that were statistically significantly associated with OS in univariate analyses. Age over 62 years, male sex, higher histological grade (G), tumor size (pT), and higher clinical stage were shown as independent prognostic factors for patients with NSCLC (Table 3). Multivariate survival analysis for patients with SCC showed that histological grade (G) and pT had an independent prognostic effect on survival (Table 3). In turn, multivariate survival analysis for AC patients showed that the male sex, living in urban areas, higher pT, and the presence of lymph node metastases (pN) were independent prognostic factors (Table 3).

Table 3. Survival analysis of patients with NSCLC (A), SCC (B), and AC (C). The analyses were performed using Cox proportional hazards model.

A						
Overall Survival						
NSCLC						
	Univariate			**Multivariate**		
Clinical Feature	***p* Value**	**HR**	**Confidence Interval 95% (HR)**	***p* Value**	**HR**	**Confidence Interval 95% (HR)**
Age ≤62 vs. >62	**0.005911**	**1.459308**	**1.114989–1.909957**	**0.001879**	**1.540898**	**1.173239–2.023770**
Sex Female vs. male	**0.000262**	**1.820726**	**1.319828–2.511725**	**0.000222**	**1.845819**	**1.333147–2.555644**
Smoking No vs. Yes	0.164272	1.325817	0.891007–1.972812			
Living in urban areas No vs. Yes	0.053255	1.575759	0.993642–2.498906			
Clinical stage I–II vs. III–IV	**0.000000**	**2.351361**	**1.782315–3.102088**	**0.009199**	**1.702469**	**1.140750–2.540783**
Histological grade G1–G2 vs. G3	**0.035460**	**1.441683**	**1.025203–2.027353**	**0.011715**	**1.564191**	**1.104626–2.214952**
pT pT1-pT2 vs. pT3-pT4	**0.000000**	**2.161526**	**1.639607–2.849582**	**0.008051**	**1.559392**	**1.122639–2.166059**
pN N0 vs. N1–N2	**0.000564**	**1.621062**	**1.231833–2.133276**	0.163298	1.285130	0.903181–1.828603
p63 ≤25 vs. >25%	0.097096	0.796109	0.608116–1.042217			
TTF-1 ≤25 vs. >25%	0.936561	0.989066	0.754490–1.296575			
Ki-67 ≤25 vs. >25%	0.968136	0.994309	0.751467–1.315628			
Cytoplasmic zyxin levels in cancer cells High vs. Low	0.506478	1.095422	0.837153–1.433368			
Nuclear zyxin levels in cancer cells Low vs. High	0.649726	1.064851	0.811928–1.396562			

Table 3. *Cont.*

B						
Overall Survival						
SCC						
	Univariate			**Multivariate**		
Clinical Feature	***p* Value**	**HR**	**Confidence Interval 95% (HR)**	***p* Value**	**HR**	**Confidence Interval 95% (HR)**
Age ≤64 vs. >64	0.083694	1.456415	0.951168–2.230043			
Sex Female vs. male	0.121189	1.595476	0.883699–2.880554			
Smoking No vs. Yes	0.748266	1.159491	0.469650–2.862597			
Living in urban areas No vs. Yes	0.502217	1.266994	0.634712–2.529139			
Clinical stage I–II vs. III–IV	**0.001125**	**2.128965**	**1.351164–3.354511**	0.247973	1.390731	0.794747–2.433645
Histological grade G1–G2 vs. G3	**0.000091**	**2.997641**	**1.729638–5.195220**	**0.002834**	**2.414826**	**1.353633–4.307953**
pT pT1-pT2 vs. pT3-pT4	**0.000534**	**2.148663**	**1.393743–3.312486**	**0.049517**	**1.682302**	**1.001099–2.827031**
pN N0 vs. N1–N2	0.908460	1.026365	0.658646–1.599380			
p63 ≤25 vs. >25%	0.365148	0.767183	0.432303–1.361474			
TTF-1 ≤25 vs. >25%	0.546339	1.199180	0.664646–2.163608			
Ki-67 ≤25 vs. >25%	0.836690	0.956480	0.626516–1.460224			
Cytoplasmic zyxin levels in cancer cells High vs. Low	0.603775	1.119886	0.730239–1.717444			
Nuclear zyxin levels in cancer cells Low vs. High	0.823766	0.953000	0.623872–1.455763			

Table 3. *Cont.*

C						
Overall Survival						
AC						
	Univariate			**Multivariate**		
Clinical Feature	***p* Value**	**HR**	**Confidence Interval 95% (HR)**	***p* Value**	**HR**	**Confidence Interval 95% (HR)**
Age ≤61 vs. >61	0.439639	1.170082	0.785611–1.742711			
Sex Female vs. male	**0.000411**	**2.195089**	**1.419128–3.395335**	**0.000119**	**2.371420**	**1.527428–3.681766**
Smoking No vs. Yes	0.331944	1.270446	0.783337–2.060456			
Living in urban areas No vs. Yes	**0.029915**	**2.244604**	**1.081842–4.657101**	**0.029913**	**2.314151**	**1.085072–4.935430**
Clinical stage I–II vs. III–IV	**0.000000**	**2.922240**	**1.945881–4.388492**	0.364952	1.311295	0.729573–2.356852
Histological grade G1–G2 vs. G3	0.880118	1.037945	0.639707–1.684099			
pT pT1-pT2 vs. pT3-pT4	**0.000001**	**2.846085**	**1.873553–4.323444**	**0.002122**	**2.191669**	**1.328609–3.615369**
pN N0 vs. N1–N2	**0.000000**	**2.883628**	**1.922023–4.326332**	**0.001392**	**2.317220**	**1.384130–3.879337**
p63 ≤25 vs. >25%	0.907950	1.032147	0.603679–1.764726			
TTF-1 ≤25 vs. >25%	0.133316	0.720412	0.469498–1.105423			
Ki-67						
≤25 vs. >25%	0.894629	1.033806	0.632080–1.690855			
Cytoplasmic zyxin levels in cancer cells High vs. Low	0.758237	1.064633	0.714493–1.586360			
Nuclear zyxin levels in cancer cells Low vs. High	0.246701	1.271330	0.846950–1.908353			

4. Discussion

Few and controversial papers related to the role of ZYX in NSCLC prompted us to investigate this issue. Moreover, to the best of our knowledge, these studies are among the few in which the assessment of ZYX expression was performed in NSCLC cases using clinical specimens.

There are many reports demonstrating that ZYX may function not only as an oncogenic protein but also as a suppressor protein in the process of carcinogenesis [17,29]. Increased levels of ZYX have been demonstrated in breast and colorectal cancers [14,15], while a suppressor role of this protein has been found in prostate and bladder cancers [17,30,31]. In NSCLC, ZYX probably functions as a suppressor protein, as demonstrated by our results.

By using Western Blot and real-time PCR, we demonstrated that the total level of ZYX in NSCLC cases was significantly decreased compared with normal lung tissue. RT-qPCR results obtained using laser microdissected sections confirmed lower *ZYX* mRNA expression in tumor cells compared with normal cells. IHC reactions also showed lower cytoplasmic ZYX expression in cancer cells compared with normal cells. The decreased levels of ZYX in cancer cells were also demonstrated by studies using an in vitro model of NSCLC cell lines. Our results are consistent with the reports of Mise et al., who demonstrated decreased expression of ZYX in cancer tumors in a mouse model of lung cancer [21]. They also observed that A549 cells with silenced ZYX expression had an increased ability to migrate [21]. This may indirectly suggest that ZYX may inhibit the epithelial-mesenchymal transition (EMT) of lung cancer cells [21]. Cadinu et al. showed lower expression of ZYX in the NSCLC cell line (HCC4017) compared with the control line [20]. Moreover, they also observed significantly lower levels of other cytoskeleton proteins in cancer cells compared with normal cells [20]. These studies suggest that decreased expression of ZYX and cytoskeletal proteins may promote the development of NSCLC. An explanation for this phenomenon may be the disruption of cell adhesion, which enables cell migration and invasion that are closely related to cancer progression.

A suppressor role of ZYX was also reported in prostate cancer [30]. Yu and Luo [30] showed that ZYX could inhibit cell migration and invasion through direct interaction with the protein known as myopodin [17,30]. These results [30] suggest that the impaired interaction of myopodin with ZYX may have a tumor-promoting effect. A similar situation may occur in NSCLC, i.e., impaired interaction of ZYX with other factors may promote the process of carcinogenesis due to reduced levels of ZYX.

In turn, Sanchez-Carbayo et al. showed that low levels of ZYX were associated with higher histological grade and higher clinical stage of bladder cancer [31]. It was suggested that impaired interaction between β-catenin and moesin, E-cadherin, or ZYX could impair the formation of cell adhesion junctions/adherens junctions and thus promote tumor progression [17,31,32]. It seems that similar interactions between ZYX and other proteins may also occur in the development of NSCLC. However, the specific mechanisms have not been described yet.

IHC reactions showed that the levels of ZYX in the cytoplasm were lower in NSCLC cells, while the nuclear expression of ZYX was higher compared with control tissue. An explanation for this phenomenon may be the translocation of ZYX from the cytoplasm to the cell nucleus, previously observed by other researchers [7,9,10,33,34]. The presence of ZYX in the nucleus may be related to its involvement in the regulation of gene expression responsible for the process of cancer transformation [7,10,33].

Moon et al., showed that treatment of SiHa cells with exogenous thymosin β4 resulted in translocation of ZYX to the cell nucleus during the first hours of incubation [34]. After some time, ZYX was translocated back to the cytoplasm [34]. They speculated that under the influence of thymosin β4, ZYX could affect cell migration by regulating actin polymerization and depolymerization. It can also be speculated that ZYX may be a transport molecule for thymosin β4, allowing it to enter the cell nucleus [34]. Thus, it can be suggested that in the case of NSCLC, ZYX may regulate actin polymerization or may function as a transport molecule for other molecules into the nucleus, thus enabling tumor progression.

The presence of ZYX in the cell nucleus may also be related to its interaction with transcription factors and regulation of gene expression that are important in carcinogenesis, including NSCLC. Choi et al. observed that the activation of the transcription factor HNF-1β by ZYX affected the migratory capacity of cells [10]. ZYX has also been shown to interact with the ZNF384 transcription factor (zinc finger protein 384) and it probably functions as a mediator for interactions between ZNF384 and p130CAS in focal adhesions [11]. The role of ZYX in transcriptional regulation was also reported by Degenhardt and Silverstein [33]. Their study [33] showed that ZYX was translocated to the cell nucleus and transcription processes were activated under the influence of the E6 protein of HPV6 [33]. In turn, Youn et al. described the role of ZYX in the regulation of retinoic acid (RA) signaling

pathway [9]. Incubation of H1299 NSCLC cells with RA resulted in translocation of ZYX to the nucleus [9]. Further analysis showed the interaction of ZYX with PTOV1 (commonly overexpressed in prostate cancer [35]) and CBP proteins (RA receptor coactivator), resulting in attenuation of the cytotoxic effect of RA [9]. This suggests that ZYX may be responsible for the resistance of cancer cells to therapy. The distribution of ZYX in cells was also reported by Grunewald et al. [36,37]. In their study on ovarian cancer and breast cancer cell lines, they observed that altered expression of LASP-1 protein, which is a focal adhesion molecule [38], resulted in changes in the amount of ZYX in focal adhesions, which affected cell proliferation and migratory abilities [36,37].

Translocation of ZYX to the cell nucleus may also affect the regulation of apoptosis. Ghosh et al. found that mechanical stimulation in the form of stretch resulted in ZYX translocation to the nucleus of vascular smooth muscle cells, which regulated the activity of specific genes [7]. At the same time, silencing of ZYX expression resulted in increased proliferation and inhibition of apoptosis induced by the stretch mechanism and Fas Ligand [7]. Hervy et al. aimed to verify the effect of ZYX on the survival of mouse embryo fibroblasts (MEFs) treated with UV-C [8]. When exposed to UV-C, translocation of ZYX to the nucleus occurred, and increased caspase-3 activity and induction of apoptosis were observed [8]. Further experiments showed that the proapoptotic effect of ZYX was dependent on CARP-1 [8]. The above results confirm that ZYX can promote apoptosis. It is known that apoptosis is one of the processes responsible for the elimination of cancer cells. In our study, the levels of nuclear ZYX in NSCLC cases were significantly higher compared with normal lung tissue, which may indicate that ZYX is translocated to the nucleus to induce apoptosis as a defense mechanism against further tumor progression. However, the expression of nuclear ZYX decreased with the increase in the histological grade of NSCLC, which was mostly found in AC cases. Although these differences were not statistically significant, the trend suggests that this hypothetical defense mechanism was ineffective. Thus, the decrease in ZYX expression, which was observed in our study, may increase the survival of tumor cells and thus promote tumor progression.

The hypothesis of a suppressor role of ZYX in NSCLC development may also be supported by the results of the analysis of the relationship between the level of this protein and tumor size. In our study, we demonstrated that cytoplasmic ZYX levels in all NSCLC cases and in the SCC subtype (which was analyzed separately) decreased with increasing tumor size. We further found that the levels of cytoplasmic ZYX in NSCLC and SCC cells decreased progressively with increasing clinical stage. The nuclear ZYX levels showed no relationship with tumor size or clinical stage. Additionally, we did not observe significant relationships between ZYX expression and histological grade (G). Although nuclear ZYX levels in NSCLC cases analyzed in total and separately for SCC and AC cases decreased slightly with increasing histological grade (G), our results were not statistically significant. The absence of correlations between ZYX levels and some clinicopathological factors may be explained by different mechanisms that cause increase or decrease in ZYX expression. Due to the lack of similar studies in NSCLC, it is not possible to compare our findings. However, they are partly in line with the observations of Sanchez-Carbayo et al., who demonstrated a correlation between low ZYX levels and higher histological grade and higher clinical stage of bladder cancer [31].

The hypothesis of a suppressive role of ZYX can be supported by survival analysis, which showed that higher levels of cytoplasmic ZYX in cancer cells were associated with longer OS. In turn, higher levels of nuclear ZYX correlated negatively with the duration of OS (except for SCC), which is contrary to the results of Hervy et al. [8]. Survival analysis using Cox proportional hazards model showed that cytoplasmic and nuclear ZYX in NSCLC, SCC and AC cells could not be considered to be independent prognostic factors for OS.

Investigation showed that in different ZYX expression groups, overall survivals of NSCLC, SCC, and AC patients did not differ dramatically from one another in each group. However, the differences were noticed for survival curves of LCC patients. The explanation

for this phenomenon might be the fact lung LCC is described as a cancer with poor prognosis [39].

Further analysis showed that the levels of both cytoplasmic and nuclear ZYX were higher in SCC cells compared with AC cells. A similar difference was observed in *ZYX* mRNA levels in SCC and AC cases and the corresponding cell lines. This suggests that ZYX expression is regulated differently in the two NSCLC subtypes. This differentiation may be due to the distinct tumor microenvironment that determines tumor properties and may influence the nature of a particular subtype of NSCLC [3]. The structure of the extracellular matrix (ECM) probably affects the expression of ZYX. Fibronectin has been shown to be part of the ECM in desmoplastic pulmonary AC, whereas keratin has been found in lung SCC [3]. Therefore, variation in ECM composition may account for the different expression of ZYX in lung SCC and AC cells. The comparison of cytoplasmic and nuclear ZYX expression between LCC and other NSCLC subtypes demonstrated different levels of this protein. Significantly higher nuclear ZYX levels were observed in SCC than in LCC cells. These facts may also be explained by different ECM composition.

The experiment was carried out on TMAs which do not constitute the whole tissue section. The next limitation is the use of only two NSCLC cell lines. Nevertheless, these cell lines represent the most common types of NSCLC, i.e., lung squamous cell carcinoma (NCI-H1703) and lung adenocarcinoma (NCI-H522). The final conclusions were stated based on the overall and cytoplasmic ZYX expression because the overall level was detected as decreased in NSCLC. However, the cytoplasmic and nuclear levels of ZYX were analyzed in relation to clinicopathological data and discussed. The roles of cytoplasmic and nuclear localizations of ZYX were not investigated with experiments.

Previous studies suggested that ZYX could act as both a promoter and suppressor protein in the process of tumor transformation, depending on the type of cancer. Our results support the conclusion that a decrease in ZYX expression may promote the formation of NSCLC. However, the role of decreased ZYX expression in NSCLC is still not completely explained.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biom12060827/s1, Figure S1: Representative IHC images of ZYX expression in different subtypes of lung adenocarcinoma. Magnification x200.

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Data Availability Statement: The protocols and datasets will be made available to other researchers on reasonable request. For protocols or datasets, contact aleksandra.partynska@umw.edu.pl.

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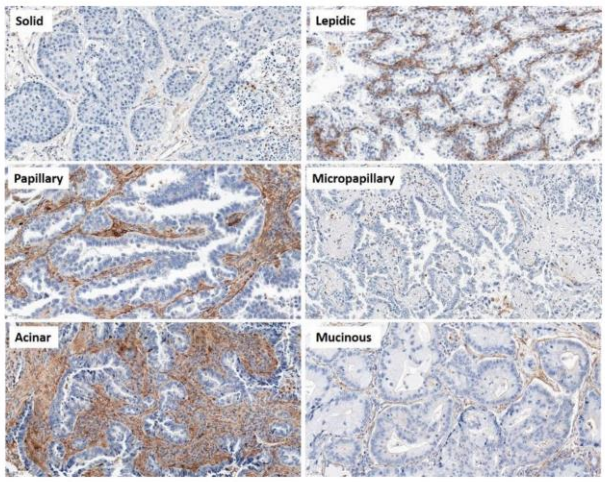

Supplementary Figure 1. Representative IHC images of ZYX expression in different subtypes of lung adenocarcinoma. Magnification x200.

Podsumowanie i wnioski

Wiele badań sugeruje udział ZYX w progresji nowotworowej. Wykazano, że ZYX może funkcjonować w dwojaki sposób, to znaczy może działać promująco jak i hamująco na onkogenezę, w zależności od rodzaju nowotworu. Wpływ zyksyny na proces nowotworzenia został podsumowany w publikacji przeglądowej.

Wyniki badań IHC, zawarte w drugiej publikacji, zademonstrowały obniżony poziom cytoplazmatycznej ZYX w komórkach NSCLC w stosunku do prawidłowych komórek płucnych. Natomiast poziom jądrowej ZYX okazał się być podwyższony w komórkach NSCLC w porównaniu do tkanki kontrolnej. Zależności te odnotowano zarówno, gdy analizowano całą kohortę NSCLC, jak i jego podtypy płaskonabłonkowy i gruczołowy osobno. Badania metodą Western Blot pokazały obniżony poziom białka ZYX w guzach NSCLC w stosunku do tkanki kontrolnej. Zauważono również obniżony poziom mRNA *ZYX* w guzach NSCLC w porównaniu do tkanki prawidłowej. Zmniejszoną ekspresję ZYX w komórkach NSCLC potwierdzają reakcje RT-qPCR wykonane na mRNA pochodzącym z wyizolowanych metodą mikrodysekcji laserowej komórek NSCLC i komórek tkanki płuc niezmienionej nowotworowo. Badania wykazały istotnie niższy poziom jądrowej ZYX w komórkach AC niż w SCC. Co więcej, w komórkach NSCLC oraz SCC istotnie niższy poziom cytoplazmatycznej ZYX występował w przypadkach guzów pT3-4 niż w pT1. Niższy poziom cytoplazmatycznej ZYX odnotowano w stadium zaawansowania klinicznego choroby III-IV niż w stadium I w przypadku komórek NSCLC i SCC. Analiza modelem proporcjonalnego hazardu Coxa wykazała, że zarówno cytoplazmatyczna, jak i jądrowa ekspresja ZYX w komórkach NSCLC nie może być traktowana jako niezależny czynnik prognostyczny. Badania na modelu *in vitro* także prezentują obniżony poziom białka ZYX w komórkach NSCLC w porównaniu do linii kontrolnej prawidłowych fibroblastów płucnych.

Końcowe wnioski:

1. Obniżony poziom ZYX w guzach NSCLC w stosunku do tkanki kontrolnej, jak i obniżony poziom ZYX w liniach komórkowych NSCLC może sugerować supresorową rolę tego białka w patogenezie NSCLC.
2. ZYX prawdopodobnie nie może być rozważana jako niezależny czynnik prognostyczny w NSCLC.

Załączniki:

Oświadczenia współautorów publikacji stanowiących podstawę Pracy Doktorskiej

Opinie Komisji Bioetycznej

Dorobek naukowy

- Publikacje naukowe i rozdział w monografii naukowej
- Doniesienia konferencyjne

Prof. dr hab. Piotr Dzięgiel
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

Wrocław, 24.04.2023 r.

Oświadczenie o współautorstwie

Oświadczam, że w pracy *Partyńska Aleksandra, Gomułkiewicz Agnieszka, Dzięgiel Piotr, Podhorska-Okołów Marzenna, The role of zyxin in carcinogenesis. Anticancer Research, 2020, Vol. 40, no. 11, s. 5981-5988, DOI: 10.21873/anticanres.14618*; mój udział polegał na końcowej korekcie manuskryptu przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Aleksandry Partyńskiej pt. „Ekspresja zyksyny w niedrobnokomórkowych rakach płuc".

Podpis

Uniwersytet Medyczny we Wrocławiu
ZAKŁAD HISTOLOGII I EMBRIOLOGII
prof. dr hab. Piotr Dzięgiel

Prof. dr hab. Marzenna Podhorska-Okołów
Zakład Badań Ultrastrukturalnych
Uniwersytet Medyczny we Wrocławiu

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M. Podhorska-Okołów

Podpis

Dr Agnieszka Gomułkiewicz
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

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Podpis

Mgr Aleksandra Partyńska
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

Wrocław, 24.04.2023 r.

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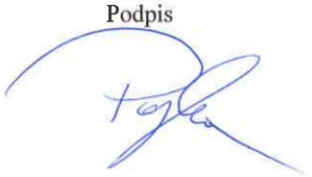
Podpis



Prof. dr hab. Piotr Dzięgiel
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

Wrocław, 24.04.2023 r.

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Oświadczam, że w pracy *Partyńska Aleksandra, Gomułkiewicz Agnieszka, Piotrowska Aleksandra, Grzegrzółka Jędrzej, Rzechonek Adam, Ratajczak-Wielgomas Katarzyna, Podhorska-Okołów Marzenna, Dzięgiel Piotr, Expression of zyxin in non-small cell lung cancer - a preliminary study. Biomolecules, 2022, Vol. 12, no. 6, art. 827, DOI: 10.3390/biom12060827;* mój udział polegał na opracowaniu częściowej koncepcji pracy, konsultacjach i opiece merytorycznej, uczestnictwie w interpretacji wyników oraz końcowej korekcie manuskryptu przed złożeniem do druku. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Aleksandry Partyńskiej pt. „Ekspresja zyksyny w niedrobnokomórkowych rakach płuc".

Podpis

Uniwersytet Medyczny we Wrocławiu
ZAKŁAD HISTOLOGII I EMBRIOLOGII
Kierownik
prof. dr hab. Piotr Dzięgiel

Prof. dr hab. Marzenna Podhorska-Okołów
Zakład Badań Ultrastrukturalnych
Uniwersytet Medyczny we Wrocławiu

Wrocław, 24.04.2023 r.

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M. Podhorska-Okołów

Podpis

Dr hab. Adam Rzechonek
Dolnośląskie Centrum Torakochirurgii
Dolnośląskie Centrum Onkologii, Pulmonologii
i Hematologii

Wrocław, 05.05.2023 r.

Oświadczenie o współautorstwie

Oświadczam, że w pracy *Partyńska Aleksandra, Gomułkiewicz Agnieszka, Piotrowska Aleksandra, Grzegrzółka Jędrzej, Rzechonek Adam, Ratajczak-Wielgomas Katarzyna, Podhorska-Okołów Marzenna, Dzięgiel Piotr, Expression of zyxin in non-small cell lung cancer - a preliminary study. Biomolecules, 2022, Vol. 12, no. 6, art. 827, DOI: 10.3390/biom12060827;* mój udział polegał na zabezpieczaniu i gromadzeniu materiału klinicznego do badań oraz gromadzeniu danych kliniczno-patologicznych pacjentów. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Aleksandry Partyńskiej pt. „Ekspresja zyksyny w niedrobnokomórkowych rakach płuc".

Podpis

Adam Rzechonek

dr hab. n. med. Adam Rzechonek
chirurg ogólny
specjalista torakochirurg
Wilczyce, ul. Dworska 21
51-361 Wrocław 10
1176870

Dr Agnieszka Gomułkiewicz
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

Wrocław, 24.04.2023 r.

Oświadczenie o współautorstwie

Oświadczam, że w pracy *Partyńska Aleksandra, Gomułkiewicz Agnieszka, Piotrowska Aleksandra, Grzegrzółka Jędrzej, Rzechonek Adam, Ratajczak-Wielgomas Katarzyna, Podhorska-Okołów Marzenna, Dzięgiel Piotr, Expression of zyxin in non-small cell lung cancer - a preliminary study. Biomolecules, 2022, Vol. 12, no. 6, art. 827, DOI: 10.3390/biom12060827;* mój udział polegał na współuczestniczeniu w izolacji RNA, współwykonywaniu reakcji odwrotnej transkrypcji i real-time PCR, konsultacjach, uczestniczeniu w analizie wyników, poprawie pierwotnej wersji manuskryptu oraz zaangażowaniu w pozyskiwanie środków finansowych na prowadzenie powyższych badań. Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Aleksandry Partyńskiej pt. „Ekspresja zyksyny w niedrobnokomórkowych rakach płuc".

Podpis

Dr Aleksandra Piotrowska
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

Wrocław, 24.04.2023 r.

Oświadczenie o współautorstwie

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Podpis

Dr Katarzyna Ratajczak-Wielgomas
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

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Podpis

Dr Jędrzej Grzegrzółka
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

Wrocław, 24.04.2023 r.

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Podpis

Mgr Aleksandra Partyńska
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

Wrocław, 24.04.2023 r.

Oświadczenie o współautorstwie

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Podpis

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

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 483/2018

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:

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 (duchowny)
mgr Luiza Műller (prawo)
dr hab. Sławomir Sidorowicz (psychiatria)
dr hab. Leszek Szenborn (pediatria, choroby zakaźne)
Danuta Tarkowska (pielęgniarstwo)
prof. dr hab. Anna Wiela-Hojeńska (farmakologia kliniczna)
dr hab. Andrzej Wojnar (histopatologia, dermatologia) przedstawiciel Dolnośląskiej Izby Lekarskiej)
dr hab. Jacek Zieliński (filozofia)

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

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

„Rola zyksyny w niedrobnokomórkowych rakach płuc"

zgłoszonym przez **mgr Aleksandrę Partyńską** uczestniczkę studiów doktoranckich w Zakładzie Histologii i Embriologii Katedry Morfologii i Embriologii Człowieka Uniwersytetu Medycznego we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła wyrazić zgodę na przeprowadzenie badania w Zakładzie Histologii i Embriologii Katedry Morfologii i Embriologii Człowieka UM we Wrocławiu pod nadzorem prof. dr hab. Piotra Dzięgiela **pod warunkiem zachowania anonimowości uzyskanych danych**.

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

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

Opinia powyższa dotyczy: projektu badawczego będącego podstawą rozprawy doktorskiej

Wrocław, dnia 6 września 2018 r.

BW

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

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

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 504/2018

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:

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 (duchowny)
mgr prawa Luiza Műller (prawo)
dr hab. Sławomir Sidorowicz (psychiatria)
dr hab. Leszek Szenborn (pediatria, choroby zakaźne)
Danuta Tarkowska (pielęgniarstwo)
prof. dr hab. Anna Wiela-Hojeńska (farmakologia kliniczna)
dr hab. Andrzej Wojnar (histopatologia, dermatologia) przedstawiciel Dolnośląskiej Izby Lekarskiej)
dr hab. Jacek Zieliński (filozofia)

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

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

„Rola zyksyny w niedrobnokomórkowych rakach płuc"

zgłoszonym przez **prof. dr hab. Macieja Zabla** zatrudnionego w Katedrze Morfologii i Embriologii Człowieka, Zakładzie Histologii i Embriologii Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła **wyrazić zgodę** na przeprowadzenie badania przez **mgr Aleksandrę Partyńską** w Zakładzie Histologii i Embriologii Katedry Morfologii i Embriologii Człowieka UMW **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 grantu dla młodych naukowców.

Wrocław, dnia 11 września 2018 r.

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

Publikacje naukowe i rozdział w monografii naukowej

Sumaryczny IF: 37,024
Sumaryczna liczba punktów MEiN: 930
Indeks Hirscha: 5

1. Urbaniak Anna, Jabłońska Karolina, Suchański Jarosław, **Partyńska Aleksandra**, Szymczak-Kulus Katarzyna, Matkowski Rafał, Maciejczyk Adam, Ugorski Maciej, Dzięgiel Piotr: *Prolactin-induced protein (PIP) increases the sensitivity of breast cancer cells to drug-induced apoptosis*. Scientific Reports 2023 vol. 13 art.6574; DOI: 10.1038/s41598-023-33707-w

   IF (2021)=4,996 Pkt MEiN=140

2. **Partyńska Aleksandra**, Piotrowska Aleksandra, Pawełczyk Konrad, Rzechonek Adam, Ratajczak-Wielgomas Katarzyna, Podhorska-Okołów Marzenna, Dzięgiel Piotr: *The Expression of Histone Acetyltransferase KAT6A in Non-small Cell Lung Cancer*. Anticancer Research 2022 Vol. 42 no. 12 s.5731-5741; DOI:10.21873/anticanres.16080

   IF (2021)=2,435 Pkt MEiN=70

3. **Partyńska Aleksandra**, Gomułkiewicz Agnieszka, Piotrowska Aleksandra, Grzegrzółka Jędrzej, Rzechonek Adam, Ratajczak-Wielgomas Katarzyna, Podhorska-Okołów Marzenna, Dzięgiel Piotr: *Expression of zyxin in non-small cell lung cancer − a preliminary study*. Biomolecules 2022 Vol. 12 no. 6 art. 827; DOI:10.3390/biom12060827

   IF (2021)=6,064 Pkt MEiN=100,00

4. **Partyńska Aleksandra**, Gomułkiewicz Agnieszka, Dzięgiel Piotr, Podhorska-Okołów Marzenna: *The role of zyxin in carcinogenesis*. Anticancer Research 2020 Vol. 40 no. 11 s. 5981-5988; DOI:10.21873/anticanres.14618

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5. Ratajczak-Wielgomas Katarzyna, Kmiecik Alicja, Grzegrzółka Jędrzej, Piotrowska Aleksandra, Gomułkiewicz Agnieszka, **Partyńska Aleksandra**, Pawełczyk Konrad, Nowińska Katarzyna, Podhorska-Okołów Marzenna, Dzięgiel Piotr: *Prognostic significance of stromal periostin expression in non-small cell lung cancer*. International Journal of Molecular Sciences 2020 Vol. 21 no. 19 art. 7025; DOI:10.3390/ijms21197025

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6. Nowińska Katarzyna, Jabłońska Karolina, Pawełczyk Konrad, Piotrowska Aleksandra, **Partyńska Aleksandra**, Gomułkiewicz Agnieszka, Ciesielska Urszula, Kątnik Ewa, Grzegrzółka Jędrzej, Glatzel-Plucińska Natalia, Ratajczak-Wielgomas Katarzyna, Podhorska-Okołów Marzenna, Dzięgiel Piotr: *Expression of irisin/FNDC5 in cancer cells and stromal fibroblasts of non-small cell lung cancer*. Cancers 2019 Vol. 11 no. 10 art. 1538; DOI:10.3390/cancers11101538

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7. Jabłońska Karolina, Nowińska Katarzyna, Piotrowska Aleksandra, **Partyńska Aleksandra**, Kątnik Ewa, Pawełczyk Konrad, Kmiecik Alicja, Glatzel-Plucińska Natalia, Podhorska-Okołów Marzenna, Dzięgiel Piotr: *Prognostic impact of melatonin receptors MT1 and MT2 in non-small cell lung cancer (NSCLC)*. Cancers 2019 Vol. 11 no. 7 art. 1001; DOI:10.3390/cancers11071001

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8. **Partyńska Aleksandra**, Jabłońska Karolina, Nowińska Katarzyna, Dzięgiel Piotr: *Rola prolaktyny oraz jej receptora w rozwoju nowotworów (The role of prolactin and its receptor in cancer development)*. Postępy Higieny i Medycyny Doświadczalnej 2019 Vol. 73 s. 232-244; DOI:10.5604/01.3001.0013.1939

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9. Nowińska Katarzyna, Ciesielska Urszula, Piotrowska Aleksandra, Jabłońska Karolina, **Partyńska Aleksandra**, Paprocka Maria, Zatoński Tomasz, Podhorska-Okołów Marzenna, Dzięgiel Piotr: *MCM5 expression is associated with the grade of malignancy and Ki-67 antigen in LSCC*. Anticancer Research 2019 Vol. 39 no. 5 s. 2325-2335; DOI:10.21873/anticanres.13349

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10. **Partyńska Aleksandra**: *Wybrane metody kształcenia praktycznego studentów wykorzystywane w naukach przyrodniczych i medycznych (Chosen methods of practical education of students in natural and medical sciences)*. W: Nowe strategie w kształceniu studentów: dobre praktyki – rekomendacje, red. Żanetta Kaczmarek i Janusz Morbitzer, Wrocław 2018, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, s. 210-227, ISBN: 978-83-7055-379-1.

    Pkt MEiN=20,00

Doniesienia konferencyjne

1. **Partyńska Aleksandra**, Gomułkiewicz Agnieszka, Piotrowska Aleksandra, Ratajczak-Wielgomas Katarzyna, Grzegrzółka Jędrzej, Rzechonek Adam, Podhorska-Okołów Marzenna, Dzięgiel Piotr*: Ekspresja zyksyny w komórkach podścieliska niedrobnokomórkowych raków płuc.* W: XI Sympozjum "Współczesna myśl techniczna w naukach medycznych i biologicznych". Wrocław, 18-19 listopada 2022. Materiały konferencyjne, Wrocław 2022, Oddział Polskiej Akademii Nauk we Wrocławiu, s. 126-128, ISBN 978-83-954493-3-8.
   Zajęcie II miejsca w sesji doniesień plakatowych

2. Glatzel-Plucińska Natalia, Olbromski Mateusz, **Partyńska Aleksandra**, Ratajczak Katarzyna, Piotrowska Aleksandra, Miążek Arkadiusz, Dzięgiel Piotr*: Białko SATB1 jako nowy cel w immunoterapii raka gruczołu piersiowego z wykorzystaniem adoptywnego transferu limfocytów T.* W: XI Sympozjum "Współczesna myśl techniczna w naukach medycznych i biologicznych". Wrocław, 18-19 listopada 2022. Materiały konferencyjne, Wrocław 2022, Oddział Polskiej Akademii Nauk we Wrocławiu, s. 65-66, ISBN 978-83-954493-3-8.

3. Glatzel-Plucińska Natalia, Olbromski Mateusz, **Partyńska Aleksandra**, Ratajczak Katarzyna, Piotrowska Aleksandra, Miążek Arkadiusz, Dzięgiel Piotr: *Białko SATB1 jako nowy cel w immunoterapii raka gruczołu piersiowego z wykorzystaniem adoptywnego transferu limfocytów T.* W: 54 Sympozjum Polskiego Towarzystwa Histochemików i Cytochemików "Gdzie jesteśmy? Dokąd zmierzamy? Techniki histochemiczne i cytochemiczne w ujęciu ponadczasowym". Bydgoszcz, 23-25 maja 2022 r. Program i książka streszczeń Bydgoszcz 2022, s. 18.

4. Nowińska Katarzyna, Jabłońska Karolina, Piotrowska Aleksandra, Pawełczyk Konrad, Ciesielska Urszula, **Partyńska Aleksandra**, Kątnik Ewa, Grzegrzółka Jędrzej, Ratajczak-Wielgomas Katarzyna, Podhorska-Okołów Marzenna, Dzięgiel Piotr: *Evaluation of the role of irisin expression in non-small cell lung cancer*. W: 53rd Symposium of the Polish Society for Histochemistry and Cytochemistry "From ultrastructure to in vivo imaging: progress in microscopical techniques". Gdańsk, 15-18 September 2019. Program, abstracts Gdańsk 2019, Polish Society

for Histochemistry and Cytochemistry; Department of Histology Medical University of Gdańsk, s. 70 poz. O50, ISBN 978-83-61216-07-0.

5. Haczkiewicz Katarzyna, **Partyńska Aleksandra**, Ratajczak Katarzyna, Dzięgiel Piotr, Podhorska-Okołów Marzenna: *Ultrastructure of breast cancer cells in the course of autophagy*. W: 53rd Symposium of the Polish Society for Histochemistry and Cytochemistry "From ultrastructure to in vivo imaging: progress in microscopical techniques". Gdańsk, 15-18 September 2019. Program, abstracts Gdańsk 2019, Polish Society for Histochemistry and Cytochemistry; Department of Histology Medical University of Gdańsk, s. 91 poz. P14, ISBN 978-83-61216-07-0.

6. **Partyńska Aleksandra**, Gomułkiewicz Agnieszka, Piotrowska Aleksandra, Podhorska-Okołów Marzenna, Dzięgiel Piotr: *Zyxin expression in non-small cell lung cancer – a preliminary study*. W: 53rd Symposium of the Polish Society for Histochemistry and Cytochemistry "From ultrastructure to in vivo imaging: progress in microscopical techniques". Gdańsk, 15-18 September 2019. Program, abstracts Gdańsk 2019, Polish Society for Histochemistry and Cytochemistry; Department of Histology Medical University of Gdańsk, s. 148 poz. P70, ISBN 978-83-61216-07-0.

7. **Partyńska Aleksandra**, Gomułkiewicz Agnieszka, Piotrowska Aleksandra, Dzięgiel Piotr: *Ekspresja zyksyny w niedrobnokomórkowych rakach płuc – badania pilotażowe*. W: X Sympozjum "Współczesna myśl techniczna w naukach medycznych i biologicznych". Wrocław, 14-15 czerwca 2019 r. Materiały konferencyjne Wrocław 2019, Oddział Polskiej Akademii Nauk we Wrocławiu, s. 90-91, ISBN 978-83-942714-9-7.

8. Andrzejewski Waldemar, Rosłanowski Adam, Kassolik Krzysztof, Grzegrzółka Jędrzej, Kmiecik Alicja, Wilk Iwona, Podhorska-Okołów Marzenna, Dzięgiel Piotr, Kosendiak Aureliusz, **Partyńska Aleksandra**, Ratajczak-Wielgomas Katarzyna: *Wpływ masażu z wykorzystaniem rolera na proces angiogenezy w mięśniach szkieletowych – doniesienie wstępne = The effect of massage using a roller on the process of angiogenesis in skeletal muscles – a preliminary report.* W: Międzynarodowy Dzień Inwalidy – XXV-edycja "Życie bez bólu. Zdrowe dzieci –

zdrowa Europa: wielka nauka dla małych pacjentów". Zgorzelec 28-30 marca 2019 r. Streszczenia Zgorzelec 2019, "Obrzeża" Oficyna Wydawnicza, s. 19-20 poz. I, 19, ISBN 978-83-88380-77-8.

9. Haczkiewicz Katarzyna, Piotrowska Aleksandra, **Partyńska Aleksandra**, Mieszała Katarzyna, Kulus Michał, Dzięgiel Piotr, Podhorska-Okołów Marzenna: *Autofagia w raku gruczołu piersiowego – badania 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, s. 18 poz. U16.

10. Jabłońska Karolina, Nowińska Katarzyna, Piotrowska Aleksandra, **Partyńska Aleksandra**, Kątnik Ewa, Pawełczyk Konrad, Podhorska-Okołów Marzenna, Dzięgiel Piotr: *Ocena prognostycznego znaczenia receptorów melatoninowych w raku płuca*. 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, s. 26 poz. U24.

11. Nowińska Katarzyna, Pawełczyk Konrad, Piotrowska Aleksandra, Ciesielska Urszula, Jabłońska Karolina, **Partyńska Aleksandra**, Kątnik Ewa, Grzegrzółka Jędrzej, Dzięgiel Piotr: *Rokownicze znaczenie ekspresji iryzyny w niedrobnokomórkowych rakach płuc*. W: IX Sympozjum "Współczesna myśl techniczna w naukach medycznych i biologicznych". Wrocław, 22-23 czerwca 2018 r. Materiały konferencyjne, Wrocław 2018, Oddział Polskiej Akademii Nauk we Wrocławiu, s. 107-109, ISBN 978-83-942714-8-0.

12. **Partyńska Aleksandra**, Gomułkiewicz Agnieszka, Piotrowska Aleksandra, Nowińska Katarzyna, Grzegrzółka Jędrzej, Dzięgiel Piotr: *Rola zyksyny w niedrobnokomórkowych rakach płuc – badania wstępne*. W: IX Sympozjum "Współczesna myśl techniczna w naukach medycznych i biologicznych". Wrocław, 22-23 czerwca 2018 r. Materiały konferencyjne Wrocław 2018, Oddział Polskiej Akademii Nauk we Wrocławiu, s. 52-53, ISBN 978-83-942714-8-0.
    Zajęcie III miejsca w sesji doniesień ustnych