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Justyna Durślewicz

Ocena wartości rokowniczej oraz korelacji kliniczno-biologicznych białka SATB1 wśród wybranych grup pacjentów onkologicznych leczonych operacyjnie

Rozprawa na stopień doktora nauk medycznych

Promotor:

prof. dr hab. Dariusz Grzanka

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Spis treści

1. Nota informacyjna i wykaz publikacji stanowiących rozprawę doktorską

Niniejsza rozprawa ma charakter spójnego tematycznie zbioru artykułów opublikowanych w czasopismach umieszczonych w ministerialnym wykazie czasopism naukowych i recenzowanych materiałów z konferencji międzynarodowych. Łączna wartość współczynnika oddziaływania (Impact Factor) publikacji stanowiących niniejszą rozprawę doktorską wynosi 5.177 oraz 200 punktów Ministerstwa Nauki i Szkolnictwa Wyższego (MNiSW). Publikacje zostały przedstawione w kolejności chronologicznej według daty wydania. Punktację podano według listy czasopism punktowanych MNiSW za rok 2021 oraz Impact Faktor według Journal Citation Reports za rok 2021.

Lista prac:

1) **Durślewicz Justyna**, Klimaszewska-Wiśniewska Anna, Jóźwicki Jakub, Antosik Paulina, Smolińska-Świtała Marta, Gagat Maciej, Kowalewski Adam, Grzanka Dariusz; Prognostic significance of TLR2, SMAD3 and localization-dependent SATB1 in stage I and II non-small-cell lung cancer patients; Cancer Control 2021 : Vol. 28, s. 1-14.
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2) **Durślewicz Justyna**, Klimaszewska-Wiśniewska Anna, Domanowska Ewa, Skoczylas-Makowska Natalia, Antosik Paulina, Zielińska Wioletta, Gzil Arkadiusz, Czajkowska Paulina, Mikołajczyk Klaudia, Grzanka Dariusz; Prognostic significance of SATB1, SMAD3, Ezrin and β-catenin in patients with pancreatic adenocarcinoma; Appl. Sci.-Basel 2022 : Vol. 12, nr 1, s. 1-26, 306.
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2. Wykaz skrótów

Skrót	Pełna nazwa w języku angielskim	Pełna nazwa w języku polskim
BURs	base unpairing regions	regiony niesparowanych zasad
CI	confidence intervals	przedziały ufności
FFPE	formalin-fixed parafin-embedded	utrwalone w formalinie i zatopione w parafinie
HR	hazard ratio	współczynnik ryzyka
IHC	immunohistochemistry	immunohistochemia
MARs	matrix attachment regions	regiony mocowania macierzy
miRs	microRNA	mikroRNA
NM	nuclear matrix	macierz jądrowa
NMPs	nuclear matrix proteins	białka macierzy jądrowej
NSCLC	non-small-cell lung carcinoma	niedrobnokomórkowy rak płuca
PAC	pancreatic cancer	rak trzustki
PDAC	pancreatic ductal adenocarcinoma	rak przewodowy trzustki
S/MAR	scaffold/matrix attachment regions	obszary mocowania rusztowania/matrycy
SATB1	special AT-rich sequence-binding protein-1	-
SATB1^{n}	nuclear immunoreactivity of SATB1	jądrowa immunoreaktywność SATB1
SATB1^{c}	cytoplasmic immunoreactivity of SATB1	cytoplazmatyczna immunoreaktywność SATB1
SCLC	small-cell lung carcinoma	drobnokomórkowy rak płuca
TCGA	The Cancer Genome Atlas	-

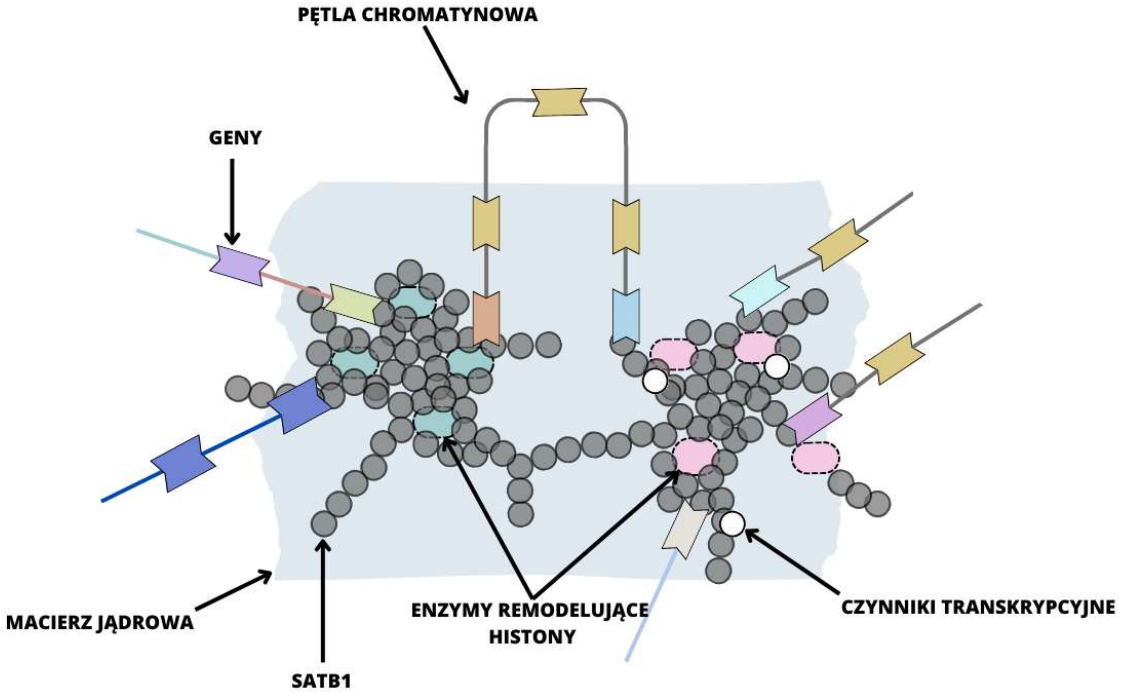
3. Wprowadzenie

3.1 Białko SATB1

Zaburzenia architektury jądra komórkowego oraz wzorów ekspresji genów są kluczowymi, odpowiednio morfologicznymi i molekularnymi zmianami występującymi we wszystkich nowotworach. Zgodnie z licznymi doniesieniami, zmiany te są silnie skorelowane z dynamiką składu polipeptydowego macierzy jądrowej (NM, ang. nuclearmatrix)[1]. Analiza profilu białkowego NM wskazuje, że chorobom nowotworowym towarzyszą modyfikacje w puli białek macierzy jądrowej (NMPs, ang. nuclear matrix proteins), które wydają się być zależne nie tylko od rodzaju i stopnia zróżnicowania tkanki, ale też typu czy stadium zaawansowania nowotworu[2]. Analiza funkcjonalna NMPs ujawniła zróżnicowaną rolę i zaangażowanie tych białek w wiele procesów komórkowych. Zidentyfikowano białka o charakterze strukturalnym, enzymy, białka szoku cieplnego, składniki kompleksów replikacyjnych, transkrypcyjnych, translacyjnych oraz remodelujących chromatynę. Ponadto, wykazano że niektóre z tych białek biorą udział w procesach takich jak dojrzewanie i transport RNA, różnicowanie oraz transdukcja sygnału[3]. Ta bogatobiałkowa struktura szkieletowa jądra komórkowego zwróciła uwagę wielu naukowców jako potencjalne źródło markerów nowotworowych i celów terapeutycznych[2][3][4][5][6].

Zmiany profilu polipeptydowego NM w przebiegu transformacji nowotworowej korelują ze zmianami miejsc kotwiczenia sekwencji S/MAR (ang. scaffold/matrix attachment regions) do NMPs, co wpływa na zmianę wielkości pętli chromatynowych (tj. przestrzennej organizacji chromatyny), a w konsekwencji na aktywność transkrypcyjną genów i fenotypową manifestację nowotworu[7]. Dotychczas zidentyfikowano kilka białek które wiążą sekwencje S/MAR i ulegają dramatycznej deregulacji w komórkach nowotworowych[4][5]. Pierwszym z nich było opisane w 1992 roku przez zespół Kohwi-Shigematsu białko macierzy jądrowej SATB1 (ang. Special AT-rich DNA Binding protein 1)[8]. Białko to organizuje chromatynę w trzeciorzędowe struktury, co umożliwia związanie z nią innych białek regulujących transkrypcję oraz enzymów modyfikujących chromatynę[9]. Zgodnie z obecną wiedzą genom zakotwiczony jest w macierzy jądrowej poprzez regiony mocowania macierzy (MARs, ang. matrix attachment regions), zaś SATB1 swoiście wiąże się z bogatymi w AT motywami regionów MAR dwuniciowego DNA, tworząc w ten sposób sieć na kształt

„klatki” wokół heterochromatyny, organizując ją tym samym w charakterystyczne pętle. Te wzbogacone w AT obszary mają powinowactwo do nieparowania zasad i są nazywane regionami niesparowanych zasad (BURs, ang. base unpairing regions). SATB1 zakotwiczony w BUR zapewnia miejsce dokowania dla białek remodelujących chromatynę i czynników transkrypcyjnych w regulacji wielu genów (Rycina 1)[10][11]. Zatem SATB1 ściśle kontroluje regulację znacznie oddalonych od siebie genów zlokalizowanych dystalnie od loci powiązanych z SATB1.



Rycina 1 SATB1 tworzy pętle chromatynowe poprzez zakotwiczenie chromatyny w macierzy jądrowej pośrednicząc w regulacji genów na dużą odległość. Zmodyfikowano na podstawie[10].

W regulacji ekspresji genów istotne znaczenie mają również modyfikacje potranslacyjne SATB1, takie jak fosforylacja i acetylacja. Modyfikacje te służą jako regulatory molekularne, nadające SATB1 zdolności do działania jako aktywator lub represor ekspresji genów[12]. SATB1 jest regulowany również przez małe niekodujące RNA zwane mikroRNA (miRs), które są posttranskrypcyjnymi regulatorami ekspresji genów. Podwyższona ekspresja miR-191 w keratynocytach naskórka indukuje starzenie się poprzez obniżenie ekspresji SATB1 oraz CDK6[13]. Z drugiej strony, miR-23a hamuje ekspresję SATB1 w kostniakomięsaku i działa jako supresor nowotworu[14]. Badania te wskazują, że SATB1 jest regulowany przez różne miR, a funkcja SATB1 zależy zarówno od modyfikacji potranslacyjnych, jak i specyfiki tkankowej.

SATB1 reguluje ekspresję wielu genów biorących udział regulacji cyklu komórkowego, proliferacji, różnicowania, adhezji, sygnalizacji i apoptozy oraz jest niezależnym markerem prognostycznym w wielu nowotworach. Podwyższona ekspresja SATB1 sprzyja licznym zmianom patologicznym w szerokiej gamie nowotworów: w tym raka piersi[11], jajnika[15], prostaty[16], płuca[17][18], nosogardła[19], przełyku[20], żołądka[21], trzustki[22][23], jelita grubego[24], wątroby[25] oraz pęcherza moczowego[26]. Uważa się, że ekspresja SATB1 związana jest z zwiększonym rozmiarem guza, występowaniem przerzutów, a jej wyciszenie prowadzi do zmniejszenia potencjału progresji wielu nowotworów. Z kolei, zgodnie z innymi doniesieniami, ekspresja SATB1 jest negatywnie skorelowana z progresją nowotworu oraz przeżyciem pacjentów w raku piersi[27], trzustki[23] i płuca[28]. Podłoże tych rozbieżności i dokładna rola SATB1 w patogenezie nowotworów pozostaje wciąż niejasna. Nie ulega wątpliwości, że SATB1 pełni istotną rolę w różnych procesach komórkowych i ścieżkach biologicznych, a jego deregulacja wiąże się z rozwojem i wzrostem nowotworów. Istnieje potencjał, aby SATB1 służył jako biomarker i cel terapeutyczny w leczeniu nowotworów.

3.2 Rak płuca

Rak płuca (ang. lung cancer) jest oskrzelopochodnym nowotworem złośliwym wywodzącym się z nabłonka dróg oddechowych[29]. Według Światowej Organizacji Zdrowia (ang. World Health Organization, WHO) jest drugim najczęściej występującym typem nowotworu i wiodącą przyczyną zgonów z powodu nowotworów na świecie[30]. Liczba zachorowań na raka płuca w 2020 roku wynosiła 2,21 mln, stanowiąc około 22,1% wszystkich przypadków nowotworów złośliwych zdiagnozowanych na świecie[31]. Pod względem umieralności zajmuje pierwszą pozycję, stanowiąc przyczynę 1,8 mln wszystkich zgonów z powodu chorób nowotworowych na świecie[32]. Wysoką śmiertelność przypisuje się oporności na chemioterapię, rozpoznaniu w zaawansowanym stadium rozwoju oraz nawrotom choroby[33]. Pomimo znacznej progresji metod postępowania terapeutycznego w ostatnich latach, 5-letni wskaźnik przeżycia względnego dla chorych z rakiem płuca wynosi zaledwie 19%. Ze względu na wysoką śmiertelność oraz stałą tendencję wzrostową współczynnika zachorowalności, rak płuca stanowi niebagatelny problem epidemiologiczny, zdrowotny, a także ekonomiczny współczesnego społeczeństwa. Głównym czynnikiem epigenetycznym odpowiedzialnym za około 80% przypadków

zachorowań na nowotwory złośliwe płuca jest palenie tytoniu. Rozwojowi raka płuca sprzyjają również takie czynniki jak: bierne palenie, zanieczyszczenie powietrza, narażenie na radon, azbest i inne substancje kancerogenne, niewłaściwa dieta, a także emisja gazów pochodzących ze spalania paliw[33][34].

Według klasyfikacji klinicznej wyodrębnia się dwa typy pierwotnych nowotworów złośliwych płuca: drobnokomórkowy (ang. small-cell lung carcinoma, SCLC) stanowiący około 15% rozpoznawanych przypadków raka płuca oraz niedrobnokomórkowy (ang. non–small-cell lung carcinoma, NSCLC) występujący u około 85% chorych[35][36][37]. Rak gruczołowy (ang. adenocarcinoma, ADC), rak płaskonabłonkowy (ang. squamous cell carcinoma, SCC) oraz wielkokomórkowy (ang. large cell carcinoma, LCC) wraz ze swoimi odmianami stanowią ponad 95% wszystkich stawianych rozpoznań NSCLC. Najczęstszą postacią, spośród głównych typów nowotworów złośliwych płuca jest gruczolakorak, występujący u około 40% chorych. Rozwija się z komórek gruczołowych wytwarzających śluz, dlatego częściej powstaje w obwodowej części płuc. Charakteryzuje się agresywnym przebiegiem oraz silną tendencją do tworzenia przerzutów odległych we wczesnych stadiach choroby. Kolejny pod względem częstości występowania jest rak płaskonabłonkowy wywodzący się z proksymalnych części oskrzeli stanowiący około 25–30% zachorowań na nowotwory złośliwe płuca. Przeważnie występuje w centralnej części płuca lub w obrębie głównych dróg oddechowych, tj. w lewym lub prawym oskrzelu. Za główny czynnik etiologiczny przyczyniający się do powstania raka płaskonabłonkowego uznaje się palenie tytoniu. Rak wielkokomórkowy jest najrzadziej występującym rakiem wśród niedrobnokomórkowych raków płuca, stanowiącym około 10% zachorowań. Ze względu na rozwój przerzutów we wczesnych stadiach choroby jego rokowanie często określane jest jako niepomyślne[38].

Zalecenia terapeutyczne dotyczące raka płuca zależne są przede wszystkim od stadium zaawansowania choroby, rozpoznania histopatologicznego, wielkości i umiejscowienia guza oraz stopnia sprawności pacjenta. Pacjenci zdiagnozowani we wczesnym stadium choroby, zwykle poddawani są resekcji chirurgicznej, która pozostaje najlepszą opcją terapeutyczną istotnie wpływającą na wieloletnie przeżycie. Ze względu na późną wykrywalność nowotworów złośliwych płuca rokowanie chorych pozostaje nadal niekorzystne[39]. Około 70% pacjentów, diagnozowanych jest w wysokim stadium zaawansowania klinicznego choroby, kiedy obecne są przerzuty. Wskaźnik 5-letniego przeżycia względnego dla chorych zdiagnozowanych we wczesnym stadium choroby wynosi 70-90%. U chorych u których

postawiono rozpoznanie w IV stadium zaawansowania klinicznego choroby, 5-letni wskaźnik przeżycia względnego dramatycznie spada do 1%[40].

3.3 Rak trzustki

Rak trzustki (ang. pancreatic cancer, PC) jest jednym z najbardziej inwazyjnych i obarczonych wysoką śmiertelnością nowotworów złośliwych na świecie. Zajmuje dwunaste miejsce wśród najczęściej diagnozowanych nowotworów, jednak ze względu na wysoki wskaźnik śmiertelności stanowi siódmą główną przyczynę zgonów z powodu raka na świecie[32]. Rak trzustki jest nowotworem złośliwym o wzrastającej częstości występowania, a wartości standaryzowanych współczynników zapadalności w populacji światowej odpowiadają współczynnikom umieralności z powodu tej choroby. Szacuje się, że do 2040 roku liczba zachorowań na raka trzustki może sięgać ponad 355 tysięcy przypadków, przewyższając raka piersi, prostaty i jelita grubego, stając się przy tym drugą przyczyną zgonów z przyczyn nowotworowych[40].

Do głównych czynników ryzyka zachorowania na raka trzustki, odpowiadającym za 30% przypadków jest palenie papierosów. Częstość rozpoznań wzrasta wraz z wiekiem. Większą zapadalność obserwuje się powyżej 55 roku życia z wyższym wskaźnikiem występowania wśród mężczyzn niż kobiet. Do czynników które w istotny sposób zwiększają ryzyko wystąpienia raka trzustki zalicza się również przewlekłe zapalenie trzustki, wieloletnią cukrzycę, otyłość i związaną z nią insulinooporność[40].

Ze względu na wysoką i wczesną inwazyjność nowotworu oraz występowanie zjawiska oporności na leczenie chemioterapeutyczne rokowanie w przypadku raka trzustki jest szczególnie niekorzystne[41]. Odsetek przeżyć pięcioletnich całej populacji chorych utrzymuje się na poziomie 7-9%, a 12-miesięczne przeżycie z rakiem trzustki nie przekracza 20%[42]. Zabieg radykalnej resekcji stanowi najskuteczniejszą metodę terapeutyczną która w chwili obecnej stanowi jedyną szansę wyleczenia. Ze względu na brak typowych i wczesnych objawów oraz późne rozpoznanie choroby, odsetek resekcyjności utrzymuje się na poziomie 20-30%. Odsetek przeżyć pięcioletnich po resekcji chirurgicznej wynosi od 15 do 25% z medianą przeżycia sięgającą 18-20 miesięcy. Odsetek przeżyć pięcioletnich u pacjentów z zaawansowanym rakiem trzustki u których stwierdza się obecność przerzutów odległych nie przekracza 5%, a średni czas przeżycia tych pacjentów wynosi 6 miesięcy[43][44].

4. Cel pracy

Wobec niewystarczającej liczby oraz braku spójności doniesień na temat klinicznej wartości białka SATB1 w NSCLC oraz PAC cele niniejszej rozprawy doktorskiej obejmowały:

1. Określenie niezależnej wartości rokowniczej SATB1 z uwzględnieniem jego subkomórkowej lokalizacji ocenionej immunohistochemicznie w kohorcie własnej oraz publicznie dostępnych danych transkryptomicznych pozyskanych z The Cancer Genome Atlas (TCGA).
2. Ocenę zależności pomiędzy tkankową ekspresją SATB1 a wybranymi danymi klinicznymi, histologicznymi oraz biologicznymi (TLR2, SMAD3, ezryna oraz β-katenina) analizowanych grup pacjentów.
3. Określenie wpływu panelu uwzględniającego SATB1 oraz powiązanych markerów na czas przeżycia całkowitego pacjentów w analizowanych grupach.
4. Ocenę wzajemnych korelacji biologicznych pomiędzy ekspresją SATB1 a funkcjonalnie powiązanymi markerami: TLR2, SMAD3, EZR oraz CTNNB1.

5. Omówienie prac włączonych do cyklu

5.1 Omówienie pracy 1: „Prognostic significance of TLR2, SMAD3 and localization-dependent SATB1 in stage I and II non-small-cell lung cancer patients"

Durślewicz Justyna, Klimaszewska-Wiśniewska Anna, Jóźwicki Jakub, Antosik Paulina, Smolińska-Świtała Marta, Gagat Maciej, Kowalewski Adam, Grzanka Dariusz; Cancer Control 2021 : Vol. 28, s. 1-14.
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Pierwszy artykuł z przedstawianego cyklu stanowi pracę oryginalną, w której podjęto próbę weryfikacji hipotez dotyczących związku pomiędzy statusem ekspresji SATB1, SMAD3 i TLR2 a cechami kliniczno-patologicznymi i rokowaniem u pacjentów z NSCLC w I-II stopniu zaawansowania klinicznego oraz wzajemnych korelacji pomiędzy ekspresją SATB1 a funkcjonalnie powiązanymi czynnikami: TLR2, SMAD3, ezryną oraz β-kateniną. W tym celu oceniono barwienie immunohistochemiczne dla wszystkich badanych markerów, wykorzystując archiwalny materiał tkankowy pochodzący od 69 pacjentów z kohorty własnej oraz dane transkryptomiczne zgromadzone w ramach projektu TCGA.

Na podstawie analiz wyników immunobarwienia SATB1 wyrażonych jako zmienna kategoryzowana uzyskana dla frakcji jądrowej stwierdzono marginalnie znaczącą statystycznie różnicę pomiędzy statusem ekspresji a typem histologicznym (p=0.068) oraz wiekiem pacjentów z NSCLC (p=0.0547). Z kolei obecność barwienia SATB1 w lokalizacji cytoplazmatycznej była związana z płcią pacjentów z grupy badanej (p=0.0547). Analizy przeżycia pokazały, że nasilony odczyn jądrowy SATB1 był korzystnym czynnikiem prognostycznym dla czasu przeżycia całkowitego pacjentów (HR 0.53, 95%CI 0.30–0.94, p=0.031) i pozostał niezależnym czynnikiem prognostycznym w analizie wieloczynnikowej skorygowanej o wiek, płeć oraz stadium zaawansowania (HR 0.49, 95%CI 0.27-0.90, p=0.022). Z kolei obecność odczynu immunohistochemicznego w frakcji cytoplazmatycznej białka SATB1 wykazywała istotny, ale odwrotny związek ze wskaźnikiem przeżycia pacjentów i była predyktorem niekorzystnego rokowania zarówno w analizie jednoczynnikowej (HR 2.04, 95%CI 1.07–3.88, p=0.031), jak również w wieloczynnikowej (HR 2.11, 95%CI 1.07–4.16, p=0.030). W oparciu o publiczną bazę danych TCGA w prezentowanej pracy badawczej wykazano związek prognostyczny pomiędzy wysokim poziomem mRNA SATB1, a dłuższym czasem przeżyciem pacjentów (p=0.0144).

Mając na uwadze możliwe interakcje między oznaczonymi białkami, przeprowadzono analizę porównawczą w której udowodniono istotną statystycznie słabą ujemną korelację pomiędzy poziomem SATB1 ocenianym w frakcji jądrowej i TLR2 (r=-0.2404; p=0.0466), słabą dodatnią korelację pomiędzy poziomem SATB1 ocenianym w lokalizacji cytoplazmatycznej (r=-0.2875; p=0. 0166) oraz umiarkowaną ujemną korelację pomiędzy poziomem SATB1 ocenianym w frakcji jądrowej i SATB1 ocenianym w lokalizacji cytoplazmatycznej (r=-0.4619; p=0.0001).

W publikacji wykazano, że wysoki poziom SMAD3 był związany z cechami kliniczno-histopatologicznymi takimi jak: stopień dojrzałości histologicznej (p=0.0265), stopień zaawansowania nowotworu (cecha T, p=0.0269) i stopnień zaawansowania klinicznego (p=0.045). Badanie ujawniło również korelację pomiędzy statusem ekspresji TLR2 a typem histologicznym NSCLC (p=0.0054). Analizy pokazały, korzystny rokowniczo związek pomiędzy wysokim poziomem SMAD3 a całkowitym czasem przeżycia pacjentów (HR 0.41, 95%CI 0.21–0.78, p=0.006) oraz niekorzystny pomiędzy wysokim poziomem TLR2 (HR 3.02, 95%CI 1.37–6.66, p=0.006) w kohorcie własnej. Przeprowadzone analizy wieloczynnikowe potwierdziły istotność wskazanych spostrzeżeń (SMAD3 – HR 0.40, 95% CI 0.20–0.78; p=0.007;TLR2 – HR 3.00, 95%CI 1.36–6.63, p= 0.007). Co istotne, analiza danych z bazy TCGA uwidoczniła odwrotny trend dla statusu ekspresji *SMAD3*, tj. wysoka ekspresja mRNA była związana ze złym rokowaniem chorych. Z kolei podobnie jak w wynikach uzyskanych z analiz kohorty własnej, wysoka ekspresja *TLR2* była związana z niekorzystnym wskaźnikiem przeżycia w kohorcie TCGA.

W ostatnim etapie pracy biorąc pod uwagę funkcjonalną zależność między badanymi czynnikami podjęto próbę określenia wpływu połączonej ekspresji SATB1, SMAD3 oraz TLR2 na przeżycie pacjentów z NSCLC. Stratyfikacja chorych w odniesieniu do łącznej ekspresji wskazanych biomarkerów pozwoliła zidentyfikować podgrupy pacjentów z największą różnicą w czasie przeżycia. W szczególności, profil ekspresji $SATB1^{n\text{-}high}/SMAD3^{high}/TLR2^{low}$ był powiązany z najkorzystniejszym przeżyciem całkowitym i przewidywał przeżycie pacjentów lepiej, niż każdy z analizowanych czynników pojedynczo.

W dyskusji artykułu wnikliwie omówiono przedstawione powyżej wyniki. W analizowanym doniesieniu naukowym wykazano, że wysoka ekspresja frakcji jądrowej była związana z korzystnym przeżyciem pacjentów z NSCLC w I-II stopniu zaawansowania klinicznego, podczas gdy obecność barwienia SATB1 w lokalizacji cytoplazmatycznej miała

znaczący związek ze złym rokowaniem. Wyniki te wydają się być szczególnie interesujące w świetle doniesień dostępnych w literaturze światowej i sugerują przeciwne znaczenie prognostyczne w zależności od lokalizacji subkomórkowej białka SATB1. Uzyskane w niniejszej pracy dane sugerują, że badane czynniki mogą mieć potencjalną przydatność kliniczną w określaniu rokowania u pacjentów z NSCLC w I i II stadium choroby.

5.2 Omówienie pracy 2: „Prognostic significance of SATB1, SMAD3, Ezrin and β-catenin in patients with pancreatic adenocarcinoma”

Durślewicz Justyna, Klimaszewska-Wiśniewska Anna, Domanowska Ewa, Skoczylas-Makowska Natalia, Antosik Paulina, Zielińska Wioletta, Gzil Arkadiusz, Czajkowska Paulina, Mikołajczyk Klaudia, Grzanka Dariusz; Appl. Sci.-Basel 2022 : Vol. 12, nr 1, s. 1-26, 306.
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Celem drugiej pracy włączonej do cyklu było ustalenie wartości rokowniczej oraz korelacji kliniczno-biologicznych SATB1, SMAD3, ezryny i β-kateniny wśród pacjentów z PAC. W celu wykrycia obecności swoistych antygenów SATB1, SMAD3, ezryny i β-kateniny wykonano barwienie immunohistochemiczne z wykorzystaniem makromacierzy tkankowych z materiałem pochodzącym od 68 pacjentów z PDAC. Wykonane zadanie badawcze obejmowało także analizę zmian w profilach ekspresji analizowanych genów na poziomie transkryptu na podstawie danych zgromadzonych w ramach projektu TCGA. Obie te oceny skorelowano z parametrami kliniczno-patologicznymi oraz całkowitym czasem przeżycia pacjentów. Dodatkowo, w celu zrozumienia funkcji oraz mechanizmów regulacji szlaków biochemicznych badanych czynników w PAC, za pomocą narzędzi bioinformatycznych skonstruowano sieć interakcji białko-białko (PPI, ang. protein-protein interactions) obejmującą SATB1, SMAD3, ezrynę, β-kateninę oraz 50 genów wykazującą pozytywną korelacje.

Z przeprowadzonych analiz uzyskanych w toku oceny immunobarwienia SATB1 wynika, że ekspresja SATB1 uzyskana dla frakcji jądrowej była znacząco zmniejszona lub utracona w próbkach PDAC w porównaniu z nienowotworowymi sąsiednimi tkankami, podczas gdy ekspresja SATB1 uzyskana dla frakcji cytoplazmatycznej była obecna w utkaniu nowotworowym, ale nie była obserwowana w żadnej z próbek kontrolnych. Na podstawie analiz wyników reakcji immunohistochemicznej SATB1 w lokalizacji jądrowej wyrażonej jako zmienna kategoryzowana wykazano istotną statystycznie różnicę pomiędzy statusem ekspresji a stopniem zróżnicowania histologicznego (p=0.03). Z kolei status ekspresji SATB1 oceniony w frakcji cytoplazmatycznej nie wykazywał istotnej korelacji z cechami kliniczno-histopatologicznymi.

Analiza zmiennych przy użyciu krzywych przeżycia Kaplana-Meiera wykazała, że odczyn jądrowy o niskiej ekspresji SATB1 wiązał się z krótszym przeżyciem całkowitym pacjentów

z PDAC, jednak różnica ta nie była istotna statystycznie (p=0.118). Z kolei wysoki odczyn immunohistochemiczny obecny w frakcji cytoplazmatycznej białka SATB1 był istotnie związany z gorszym przeżyciem pacjentów (p=0.036). Ponadto, analizy wykazały, że wysoka ekspresja SATB1 zlokalizowana w cytoplazmie była predyktorem niekorzystnego rokowania zarówno w analizie jednoczynnikowej (HR 1.87, 95%CI 1.03-3.40, p=0.039), jak również w wieloczynnikowej (HR 1.86, 95%CI 0.93-3.74, p=0.08). Korzystając z publicznej bazy danych TCGA w niniejszej pracy badawczej wysokim poziomem mRNA SATB1 okazał się korzystnym niezależnym czynnikiem prognostycznym w analizie wieloczynnikowej (HR 0.50, 95%CI 0.32-0.78, p=0.002).

W analizach dotyczących kohorty własnej, stwierdzono słaby, ujemny i istotny związek pomiędzy ekspresją β-kateniny, a SATB1 ocenianym w jądrze komórkowym (r=-0.291, p=0.015) oraz umiarkowaną, pozytywną i istotną asocjację pomiędzy β-kateniną, a SATB1 zlokalizowanym w cytoplazmie (r=0.321, p=0.007). Współczynnik korelacji Spearmana ujawnił słabą dodatnią i istotną zależność pomiędzy ekspresją *SATB1* a *CTNNB1* (r=0.244, p=0.001) w kohorcie TCGA.

Omawiane badanie ujawniło związek pomiędzy statusem ekspozycji ezryny, a stopień zaawansowania nowotworu (cecha T, p=0.048). Nie wykazano innych istotnych korelacji pomiędzy statusem badanych markerów ocenionych za pomocą metody immunohistochemicznej, a cechami kliniczno-patologicznymi w badanej grupie pacjentów. Dalsze analizy ujawniły niekorzystną rokowniczo zależność pomiędzy wysokim poziomem SMAD3, a całkowitym czasem przeżycia pacjentów (HR 2.40, 95%CI 1.31–4.40, p=0.005). Analiza wieloczynnikowa metodą regresji Coxa potwierdziła istotność wskazanych spostrzeżeń (HR 3.08, 95%CI 1.52–6.23, p=0.002) dotyczących ekspozycji SMAD3.

Z kolei w kohorcie TCGA wykazano korelacje pomiędzy ekspresją *SMAD3*, a stopniem złośliwości histologicznej (p=0.012), stopniem zaawansowania nowotworu (cecha T, p=0.026) oraz stadium TNM (p=0,008), ekspresją *EZR*, a stopniem złośliwości histologicznej (p=0.02), stopniem zaawansowania nowotworu (cecha T, p=0.0157) oraz stadium TNM (p=0,0007) oraz ekspresją *CTNNB1*, a stopniem złośliwości histologicznej (p=0.001), stopniem zaawansowania nowotworu (cecha T, p=0.009), stopniem rozprzestrzenienia nowotworu do regionalnych węzłów chłonnych (p=0.0002) oraz stadium TNM (p=0.0001). Ponadto, *SMAD3* oraz *EZR* okazały się niekorzystnymi, niezależnymi

markerami prognostycznymi w kohorcie TCGA (*SMAD3* – HR 1.57, 95% CI 0.99–2.49; p=0.05; *EZR* – HR 2.50, 95%CI 1.07–5.83, p=0.033).

Mając na uwadze funkcjonalne zależności pomiędzy badanymi czynnikami dokonano stratyfikacji chorych w odniesieniu do łącznej ekspresji badanych biomarkerów. Profil uwzględniający wysokie współwyrażanie $SATB1^{c}$, SMAD3, Ezryny i β-kateniny okazał się silnym niezależnym czynnikiem prognostycznym związanym z niekorzystnym rokowaniem pacjentów w kohorcie własnej (HR 7.32, 95%CI 2.05–26.21, p=0.002). Z kolei profil $SATB1^{n\text{-}high}SMAD3^{low}Ezryna^{low}$β-catenina był powiązany z korzystnym przeżyciem całkowitym (HR 0.11, 95%CI 0.01–0.93, p=0.04). Podobnie, w zbiorze danych TCGA, panel uwzględniający ekspresję *SATB1*, *SMAD3*, *EZR* oraz *CTNNB1* okazał się silnym niezależnym czynnikiem prognostycznym (HR 3.28, 95%CI 2.02–5.33, p<0.0001). Zgodnie z przeprowadzoną analizą wzbogacenia funkcjonalnego, przewidywana strategia biologiczna związana z koekspresją badanych czynników obejmowała znaczące wzajemne powiązania z głównymi regulatorami związanymi z procesem nowotworzenia.

W dyskusji artykułu omówiono przedstawione wyniki, zwracając szczególną uwagę na konieczność oceny SATB1 z podziałem na barwione frakcje komórkowe. Podsumowując, uzyskane w niniejszej pracy dane wskazują, że badane czynniki mogą mieć potencjalną przydatność kliniczną w określaniu rokowania u pacjentów z PAC.

6. Publikacje będące przedmiotem rozprawy doktorskiej

6.1 Artykuł oryginalny 1

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Prognostic Significance of TLR2, SMAD3 and Localization-dependent SATB1 in Stage I and II Non–Small-Cell Lung Cancer Patients

Justyna Durślewicz, MSc[1], Anna Klimaszewska-Wiśniewska, PhD[1], Jakub Jóźwicki, MD, PhD[1], Paulina Antosik, PhD[1], Marta Smolińska-Świtała, PhD[1], Maciej Gagat, PhD[2], Adam Kowalewski, MD[1,3], and Dariusz Grzanka, MD, PhD[1]

Abstract

This study aimed to explore the prognostic value of SATB1, SMAD3, and TLR2 expression in non–small-cell lung carcinoma patients with clinical stages I-II. To investigate, we evaluated immunohistochemical staining to each of these markers using tissue sections from 69 patients from our cohort and gene expression data for The Cancer Genome Atlas (TCGA) cohort. We found that, in our cohort, high expression levels of nuclear SATB1^{n} and SMAD3 were independent prognostic markers for better overall survival (OS) in NSCLC patients. Interestingly, expression of cytoplasmic SATB1^{c} exhibited a significant but inverse association with survival rate, and it was an independent predictor of unfavorable prognosis. Likewise, TLR2 was a negative outcome biomarker for NSCLC even when adjusting for covariates. Importantly, stratification of NSCLCs with respect to combined expression of the three biomarkers allowed us to identify subgroups of patients with the greatest difference in duration of survival. Specifically, expression profile of SATB1$^{n\text{-}high}$/SMAD3^{high}/TLR2^{low} was associated with the best OS, and it was superior to each single protein alone in predicting patient prognosis. Furthermore, based on the TCGA dataset, we found that overexpression of SATB1 mRNA was significantly associated with better OS, whereas high mRNA levels of SMAD3 and TLR2 with poor OS. In conclusion, the present study identified a set of proteins that may play a significant role in predicting prognosis of NSCLC patients with clinical stages I-II.

Keywords

non-small-cell lung cancer, prognostic factor, SATB1, SMAD3, TLR2

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Introduction

According to the World Health Organization (WHO), lung cancer is the second most frequent type of cancer and the leading cause of cancer mortality worldwide, being responsible for approximately 11.6% of the total number of new cancer cases and 1.8 million deaths in 2018.[1] This high mortality rate is often attributed to disease recurrence, resistance to chemotherapy, and advanced-stage diagnosis. Despite significant developments in oncological management in recent years, the 5-year relative survival rate for people with all types of lung cancer is just 19% (men 16% and women 22%).[2] Therapeutic recommendations depend primarily on tumor stage, histology, size, and position of the cancer, together with patient-specific factors (e.g., age, comorbidity, and pulmonary function).[3] Cigarette smoking is the most important risk factor for lung cancer. Globally, cigarette smoking is linked to approximately 80% of lung cancer deaths. Additional factors contribute to lung cancer development such as secondhand smoking, air pollution, exposure to radon, asbestos, and other carcinogens, poor diet, and indoor emission of fuel burning.[4]

[1]Department of Clinical Pathomorphology, Faculty of Medicine, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Toruń, Poland
[2]Department of Histology and Embryology, Faculty of Medicine, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Toruń, Poland
[3]Department of Tumor Pathology and Pathomorphology, Oncology Centre, Prof Franciszek Łukaszczyk Memorial Hospital, Bydgoszcz, Poland

Corresponding Author:
Justyna Durślewicz, Department of Clinical Pathomorphology, Faculty of Medicine, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Skłodowskiej Curie 9, 85-094 Bydgoszcz, Toruń 87-100, Poland.
Email: justyna.durslewicz@cm.umk.pl

There are 2 main types of lung cancer: small-cell lung carcinoma (SCLC, approx. 15% cases) and non–small-cell lung carcinoma (NSCLC, approx. 85% cases).[5,6] NSCLC is further classified into 3 main subtypes: squamous cell carcinoma (SCC) (25–30%), adenocarcinoma (ADC) (40%), and large cell carcinoma (LCC) (10–15%), and several other less common subtypes, such as adenosquamous carcinoma and sarcomatoid carcinoma.[7] Patients with early-stage NSCLC usually undergo surgical resection, which remains the best therapeutic option for long-term survival. Unfortunately, around two thirds of cases undergo late-stage diagnoses, when the cancer has already metastasized. While the 5-year relative survival rate for operable early-stage disease is up to 70–90%, it drops dramatically to around 1% for stage IV NSCLC. The median overall survival (OS) of these patients is around 10 months depending on treatment, histology type, and other factors.[8–10]

Given that NSCLC represents a major health problem accounting for large numbers of deaths, the identification of prognostic biomarkers for these patients is crucial to enhance survival. A number of studies have shown that several nuclear matrix proteins (NMPs) are dramatically deregulated in various cancers.[11–24] Undoubtedly, a greater understanding of the relationship between this group of proteins and cancer can be very useful from a clinical point of view.

Special AT-rich binding protein 1 (SATB1) is a higher-order chromatin organizer and global transcriptional regulator.[11] This 763-amino acid protein identified in thymocytes is encoded by the SATB1 human gene located on chromosome 3p23. SATB1 is a known factor that binds to AT-rich sequences known as base unpairing regions (BURs) in the matrix attachment regions (MARs) of DNA. SATB1 anchored to BURs provides a "docking site" on the nuclear matrix for chromatin remodeling/modifying enzymes and transcription factors. Strikingly, SATB1 regulates a number of genes, even those located on distant chromosomes, and therefore, it is referred to as a "genome organizer."[12] Under physiological conditions, SATB1 is involved in T-cell development, cellular homeostasis, early erythroid differentiation, and responses to various stimuli.[13] Besides physiological processes, this protein is thought to be an important factor in numerous malignancies. Abnormal expression of SATB1 has been reported in various types of cancers, including breast,[14] gastric,[15] lung,[16,17] laryngeal squamous cell carcinoma,[18] colorectal,[19] endometrial,[20] prostate,[21] liver[22] ovarian,[23] and bladder cancers.[24] Despite being differentially expressed, a comprehensive understanding of the role of SATB1 protein in NSCLC is hampered by the limited number of studies on the topic, a large heterogeneity between and within histological subtypes, as well as research discrepancies between cell lines and clinical NSCLC tumors.[16,25-27] It is, therefore, urgent to study the significance of SATB1 in additional NSCLC cohorts and to uncover potential culprits of NSCLC that may overlap with SATB1 to affect patient prognosis.

SMADs are a family of intracellular proteins that transmit signals from the transforming growth factor-β (TGFβ) superfamily of receptors.[28] There are 3 distinct subgroups of SMADs based on their different roles in TGFβ family signal transduction: R-SMADs (receptor-regulated), which include SMADs 1, 2, 3, 5, and 8, Co-SMADs (common partner), that is, SMAD4, and I-SMADs (inhibitory), which comprises SMAD6 and SMAD7.[29,30] As numerous reports suggest, SMAD signaling seems to be relevant to the pathogenesis of several cancers.[31–33] However, the role of SMAD3 in tumorigenesis is not clear, as it has been shown to function as both a tumor suppressor and prometastatic factor.[34]

Toll-like receptors (TLRs) are a family of immune receptors expressed by antigen presenting cells, fibroblasts, epithelial, and cancer cells.[35] In both normal and tumor cells, they play important roles in the regulation of inflammatory responses, cell proliferation, and apoptosis.[36,37] TLRs can recognize a variety of pathogen-associated molecular patterns (PAMPs) to induce various immune responses. According to cellular localization, TLRs are divided into 2 major subtypes: extracellular (TLR1, TLR2, TLR5, TLR6, and TLR10) and intracellular (TLR3, TLR7, TLR8, and TLR9).[38,39] In the cell line studies, TLR2 was suggested to be a potential therapeutic target in lung adenocarcinoma,[40] as well as a specific mediator between lung cancer cells and mesenchymal stem cells present within the tumor microenvironment, facilitating cross-talk leading to the promotion of tumor-supportive phenotypic changes of mesenchymal cells.[41] However, TLR2 represents a double-edge sword and may also act as tumor suppressor.[42] Uncovering the prognostic significance of TLR2 in NSCLC is therefore expected to provide better understanding of its role in the biology of this tumor.

For the purpose of the present study, we selected solely SMAD3 and TLR2 since the convergence of these proteins with one another,[43] as well as with SATB1 signaling,[44,45] has been previously reported; however, there are no studies on their joint evaluation in NSCLC samples. According to the report by Mikami et al., TGFβ receptor-SMAD3/4 signaling pathway is positively involved in TLR2 induction via a dual mechanism involving functional cooperation with the NF-κB pathway and MAPK phosphatase 1 (MKP-1)-dependent inhibition of p38 MAPK, a negative regulator for TLR2 induction. It showed that TβR-Smad3/4 signaling acts as a positive regulator for host defense and immune response by increasing the expression of TLR2 during respiratory bacterial infections.[43] Lung cancer patients often present with pulmonary bacterial infections, and this coincides with a poor prognosis.[46–48] Although the underlying mechanisms for pulmonary infection-triggered lung cancer development are still not fully understood, TLR signaling seems to play an important role. For instance, Ye et al. revealed that NSCLC cells were competent and active in sensing Gram-negative bacteria through TLRs, which fueled their aberrant metabolic features to promote tumor outgrowth and metastasis.[49,50] Besides, SATB1, SMAD3, and TLR2 may be also related

Table 1. Immunoreactivity results for SATB1, SMAD3, TLR2 in association with clinicopathological characteristics of patients with NSCLC.

		SATB1^n			SATB1^c			SMAD3			TLR2		
Cases (n = 69)		+	-	*P*-value	+	-	*P*-value	+	-	*P*-value	+	-	*P*-value
Histological type													
ADC	27	9	18	.068	7	20	.6344	7	20	.0629	7	20	.0054
SCC	37	23	14		6	31		15	22		1	36	
LCC	5	2	3		1	4		4	1		0	5	
Gender													
Female	22	9	13	.4403	8	14	.0502	9	13	.792	3	19	.7034
Male	47	25	22		6	41		17	30		5	42	
Age													
<62	37	14	23	.0547	8	29	>.9999	18	19	.0506	4	33	>.9999
>63	32	20	12		6	26		8	24		4	28	
Histologic grade													
G2	19	10	9	.7918	5	14	.5083	3	16	.0265	4	15	.2025
G3	50	24	26		9	41		23	27		4	46	
pT status													
T1	25	12	13	.9411	3	22	.368	10	15	.0269	1	24	.1514
T2	27	14	13		6	21		14	13		3	24	
T3	17	8	9		5	12		2	15		4	13	
pN status													
N0	63	33	30	.1981	14	49	.3348	25	38	.3978	8	55	>.9999
N1	6	1	5		0	6		1	5		0	6	
Stage													
I	39	19	20	>.9999	7	32	.7637	19	20	.0450	4	35	.7204
II	30	15	15		7	23		7	23		4	26	

SATB1^n = nuclear immunoreactivity of SATB1; SATB1^c = cytoplasmic immunoreactivity of SATB1; HR = hazard ratio; CI = confidence intervals. Significant *P*-values ($P < .05$) are indicated in bold.

by their cellular and tissue functions, as each of these proteins is known to regulate the dynamic equilibrium of apoptosis, invasion, migration, proliferation, immune modulation, and inflammation,[34,42,51] the disturbance of which is strongly implicated in lung carcinogenesis. Moreover, as far as we are aware, at the protein level, TLR2 has not been previously evaluated as a biomarker candidate for the prediction of clinical outcome in NSCLC. Likewise, SMAD3 is also underexplored in this group of cancer patients. All these make SATB1, SMAD3, and TLR2 interesting candidates to be explored as the individual, and especially combined biomarkers for prognostication of NSCLC patients.

Therefore, the current study was designed to evaluate the immunohistochemical (IHC) expression of SATB1, SMAD3, and TLR2 in 69 formalin-fixed paraffin-embedded tissue samples (FFPE) from NSCLC patients with clinical stages I to II. The research included the reference of obtained results to OS of patients, clinicopathological data, and also the analysis of the correlation between the chosen proteins. Importantly, the combined prognostic value of these 3 proteins was also evaluated. Finally, we examined mRNA expression of these markers in the context of patient survival by utilizing the TCGA dataset.

Materials and methods

The study was conducted on archival FFPE tissue samples collected between 2010 to 2014 from 69 patients diagnosed with NSCLC in Franciszek Łukaszczyk Oncology Center of Bydgoszcz. Histopathological evaluation of each tumor sample was performed by 2 independent pathologists for the purpose of selecting a representative study group at the Department of Clinical Pathomorphology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń. All tumors were reclassified according the standardized TNM eighth edition classification of The American Joint Committee on Cancer (AJCC) criteria. To avoid excessive study complexity, cohort included ADC, SCC, and LCC, while all other histological types were excluded from the series. The inclusion and exclusion criteria is summarized in Supplementary Figure 1. The study protocol has been approved by The Ethics Committee of Nicolaus Copernicus University in Toruń, Ludwik Rydygier Collegium Medicum in Bydgoszcz (approval number KB 336/2018). All methods used were performed in accordance with applicable principles of good laboratory practice.

FFPE tissue blocks with representative tumor areas were cut using a manual rotary microtome (Accu-Cut, Sakura,

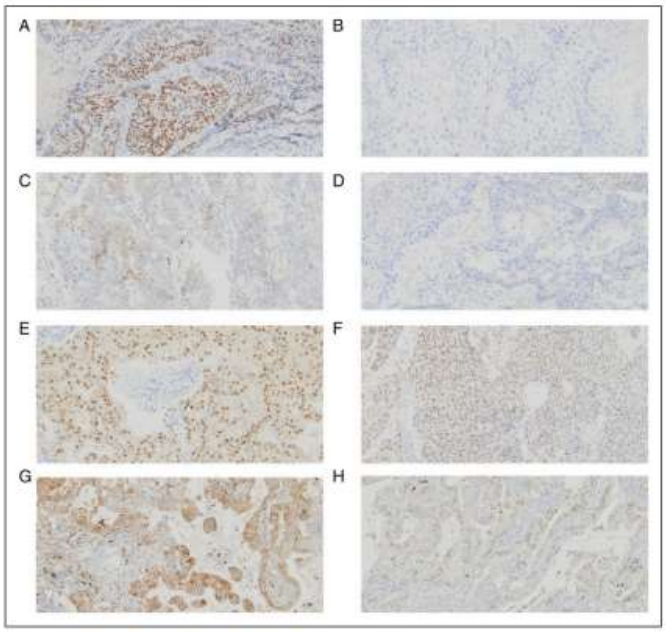

Figure 1. Immunohistochemical analysis of SATB1^{n}, SATB1^{c}, SMAD3, and TLR2 expression in NSCLC tissues (primary magnification ×20). (A) Strong positive (+3) nuclear staining for SATB1, (B) Negative expression of nuclear staining for SATB1, (C) Positive cytoplasmic staining for SATB1, (D) Negative expression of cytoplasmic SATB1, (E) Strong positive (+3) staining for SMAD3, (F) Negative expression of SMAD3, (G) Strong positive (+3) staining for TLR2, (H) Negative expression of TLR2.

Torrance, CA, USA) to 4.0 μm thick. Sections were placed on high-adhesive glass slides (SuperFrost Plus; Menzel-Glaser, Braunschweig, Germany) and dried at 60°C for 1 h. IHC staining was performed using DakoAutostainer Link 48 (Dako, Agilent Technologies, USA) or BenchMark® Ultra automated slide processing system (Ventana Medical Systems, Tucson, AZ, USA). Standardization and optimization of the IHC method were performed using instructions provided by the antibody manufacturers, and data are available in the Human Protein Atlas (http://www.proteinatlas.org).[52]

IHC staining of SATB1 and SMAD3 was performed using Dako Autostainer Link 48 automated slide staining platform (Dako) and the FLEX + visualization system. Tissue sections were deparaffinized and rehydrated prior to antigens retrieval using a high-pH buffer (Dako, Agilent Technologies, USA) for 20 min in PT Link pre-treatment module (Dako) at 95–98°C. Next, slides were treated with 3% H_2O_2 for 10 min at room temperature (RT) to inhibit endogenous peroxidase activity. Then, the preparations were incubated with 3% bovine serum albumin (BSA) solution for 15 min at RT to block non-specific antibody binding sites. The incubation with anti-SATB1 antibody (1:200; cat. no: ab109122, Abcam, Cambridge, MA, USA) and anti-SMAD3 antibody (1:100; cat. no: ab28379) was performed for 30 min at RT. Next, the sections were incubated with the secondary horseradish peroxidase (HRP, Dako) labeled antibody for 20 min at RT. Subsequently, 3,3-diaminobenzidine (DAB) was used to enable localization of the antigen–antibody complex. The tissue sections were counterstained in hematoxylin and washed with PBS buffer. Then, slides were dehydrated in increasing ethanol concentrations (80, 90, 96, and 99.8%), and finally, tissue sections were cleared in xylenes (I–IV), mounted using mounting medium, and examined.

IHC staining of TLR2 was performed using BenchMark® Ultra automated slide processing system (Ventana Medical Systems, Tucson, AZ, USA). Slides were deparaffinized and rehydrated in EZ Prep solution (Ventana Medical Systems) for 8 min at 72°C. Antigen retrieval was achieved in a high-pH Cell Conditioning (CC1) solution for 64 min. Next, incubation with the primary anti-TLR2 antibody (1:200; cat. no: ab24192, Abcam) was performed for 32 min. Antibody detection was performed using VentanaUltraView DAB Detection Kit (Ventana Medical Systems). The tissue sections were counterstained with Hematoxylin for 12 min and one drop of Bluing Reagent for 4 min. Finally, tissue sections were washed in tap water followed by dehydration in increasing ethanol concentrations (80, 90, 96, and 99.8%). Xylene was

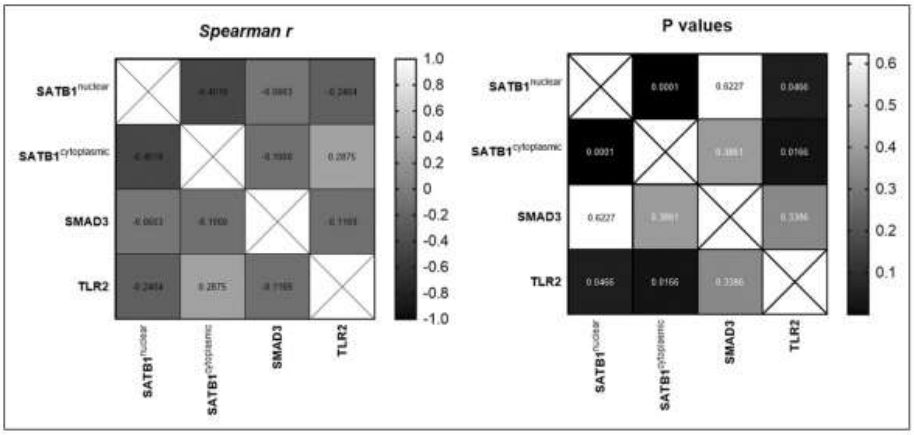

Figure 2. Correlation between SATB1^{n}, SATB1^{c}, SMAD3, and TLR2 expression in lung cancer tissues. Correlation values are presented in a heat map (Spearman correlation test).

used to clear the sections, followed by mounting medium and coverslips prior to observation.

Protein expression was analyzed using an ECLIPSE E400 microscope (Nikon Instruments Europe, Amsterdam, Netherlands) at 20× and 40× magnification. All sections were reviewed separately by 2 independent pathologists without knowledge of the patient's clinical data. The scoring system for SATB1 (SATB1^{n}) and SMAD3 nuclear, as well as TLR2 cytoplasmic immunoreactivity, was determined by adding the multiplication of the fraction of stained cells (FSC) and the percentage of cells at each staining intensity level with the staining intensity ordinal value (scored from 0 for "no staining" to 3+ for "strong staining"), according to a modified H-score with the formula: [1 × (FSC × % cells 1+) + 2 × (FSC × % cells 2+) + 3 × (FSC × % cells 3+)], whereby FSC was calculated based on the number of stained cells per 1000 cells of the same type. The final staining score, ranging from 0 to 300, was then segregated into positive (high) and negative (low) expression on the basis of a specific discriminatory threshold established by the Evaluate Cutpoints software.[53] The cut-off values for positive and negative SATB1^{n}, SMAD3, and TLR2 were as follows: <1; ≥1, <230; ≥230, and <1; ≥1, respectively. For evaluation of cytoplasmic SATB1 (SATB1^{c}), slides were scored as either positive or negative based on the presence (+) or absence (−) of cytoplasmic tumor cell staining.

In our analyses, we also examined the prognostic significance of SATB1, SMAD3, TLR2 mRNA levels in The Cancer Genome Atlas (TCGA) cohort. The survival and gene expression data for the cohort of 630 NSCLC patients were obtained from www.cBioPortal.org and UCSC Xena Browser (http://xena.ucsc.edu/). The RNA-sequencing (RNA-seq) datasets were normalized using the DESeq2 method. The data was split into low-level and high-level expression groups according to cut-off points established in the Evaluate Cutpoints software.[53] Our analyses only included stage I and II cases.

Statistical analyses were performed using GraphPad Prism v 7.01 (GraphPad Software, La Jolla, CA, USA) and SPSS version 26.0 software (IBM Corporation, Armonk, NY, USA). A two-tailed Chi-squared test or Fisher's exact test was used to assess the significance among the clinical factors and the H-scores evaluated by pathologists. Spearman's correlation coefficient was used to assess the correlations between the expression of SATB1, SMAD3, and TLR2. Survival curves were plotted using the Kaplan–Meier method, and the differences were evaluated using a log-rank test, counting OS time from the date of operation to the date of death of any cause or the date of last follow-up. The proportionality assumption was verified by graphical examination and by testing for significant interactions when each variable was entered as a time-based covariate. Univariate and multivariate survival analyses were performed with Cox proportional hazard regression for variables that satisfied proportional hazards assumption. The hazard ratios (HRs) and 95% confidence intervals (95% CIs) were also calculated. Multivariate Cox proportional hazards models were built for each tumor marker after data were adjusted for covariates, including gender (male *vs* female), age (≤62 years *vs* >62 years), and AJCC pathological stage (stage I *vs* stage II). A *P*-value of ≤.05 was considered statistically significant.

Results

A total of 69 patients (47 male; 68.1% and 22 female; 31.9%) diagnosed with NSCLC were included in this study with a

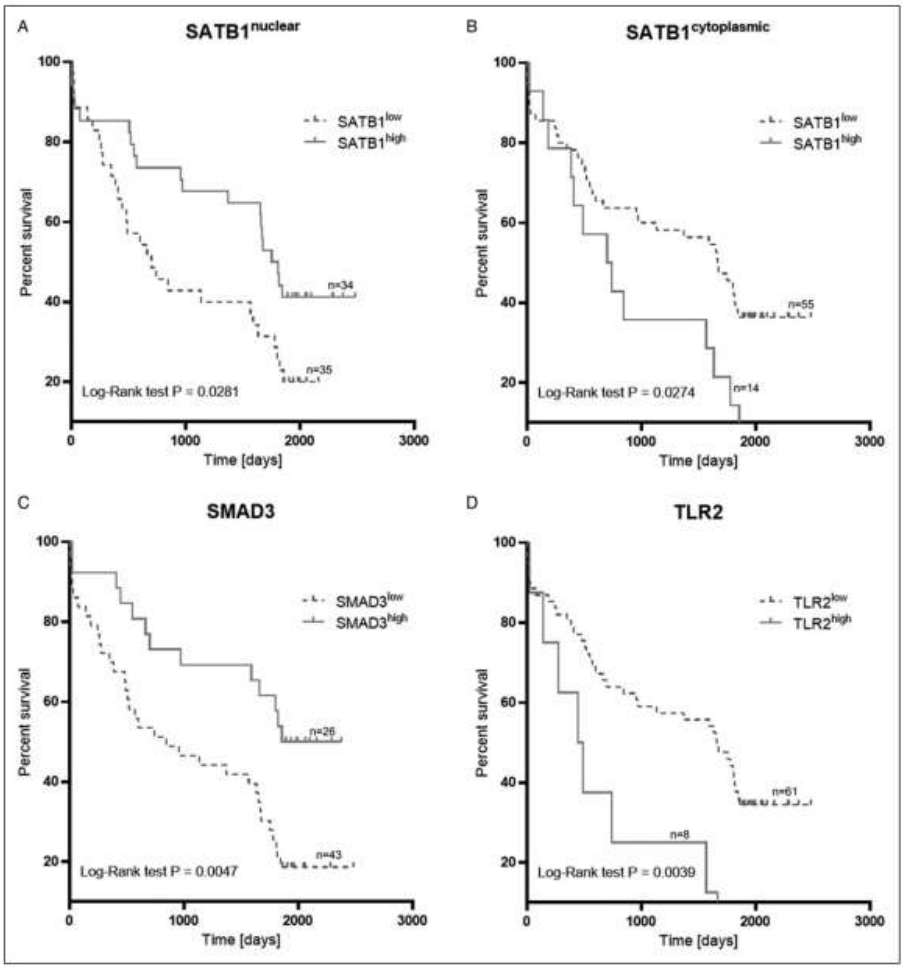

Figure 3. Overall survival analysis according to the expression of SATB1^{n} (A), SATB1^{c} (B), SMAD3 (C), and TLR2 (D).

mean age at diagnosis of 63 (range 46–82 years). The most common histological type was SCC (n=37; 53.6%), followed by ADC (n=27; 39.1%) and LCC (n=5; 7.2%). According to histological differentiation, tumors were divided into G2: moderately differentiated (intermediate grade) and G3: poorly differentiated (high grade). There were 17 pT3 (24.6%) cases, 27 pT2 (39.1%) cases, and 25 pT1 (36.2%) cases. Most tumors were diagnosed at stage I (56.5%) while 30 cases (43.5%) were diagnosed at stage II. Clinical stages IA, IB, IIA, and IIB were found, respectively, in 22 (31.9%), 15 (21.7%), 9 (13%), and 23 (33.3%) patients. Postsurgical survival data was available for all patients. The median follow-up time was 1990 days and 48 (69.6%) patients died during follow-up. Clinicopathological characteristics of patients of this cohort is summarized in Table 1.

IHC staining of SATB1 was detected in the nuclear and cytoplasmic compartments of NSCLC cells (Figure 1A–1D). Positive nuclear immunoreactivity of SATB1 was found in 34 (49.28%) NSCLC cases, whereas the remaining 35 (50.72%) were negative. Cytoplasmic staining of SATB1 was present in 14 (20.29%) cases. The positive expression of SATB1^{n} trending towards an association with histological type (P = .068). Positive expression of SATB1^{n} was more common in SCC (n=23; 62.16%) than in LCC (n=2; 40%) and ADC (n=9; 33.33%). Moreover, positive expression of SATB1^{n} was more frequently detected in older (62.5%) than younger people

(37.83%), although this was not a significant association (P = .0547). In turn, expression of SATB1^{n} was not associated with gender, histological grade, stage, pT, and pN status (P > .05). The association of SATB1^{c} expression with gender was of borderline significance (P = .0502). Positive expression of SATB1^{c} was more common in female (36.36%) than male (12.77%). Expression of SATB1^{c} was not correlated with histological type, histological grade, age, stage, pT, and pN status. The relationship between SATB1 expression and NSCLC clinicopathological features is summarized in Table 1.

IHC staining of SMAD3 was detected in the nuclear compartments of NSCLC cells of 26 (37.68%) NSCLC cases (Figure 1E–1F). The relationship between SMAD3 expression and clinicopathological features was analyzed and demonstrated the association with histological grade (P = .0265), pT status (P = .0269), and tumor stage (P = .045). SMAD3 overexpression was more frequently detected in poorly (n=23; 46.00%) differentiated tumors than in moderately differentiated ones (n=3; 15.79%) (P = .00265). The ratio of SMAD3 overexpression was also significantly higher in patients with pT2 (n=14; 51.85%) NSCLCs than in those with pT1 (n=10; 40.00%) and pT3 (n=2; 11.76%) tumors (P = .0269). Moreover, the overexpression of SMAD3 was more frequently detected in stage I (n=19; 48.72%) tumors than in those with stage II (23.33%) (P = .0450). The prevalence of SMAD3 overexpression was higher in LCC (80%) than in SCC (40.54%) and ADC (25.93%) although this was not a significant association (P = .0629). In addition, high SMAD3 levels were trending towards a correlation with age (P = .0506), being more frequently detected in younger (48.65%) than older people (25%). The expression status of SMAD3 was not associated with gender and pN status. The relationship between SMAD3 expression and NSCLC clinicopathological features is summarized in Table 1.

IHC staining of TLR2 was detected in the cytoplasmic compartments of 8 (11.6%) NSCLC cells (Figure 1G–1H). The ratio of TLR2 overexpression was more common in ADC (n = 7; 25.93%) than in SCC (n = 1; 2.7%) and LCC (n = 0; 0%) (P = .0054). The expression status of TLR2 was not correlated with gender, age, histological grade, stage, pT, and pN status. Representative images of IHC staining for all histological types are demonstrated in Supplementary Figure 2.

A weak negative and significant association was confirmed between the expression of SATB1^{n} and TLR2 (P = .0466, Spearman coefficient r = −.2404). Furthermore, weak positive and significant association was found between SATB1^{c} and TLR2 expression (P = .0166, Spearman coefficient r = .2875). In addition, a moderately negative association was confirmed between the expression of SATB1^{n} and SATB1^{c} (P = .0001, Spearman coefficient r = −.4619). In the entire cohort, the expression of SMAD3 was not significantly correlated with the expression of SATB1 and TLR2 (Figure 2).

Table 2. Univariate analysis of prognostic factors by Cox proportional hazard model (n = 69).

	Univariate analysis			
Variable	HR	95% CI		*P*-value
SATB1^{n}	.53	.30	.94	**.031**
SATB1^{c}	2.04	1.07	3.88	**.031**
SMAD3	.41	.21	.78	**.006**
TLR2	3.02	1.37	6.66	**.006**
Gender	1.02	.56	1.87	.96
Age	1.03	.99	1.07	.17
Stage	.84	.48	1.48	.55
pT status	.68	.36	1.29	.24
pN status	1.7	.67	4.32	.27

SATB1^{n} = nuclear immunoreactivity of SATB1; SATB1^{c} = cytoplasmic immunoreactivity of SATB1; HR = hazard ratio; CI = confidence intervals. Significant *P*-values (P < .05) are indicated in bold.

Table 3. Multivariate analysis of prognostic factors by Cox proportional hazard model (n = 69).

	Multivariate analysis: SATB1^{n}					Multivariate analysis: SATB1^{c}			
Variable	HR	95.0% CI		*P*-value	Variable	HR	95.0% CI		*P*-value
SATB1^{n}	.49	.27	.90	**.022**	SATB1^{c}	2.11	1.07	4.16	**.030**
Gender	.90	.49	1.66	.74	Gender	.86	.46	1.61	.64
Age	1.23	.69	2.22	.48	Age	1.08	.61	1.90	.80
Stage	1.19	.67	2.10	.55	Stage	1.12	.63	1.98	.70
	Multivariate analysis: SMAD3					Multivariate analysis: TLR2			
Variable	HR	95.0% CI		*P*-value	Variable	HR	95.0% CI		*P*-value
SMAD3	.40	.20	.78	**.007**	TLR2	3.00	1.36	6.63	**.007**
Gender	1.01	.55	1.85	.98	Gender	1.03	.56	1.88	.93
Age	.93	.52	1.65	.80	Age	1.00	.57	1.77	.10
Stage	.94	.52	1.70	.83	Stage	1.17	.66	2.07	.59

SATB1^{n} = nuclear immunoreactivity of SATB1; SATB1^{c} = cytoplasmic immunoreactivity of SATB1; HR = hazard ratio; CI = confidence intervals. Likelihood ratio P, adjusted for gender, age and stage. Significant *P*-values (P < .05) are indicated in bold.

Kaplan–Meier survival analysis indicated that NSCLC patients with a high level of SATB1^{n} expression (median OS 1781 days) had higher OS rates (log-rank test $P = .028$) than those with SATB1^{n} low expression level (median OS 701 days). NSCLC patients with the presence of SATB1^{c} expression (median OS 722.5 days) had lower OS rates (log-rank test $P = .0274$) than those with low-level expression (median OS 1668 days). Kaplan–Meier analysis also revealed the significance of SMAD3 and TLR2 expression for NSCLCs. We found that high expression of TLR2 correlated with decreased OS rates (log-rank test $P = .0039$), and high expression of SMAD3 correlated with increased OS rates (log-rank test $P = .0047$) (Figure 3). Median OS periods for TLR2^{high} and TLR2^{low}, as well as SMAD3^{high} and SMAD3^{low} were 467 days/1662 days and 2116 days/846 days, respectively.

Univariate analysis demonstrated that positive SATB1^{n} expression was significantly associated with a better survival prognosis (HR .53, 95%CI .30–.94, $P = .031$), and it persisted as an independent prognostic factor for improved OS in multivariate analysis after adjustment for age, gender, and stage (HR .49, 95%CI .27–.90, $P = .022$). In the case of SATB1^{c}, the univariate Cox analysis revealed its presence predicted an unfavorable OS (HR 2.04, 95%CI 1.07–3.88, $P = .031$). When examined in multivariate analysis, SATB1^{c} remained as an independent prognostic factor in terms of OS (adjusted HR 2.11, 95%CI 1.07–4.16, $P = .030$). Likewise, TLR2 was a significant predictor of poor OS in both univariate (HR 3.02, 95%CI 1.37–6.66, $P = .006$) and multivariate (adjusted HR 3.00, 95%CI 1.36–6.63, $P = .007$) analysis. Furthermore, univariate analysis showed a longer OS was significantly correlated with high SMAD3 expression (HR .41, 95%CI .21–.78, $P = .006$), a result that was maintained during multivariate analysis following adjustment for covariates (HR = .40, 95% CI .20–.78; $P = .007$). Results for univariate and multivariate analysis are summarized in Table 2 and Table 3, respectively.

Table 4. Univariate analysis of prognostic factors by Cox proportional hazard model for combined expression of proteins (n = 69).

Variable	Univariate analysis: SATB1/SMAD3/TLR2			
HR	95% CI		*P*-value	
Others	Ref			
SATB1$^{n\text{-}high}$/SMAD3^{high}/TLR2^{low}	.19	.06	.62	**.006**
SATB1$^{n\text{-}low}$/SMAD3^{low}/TLR2^{high}	2.34	.90	6.11	.082

SATB1^{n} = nuclear immunoreactivity of SATB1; SATB1^{c} = cytoplasmic immunoreactivity of SATB1; HR = hazard ratio; CI = confidence intervals. Significant *P*-values ($P < .05$) are indicated in bold. Cases designated 'others' grouped the remaining combinations of expression patterns.

Having established the significance of SATB1, SMAD3, and TLR2 as single prognostic markers in our cohort of NSCLC patients, we also examined the impact of their combined expression on OS. As shown by Kaplan–Meier analysis, the best OS was observed for patients whose NSCLCs simultaneously expressed SATB1^{n} and SMAD3 at high level and TLR2 at low level. In turn, patients whose NSCLCs had opposite expression profile of the 3 proteins had dramatically shorter OS (undefined *vs* 490 days; $P < .0001$) (Figure 4A). A univariate analysis of a combined 3-protein panel of SATB1$^{n\text{-}high}$/SMAD3^{high}/TLR2^{low} was associated with better survival prognosis (HR .19, 95%CI .06–.62, $P = .006$) (Table 4) and was a potent independent prognostic marker for NSCLC patients when examined in a multivariate analysis (adjusted HR .19, 95%CI .06–.63, $P = .007$) (Table 5). The worst OS was seen for SATB1$^{n\text{-}low}$TLR2^{high} co-expressing tumors with a particularly short median OS (445 days), while the patients with the opposite expression profile had median OS of 1807 days ($P = .0003$) (Figure 4B).

The analysis of prognostic significance of SATB1 mRNA levels in the TCGA cohort revealed that SATB1 overexpression was significantly associated with better OS of

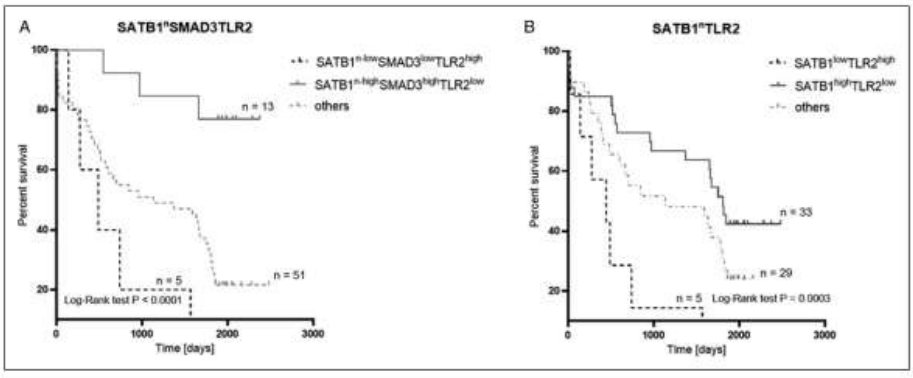

Figure 4. Overall survival analysis according to the combination of the protein panel: SATB1^{n}SMAD3TLR2 (A), SATB1^{n}TLR2 (B).

NSCLC patients ($P = .0144$). The median OS times were 1830 and 2639 days for low and high expression groups, respectively. Furthermore, the TCGA dataset showed that SMAD3 overexpression was associated with significantly shorter OS (2620 days *vs* 1830 days; $P = .0214$). Finally, Kaplan–Meier survival analysis revealed that high TLR2 expression was significantly associated with better survival time (1841 days) of NSCLC patients in comparison to those with its low expression level (634 days; $P = .0248$) (Figure 5).

Discussion

NSCLC is among the most frequently diagnosed malignancies and the leading cause of cancer-related death worldwide. Early diagnosis and treatment of NSCLC is a prerequisite for increasing survival. Those patients with early-stage NSCLC but identified as low risk require less aggressive therapy, while those classified as high risk might be good candidates for adjuvant therapy. Therefore, it is essential to establish diagnostic and prognostic markers that can identify early-stage NSCLC patients who require more aggressive therapy.

In the past few years, research has been conducted to assess the expression level and role of SATB1 in many kinds of human tumors, including lung cancer. However, there are contradictory results about SATB1 expression levels, especially when examining prognostic and clinicopathological features. Several studies have shown discrepancies regarding SATB1 expression in lung tumors compared to normal lung tissues.[16,25,27] Due to the lack of data on SATB1 expression in normal bronchial tissues in ourcohort, it was not possible to verify these findings.[17,54–56] Several attempts have also been made to investigate the correlation between SATB1 expression and clinicopathological features of NSCLC patients. In our investigation, we observed cytoplasmic and nuclear staining of the SATB1 protein in cancer cells. Nuclear SATB1 positivity was 49.28% and higher than the rates reported by Glatzel-Plucinska et al. and Selinger et al.[16,25] We presume that the discrepancy between the positivity rates may be due to differences in the experimental design, for example, evaluation of IHC reactions, selected cut-offs, antibody clones, and the subjectivity of the pathologists interpretation. Several studies from other authors have reported that SCCs show markedly higher SATB1 expression level compared to ADC.[16,25,27] A similar trend was also reported in our study for $SATB1^{n}$, but the data were not significant ($P = .068$). In our cohort, the $SATB1^{n}$ positivity rate occurred more frequently in SCC (62.16%) than ADC (33.33%). SCCs and ADCs differ in gene expression profile, cellular origin, and also targeting mutations, and this may be responsible for the differences in the frequency of SATB1 positive cases. Furthermore, a congruous relationship has also been demonstrated by Glatzel-Plucinska et al. in SCC and ADC cell lines. Moreover, the previous research has shown that SATB1 expression was associated with the degree of tumor differentiation in clinical ADC and SCC samples, although this could not be confirmed in our study, possibly due to the smaller cohort size. In addition, our study included only moderately and poorly

Table 5. Multivariate analysis of prognostic factors by Cox proportional hazard model for combined expression of proteins (n = 69).

Variable	Multivariate analysis: SATB1/SMAD3/TLR2			
	HR	95% CI		*P*-value
Others	Ref			
$SATB1^{n\text{-}high}/SMAD3^{high}/TLR2^{low}$	.19	.06	.63	**.007**
$SATB1^{n\text{-}low}/SMAD3^{low}/TLR2^{high}$	1.87	.68	5.17	.225
Gender	1.00	.54	1.85	.989
Age	1.03	.99	1.07	.156
Stage	.62	.34	1.13	.116

$SATB1^{n}$ = nuclear immunoreactivity of SATB1; $SATB1^{c}$ = cytoplasmic immunoreactivity of SATB1; HR = hazard ratio; CI = confidence intervals. Likelihood ratio P, adjusted for gender, age and stage. Significant *P*-values ($P < .05$) are indicated in bold. Cases designated 'others' grouped the remaining combinations of expression patterns.

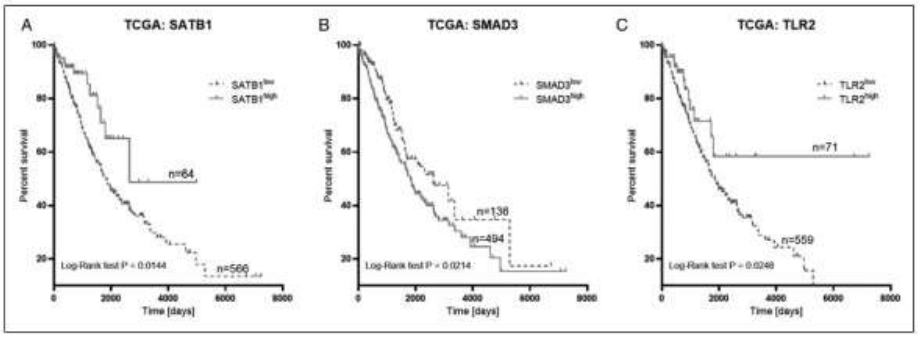

Figure 5. Overall survival analysis of SATB1 (A), SMAD3 (B), TLR2 (C) mRNA levels in TCGA cohort.

differentiated tumors. Selinger et al. noticed that a high SATB1 level was associated with an early disease stage. The patients in our cohort had stage I or II disease; therefore, we could not make comparisons with more advanced clinical stages. Several studies have found a significant associations between SATB1 expression and OS of NSCLC patients. Our cohort study showed that patients with high $SATB1^n$ expression had better median OS than did patients with $SATB1^n$ underexpression (1781 *vs* 701 days). Moreover, and based on the TCGA dataset, we found that SATB1 overexpression was also significantly associated with better OS of NSCLC patients. Furthermore, we revealed that an elevated SATB1 expression was an independent prognostic factor that predicted superior survival in NSCLC patients, which was also confirmed by Glatzel-Plucinska et al.[16] Additionally, Selinger et al.[25] have demonstrated that a loss of SATB1 expression was a negative prognostic factor for patients with SCC. However, our examination of cytoplasmic SATB1 showed these patients had lower OS rates than those with $SATB1^c$ underexpression. Furthermore, we demonstrated that high $SATB1^c$ expression was an independent prognostic factor which predicted poor OS. To the best of our knowledge, this is the first study to show that $SATB1^c$ was significantly associated with OS of NSCLC patients. The findings may imply that depending on subcellular localization, SATB1 has an opposite prognostic significance in terms of OS in early-stage NSCLC patients. Interestingly, Selinger et al. also observed cytoplasmic staining of SATB1, but this staining pattern was not associated with survival or any other clinicopathological parameters. Other cited authors omitted the estimation of the cytoplasmic fraction of SATB1 or combined it with the nuclear fraction, which seems unjustified in view of our results. Undoubtedly, our results suggested an association of both nuclear and cytoplasmic SATB1 expression with patient OS, highlighting the role of SATB1 in tumor progression.

SMAD is a critical intracellular mediator of TGFβ signaling from the cell surface to the nucleus, and the subject of this investigation, SMAD3, is involved in regulating gene activity, cell proliferation, differentiation, and cell death. A number of previous studies demonstrated that SMAD3-mediated TGFβ signaling is implicated in tumor angiogenesis, tissue invasion, and metastasis. Scientists agree that the overexpression of SMAD3 is involved in the regulation of various physiological, as well as pathological processes, including carcinogenesis. However, its role in tumorigenesis is not clear.[57,58] The results of the present study indicated that SMAD3 was overexpressed in 37.68% of NSCLC cases. Our study further demonstrated that SMAD3 expression was significantly correlated with histopathological grade, pT status, and stage, but not with gender and pN status. Moreover, the overexpression of SMAD3 was trending towards a correlation with histological type and age. In our cohort, it was more likely to have SMAD3 positivity in poorly differentiated cancer cells than in those that were moderately differentiated, which could indicate that SMAD3 is involved in the progression of NSCLC. On the other hand, our results revealed an association of SMAD3 with favorable prognostic variables, such as smaller tumor size and more frequent occurrence in stage I tumors. In recent years, several studies have been carried out to examine the relationship between SMAD3 and the prognosis of cancer patients.[33,59,60] In contrast to the cited results, we found that patients with high SMAD3 expression had better median OS than did patients with SMAD3 underexpression. Cox regression analyses showed that overexpression of SMAD3 was a favorable independent prognostic factor for OS. This discordance between studies may be due to different study population or ethnicity-related differences in NSCLC biology. Moreover, Niu et al. used tissue microarrays (TMAs), but the heterogeneity of whole tumor samples could confound microarray analysis, so in our study we stained whole-tissue sections. Notably, in our report, we included only patients in stage I to II of NSCLC, whereas Marwitz et al. did not provide patient stage information from their cohort, and Niu et al. presented stage data for 18 (15.13%) patients only, all of whom had stage III and IV lung cancer. Therefore, it is most probable that in NSCLC, SMAD3 may act as a negative regulator of carcinogenesis and improve patient survival in early disease stages, while it functions in the opposite manner in advanced cancers, resulting in diminished survival in patients. It is not unexpected since, based on experimental model systems, a similar duality of function under different tumor stages has been reported for TGFβ, also in the case of lung cancer. In the experimental studies on other tumor types, this paradoxical effect of TGFβ has been shown to be mediated through , for example, SMAD3.[61] On the other hand, our analyses based on TCGA have shown that the expression of SMAD3 mRNA was significantly negatively correlated with patient survival, which is in agreement with Niu et al.[59,60] Pan et al.[62] revealed that SMAD3 was a tumor suppressor for post-progression survival but an oncogene for progression-free survival. In turn, Zeng et al.[63] showed that increased SMAD3 mRNA levels were not related to OS in NSCLC patients from the CBioPortal cohort. These results suggest an inverse role for SMAD3 protein and mRNA in the clinical behavior of NSCLC. A mismatch between mRNA and protein levels has been widely reported in the literature.[64–66] The difference between mRNA and protein expression with respect to prognostic significance confirms the importance of comprehensive tumor analyses. However, it cannot be assumed that the amount of mRNA is directly correlated with protein expression. Noteworthily, posttranscriptional and posttranslational mechanisms may influence protein levels, and increased mRNA levels may produce only small amounts of detectable proteins.[67] Based on our results, we can conclude that SMAD3 protein is perhaps functionally associated with better prognosis in early-stage NSCLC, whereas the contrary is true for SMAD3 mRNA. However, our conclusions are constrained by the fact that presented results come from disparate research population, that is, for proteins from our own cohort and mRNAs from the TCGA cohort. Definitely, to validate this concept, it is necessary to estimate mRNA and protein expression in one cohort of patients.[68]

TLR2 is a member of the TLR family, mainly expressed by immune cells, but as they are also expressed on tumor cells. Thus, in recent years, a lot of scientific interest has focused on TLR expression and their functions in cancer cells, and a growing body of evidence underscores the correlation between TLR expression and cancer prognosis. In recent years, numerous studies have indicated that TLR2 is expressed on neoplastic cells from several solid tumors, such as brest,[69] gastrin,[70] colon,[71] oral,[72] and pancreatic cancer.[73] In addition, it has been revealed that activation of TLR2 promotes cancer progression and metastasis through different cell-intrinsic mechanisms,[74] and its expression closely associates with patient prognosis.[69,70,75] To our knowledge, the present study is the first to investigate the correlation between TLR2 protein expression and clinicopathological features in patients with clinical stages I to II NSCLC. In our cohort, TLR2 was overexpressed in 11.6% of NSCLC cases and TLR2 positivity rate more frequently occurred in ADC (25.9%) than SCC (2.7%) and LCC (0%). These results suggest that ADC tumors express higher levels of TLR2 than SCC and LCC. A similar relationship was observed by Gergen et al., but in this case, it was found in ADC and non-adenocarcinoma cell lines.[54] Furthermore, several authors have investigated the correlation between TLR2 expression and patient survival.[69,70,73,75,76] Bauer et al. provided evidence for a significant association between high mRNA expression of TLR2 in TCGA cohort of NSCLC patients. According to their report, expression of TLR2 is associated with improved survival outcomes in NSCLC, and our analysis of TCGA data supports this finding. However, and contrary to mRNA expression, we found using IHC that high TLR2 protein levels were significantly associated with worse OS of NSCLC patients. Moreover, we demonstrated that TLR2 was a significant predictor of poor OS in both univariate and multivariate analyses. These results suggest opposite role for TLR2 protein and mRNA in the clinical behavior of stage I to II NSCLCs. To confirm this supposition, it is necessary to estimate mRNA and protein expression in the same cohort of patients. These results suggest an opposite role for TLR2 protein and mRNA in the clinical behavior of stage I to II NSCLCs. Notably, TLR2 is not only expressed in tumor cells but also in immune cells, endothelial and epithelial cells, serving as internal staining controls. IHC staining enables for quantitative evaluation of proteins in a morphological and subcellular context, an advantage of this method over gene expression analyses. To confirm this supposition, similar to our findings with SMAD3, it is necessary to estimate mRNA and protein expression in the same cohort of patients following laser tissue microdissection.

Lastly, given the functional relationship between SMAD3 and SATB1,[77] as well as a potential link between the latter and TLR2,[44] we attempted to determine the effect of the combined expression of these proteins on OS of NSCLC patients. Kaplan–Meier survival analysis demonstrated that the subset of patients with tumors that co-expressed high levels of SATB1 and SMAD3, and simultaneously low levels of TLR2 had the best OS, and the combined expression of the 3 markers better predicted patient survival than looking at each marker individually. Furthermore, a combined 3-protein panel of $SATB1^{n-high}/SMAD3^{high}/TLR2^{low}$ emerged as a powerful independent prognostic factor associated with better outcome. Therefore, our analyses showed that utilizing IHC to examine the combined expression of $SATB1^{n}$, SMAD3, and TLR2 could be more helpful for predicting the prognosis of NSCLC patients than single markers. Given that our study group is limited in number, it is necessary to confirm this finding in large-scale studies.

In summary, our cohort study revealed that nuclear SATB1 expression was associated with favorable patient survival, whereas cytoplasmic SATB1 had a significant correlation with poor outcome. Interestingly, nuclear and cytoplasmic SATB1 appeared to be independent markers either for better or worse prognosis, respectively. These data suggest that a differential subcellular expression of SATB1 plays an important role in the pathology and/or progression of NSCLC, and clinically, it has an inverse prognostic significance. Furthermore, herein we found that high expression levels of SMAD3 and TLR2 were independent prognostic markers associated with favorable and poor survival, respectively. Importantly, stratification of NSCLCs with respect to combined expression of the 3 biomarkers allowed us to identify subgroups of patients with the greatest difference in survival. Specifically, the expression profile of $SATB1^{n-high}/SMAD3^{high}/TLR2^{low}$ was associated with the best OS, and it was superior to each single protein alone in predicting patient prognosis. Based on the TCGA dataset, we revealed that overexpression of SATB1 mRNA was significantly associated with better OS, while high mRNA levels of SMAD3 and TLR2 were negatively correlated with patient survival. Overall, each of these proteins may have a potential clinical utility for determining a patient's prognosis in stage I and II NSCLC patients by themselves, although when a combined 3 protein panel is used; this provides a stronger indication of OS than each marker alone. However, our results should be interpreted cautiously due to existence of some limitations. This study needs validation with a larger patient cohort, and in prospective and multicenter studies.

Notations

ADC - adenocarcinoma
AJCC - The American Joint Committee on Cancer
BUR - base unpairing region
FFPE - paraffin-embedded tissue samples
IHC - immunohistochemical
LCC - large cell carcinoma
MAR - matrix attachment region
NMP - nuclear matrix protein
NSCLC - non–small-cell lung carcinoma
OS - overall survival

PAMP - pathogen-associated molecular pattern
SATB1 - special AT-rich binding protein 1
SCC - squamous cell carcinoma
SCLC - small-cell lung carcinoma
TGFβ - transforming growth factor-β
TLR - toll-like receptor
WHO - World Health Organization

Declaration of Conflicting Interests

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Ethics Statement

The study protocol has been approved by The Ethics Committee of Nicolaus Copernicus University in Toruń, LudwikRydygier Collegium Medicum in Bydgoszcz (approval number KB 336/2018).

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ORCID iDs

Justyna Durślewicz https://orcid.org/0000-0001-7301-5324
Anna Klimaszewska-Wiśniewska https://orcid.org/0000-0002-8493-3806
Jakub Jóźwicki https://orcid.org/0000-0002-2771-413X

Supplemental Material

Supplemental material for this article is available online.

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6.2 Artykuł oryginalny 2

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Prognostic Significance of SATB1, SMAD3, Ezrin and β-Catenin in Patients with Pancreatic Adenocarcinoma

Justyna Durślewicz [1,*], Anna Klimaszewska-Wiśniewska [1], Ewa Domanowska [1], Natalia Skoczylas-Makowska [1], Paulina Antosik [1], Wioletta Zielińska [2], Arkadiusz Gzil [1], Paulina Czajkowska [2], Klaudia Mikołajczyk [2] and Dariusz Grzanka [1]

[1] Department of Clinical Pathomorphology, Faculty of Medicine, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, 85-094 Bydgoszcz, Poland; ania.klimaszewska@op.pl (A.K.-W.); ewdom@cm.umk.pl (E.D.); n.makowska@cm.umk.pl (N.S.-M.); paulina.antosik@cm.umk.pl (P.A.); arkadiuszgzilresearch@gmail.com (A.G.); d_grzanka@cm.umk.pl (D.G.)

[2] Department of Histology and Embryology, Faculty of Medicine, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, 85-092 Bydgoszcz, Poland; w.zielinska@cm.umk.pl (W.Z.); paulina.czajkowska25@gmail.com (P.C.); klaudia.mikolajczyk@cm.umk.pl (K.M.)

* Correspondence: justyna.durslewicz@cm.umk.pl; Tel.: +48-52-585-42-00

Abstract: The present study aimed to explore the role of SATB1, SMAD3, Ezrin and β-catenin as individual and combined biomarkers for the survival prediction in pancreatic adenocarcinoma (PAC). Notably, this study distinguished for the first time a potential prognostic value of SATB1 corresponding to its subcellular localization in PAC. Immunohistochemical staining on tissue macroarrays, as well as RNA-seq data from public sources, were investigated, and the results correlated with overall survival (OS) and clinicopathological features. The connectivity between the examined factors, as well as their common signaling pathways, were demonstrated by the functional enrichment analysis. Herein, the prognostic ability of cytoplasmic SATB1 in OS analysis was even superior to nuclear SATB1. Both staining patterns tended to have opposite roles in the prognosis of PAC: $SATB1^{c}$ was an independent prognostic factor for poor OS, whereas $SATB1^{n}$ expression reached no statistical significance, but Kaplan–Meier curves separated patients with low expression and adverse prognosis from patients with high expression and favorable prognosis. High levels of SATB1 mRNA appeared as an independent prognostic indicator for better OS. Furthermore, individual expression of SMAD3 or Ezrin, as well as combined expression of SATB1/SMAD3/Ezrin/β-catenin, were associated with OS independently of conventional risk factors, both in our cohort and TCGA dataset. In our series, patients with tumors harboring combined expression of $SATB1^{n\text{-}high}$/$SMAD3^{low}$/$Ezrin^{low}$/$\beta\text{-catenin}^{low}$ experienced the highest survival rates, while those with $SATB1^{c\text{-}present}$/$SMAD3^{high}$/$Ezrin^{high}$/$\beta\text{-catenin}^{high}$ had the worst survival. In conclusion, protein and/or mRNA expression levels of SATB1, SMAD3, Ezrin and β-catenin may serve as potential prognostic biomarkers for PAC, both as single predictors and even better when combined.

Keywords: pancreatic ductal adenocarcinoma; SATB1; SMAD3; Ezrin; β-catenin; prognostic factor

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1. Introduction

Pancreatic cancer is one of the most aggressive and lethal malignant neoplasms worldwide. According to the Global Cancer Statistics 2020, pancreatic cancer is ranked as the 7th leading cause of cancer-related deaths, accounting for 4.7% of all diagnosed malignancy cases with increasing frequency [1]. Despite significant advances in the understanding of potential risk factors that cause pancreatic cancer and newly available tools for early diagnosis and treatment, the general 5-year survival rate for patients is lower than 9% [2]. There are two main types of pancreatic cancer: pancreatic adenocarcinoma, known as pancreatic ductal adenocarcinoma (PDAC), which originates from the epithelium of a duct, and pancreatic neuroendocrine tumor arising from the abnormal growth of endocrine

(hormone-producing) cells in the pancreas called islet cells [3]. PDAC is a type of exocrine pancreatic cancer and the most common type of pancreatic malignancies (more than 85% of all cases). This type is known for its extremely poor prognosis, with an overall 1-year mortality rate of 24% [2]. Undoubtedly, there is an urgent need for the development of a novel, more effective therapeutic regimen. Therefore, the identification of key oncogenic regulators and understanding of pathways involved in the pathogenesis and progression of pancreatic cancer is of high importance. A greater understanding of the relationship between biomarkers expression and the clinicopathological characteristics as well as patient survival would be useful from the clinical point of view.

Ezrin is a protein encoded by the EZR human gene. It is a principal member of the ezrin–radixin–moesin (ERM) family, which functions as a general cross-linker between membrane proteins and actin filaments. Ezrin exists in two conformations—dormant and active. The former exists in a closed form that mostly resides in the cytoplasm, and the latter is an open form localized mainly at the plasma membrane. It is widely established that Ezrin is frequently overexpressed in invasive cancers, which is related to a poor prognosis. The results of several studies suggest that Ezrin may regulate various signaling pathways and molecules involved in tumor progression [4–6].

SMAD family member 3 (SMAD3) acts through transforming growth factor-beta (TGF-β). The protein participates in the regulation of gene activity, cell proliferation, differentiation and death [7]. However, the SMAD3-mediated TGF-β signaling pathway is also involved in the induction of tumor angiogenesis and the promotion of tissue invasion and metastasis [8,9].

The many cellular processes, including organ development, differentiation and tissue homeostasis, are controlled by the Wnt/β-catenin signaling pathway. Aberrant Wnt/β-catenin signaling can lead to developmental defects and cancer progression. β-catenin (encoded by CTNNB1 gen) is a key molecule in this pathway. Stabilized β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it binds the TCF/LEF family members and induces the transcription of target genes. Many studies have reported that overexpression of β-catenin is associated with several human cancers [10–12]. However, the role of β-catenin expression in PDAC is somewhat controversial [13].

Special AT-rich sequence-binding protein 1 (SATB1) is a higher-order chromatin organizer and a global transcriptional regulator. SATB1 may regulate whole sets of genes, even those located on distant chromosomes, by altering the functional organization of the DNA sequence. In addition, SATB1 is engaged in post-transcriptional modifications, such as phosphorylation or acetylation, conferring its ability to act as a repressor or activator of gene expression [14]. SATB1 is known to play a vital role in the differentiation, embryonic development and maturation of thymocytes [15]. Furthermore, our team has revealed that the SATB1/F-actin complex is involved in the active cell death of both tumor [16] and non-tumor cells [17]. However, it may also contribute to tumor progression and metastasis. Many recent studies have shown that the abnormal expression of SATB1 is frequently associated with clinicopathological features and patient survival, but its clinical value in PDAC is still underexplored [18–24].

Similar to other malignancies, uncontrolled cell proliferation is induced in PDAC by the alternating function of different intracellular signaling pathways' components. As demonstrated in previous studies, SATB1, SMAD3, Ezrin and β-catenin play an important role in regulating cell proliferation, migration and apoptosis, which are strongly implicated in carcinogenesis. In this study, we selected these proteins due to the fact that they may be related by their respective roles and overlapping signaling cascades in tumor cells; however, their joint expression has not been previously evaluated in clinical samples of PDAC. Therefore, the aim of this research was to explore the prognostic value of the single and combined expression of SATB1, SMAD3, Ezrin and β-catenin in PDAC. Protein expression was evaluated by immunochemistry in the institutional tissue macroarrays (TMAs), while mRNA expression used publicly available TCGA data. Both were correlated with clinicopathological parameters and patient outcome (overall survival, OS). Finally,

protein–protein interaction (PPI) network for β-catenin, Ezrin, SATB1, SMAD3 and their 50 neighbors was constructed and functionally annotated to predict biological functions and pathways possibly related to examined factors in PAC.

2. Materials and Methods

2.1. Patients and Tissue Specimens

The research was performed on formalin-fixed paraffin-embedded tissue (FFPE) specimens from 68 patients with PDAC who were operated at the Department of Liver and General Surgery, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun (Poland). Histopathological diagnosis for each tumor sample was confirmed by two independent pathologists, based on the hematoxylin and eosin (HE) staining in the Department of Clinical Pathomorphology, Collegium Medicum in Bydgoszcz. All tumors were reclassified according to the standardized TNM 8th edition classification of The American Joint Committee on Cancer (AJCC) criteria. To avoid excessive study complexity, the cohort included only adenocarcinomas, and all other histological types were excluded from the series. All clinical data were systematized and analyzed in detail. The study group included 68 patients (34 female, 34 male) with an average age of 63 years (range 43–81). The most common tumor location was the head of the pancreas (n = 60, 88.24%). In turn, pancreatic body and tail tumors were less frequent (n = 8, 11.77%). Considering a histological grade, 5 samples were classified as differentiated (G1; 7.35%), 55 as moderately differentiated (G2; 80.88%), and 8 as poorly differentiated (G3; 11.77%) cancer tissue. Regarding pathologic T stage, the study group consisted of 10 T1 (14.71%), 43 T2 (63.24%), 8 T3 (11.77%), 2 T4 (2.94%) and 5 Tx (7.35%) cases. Thirty-six (52.94%) patients were characterized by a positive and 30 (44.12%) by a negative lymph node status (no data for 2 patients). Perineural invasion was present in 42 (61.77%) patients, while vascular invasion was confirmed for 23 (37.10%) patients. Data concerning postsurgical survival were available for 62 patients, and the median follow-up time was 1427 days. The study was carried out using the same tissue specimens as in our previously published study [25], whereby the final follow-up was extended to 6 September 2020. The control tissue (n = 64) was from 54 of the 68 PDAC patients with the adjacent normal pancreatic tissue and another 10 specimens from normal peritumoral tissue of other PDAC patients. The study protocol has been approved by The Ethics Committee of Nicolaus Copernicus University in Torun, Ludwik Rydygier Collegium Medicum in Bydgoszcz (approval number KB 342/2020).

2.2. Tissue Macroarrays and Immunohistochemical Staining

Representative tumor areas (tumor areas with at least 80% of tumor cells) and the adjacent areas of histologically normal tissue were selected in order to obtain tissue macroarray. Tissue macroarrays were obtained by transferring representative large tissue fragments from donor blocks into a new recipient block. One recipient block included 5 different tumor samples from donors' paraffin blocks. For verification tumor cells in tissue macroarray blocks, HE staining was performed. IHC studies were carried out on FFPE specimens at the Department of Clinical Pathomorphology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, Poland. Selected paraffin blocks were cut into 3–4 µm thick sections using a manual rotary microtome (Accu-Cut, Sakura, Torrance, CA, USA). Subsequently, paraffin sections were placed on extra adhesive slides (SuperFrost Plus; Menzel-Glaser, Braunschweig, Germany) and dried at 60 °C for 1 h. Next, sections were deparaffinized and rehydrated. Antigen retrieval was performed using a high-pH buffer (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) at 95–98 °C for 20 min in an automated PT-link system (Dako). Automated immunostaining for anti-SATB1 antibody (ab109122, Abcam, Great Britain), anti-SMAD3 antibody (ab28379, Abcam), anti-Ezrin antibody (ab41672, Abcam) and anti-β-catenin antibody (ab32572, Abcam) was carried out with EnVision FLEX+ HRP reagents (Dako). Endogenous peroxidase activity, as well as the nonspecific binding sites, were blocked by incubation with 3% H_2O_2 for 10 min at room temperature (RT) and 3% bovine serum albumin (BSA) for 15 min at RT, respec-

tively. Tissue sections were then incubated with primary rabbit polyclonal anti-SATB1 antibody (1:200), primary rabbit polyclonal anti-SMAD3 antibody (1:500), primary rabbit polyclonal anti-Ezrin antibody (1:100) and primary rabbit monoclonal anti-β-catenin antibody (1:100) for 30 min at RT. Finally, slides were incubated with EnVisionFlex+ Anti-Mouse/Rabbit HRP-Labeled Polymer (Dako) for 20 min. The staining signal was developed with 3,3′diaminobenzidine (DAB) solution. Tissues sections were counterstained with hematoxylin and dehydrated in ethanol of increasing concentration (80, 90, 96, 99.8%), then cleared in a series of xylenes (from I to IV). Finally, the slides were sealed with Dako mounting medium.

2.3. Evaluation of Immunohistochemistry Staining

The IHC evaluation of protein expression was performed by two independent pathologists in the light ECLIPSE E400 microscope (Nikon Instruments Europe, Amsterdam, Netherlands) at 20× original objective magnification. Each core was evaluated randomly in three areas with obvious lesions.

The immunoexpression of studied proteins was evaluated according to the modified Index Remmele–Stegner (IRS) by multiplying the percentage of positively stained cells/areas and staining intensity. The percentage of positive cells/area was scored in the following way: (0)—less than 10%, (1)—11–20%, (2)—21–50%, (3)—51–80% and (4)—equal or more than 81%, and the intensity of the stain was scored using the following criteria: (0)—negative; (1)—weak staining; (2)—moderate staining; and (3)—strong staining. The final staining result, ranging from 0 to 12, was divided into two expression groups based on a specific discrimination threshold set by the Evaluate Cutpoints software [26]. The cut-off values for high and low expression of nuclear SATB1 ($SATB1^{n}$), SMAD3, Ezrin and β-catenin were as follows: <1; ≥1, <8; ≥8, <8; ≥8, <4; ≥4, respectively. Cytoplasmic SATB1 ($SATB1^{c}$) expression was scored as positive or negative based on the presence (+) or absence (−) of cytoplasmic tumor cell staining. Combined prognostic values of candidate biomarkers were evaluated in the following expression groups: cases with the co-expression of $SATB1^{n\text{-}low}SMAD3^{high}Ezrin^{high}$β-$catenin^{high}$ were analyzed in comparison with the opposite expression pattern ($SATB1^{n\text{-}high}SMAD3^{low}Ezrin^{low}$β-$catenin^{low}$), whereas "other" defined all else expressive cases (the reference group); a similar scheme was followed for the co-expression of $SATB1^{c\text{-}present}SMAD3^{high}Ezrin^{high}$β-$catenin^{high}$.

2.4. In Silico Analysis

To support our results and confront them with online available data, we additionally assessed *SATB1*, *SMAD3*, *EZR* and *CTNNB1* expression in the TCGA cohort. Gene expression data for The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) cohorts from 177 patients with pancreatic adenocarcinoma (146 with ductal histology) and 165 of non-cancerous pancreatic tissues were obtained from www.cBioPortal.org and UCSC Xena Viewer (http://xena.ucsc.edu/, accessed on 3 July 2020). The RNA sequencing datasets (RNA-seq) were normalized via DESeq2 normalization, and data were divided into low- and high-expression groups according to the cutpoints established in Evaluate Cutpoints software. The cut-off values for positive (high) and negative (low) expression of *SATB1*, *SMAD3*, *EZR*, *CTNNB1* mRNA were as follows: <10.99; ≥10.99, <12.67; ≥12.67, <14.35; ≥14.35, <14.68; ≥14.68, respectively. We created the expression combination of the factors tested. Cases with the co-expression of $SATB1^{low}SMAD3^{high}EZR^{high}CTNNB1^{high}$ were analyzed in comparison with the opposite expression pattern, whereas "other" defined all else expressive cases.

2.5. Protein-Protein Interaction (PPI) Network Construction and Functional Enrichment Analysis

The 50 neighboring genes most relevant to the queried genes (*CTNNB1*, *EZR*, *SATB1* and *SMAD3*) were filtered through the Search Tool for the Retrieval of Interacting Genes (STRING) database (https://string-db.org, accessed on 14 September 2021) and further used to construct a network map, which was visualized via Cytoscape software version

3.8.2. The cut-off criteria to construct the protein–protein interaction (PPI) network was set as confidence score ≥0.4 and the maximum number of interactors = 50. The most significant modules in the PPI network were identified using the Cytoscape plugin Molecular Complex Detection (MCODE) [27]. The screening options were set as degree cut-off = 2, max. depth = 100, k-core = 2 and node score cut-off = 0.2. The top 10 genes in the network were screened as hub genes using the degree method with the CytoHubba plugin [28] in Cytoscape software. Pathway analysis and visualization were performed using the Reactome Pathway database (https://reactome.org, accessed on 14 September 2021) [29]. To find KEGG Pathway and Gene Ontology (GO) categories (cellular component (CC), biological process (BP) and molecular function (MF)) shared by the analyzed genes, the Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov, accessed on 14 September 2021) [30] was applied. The KEGG BRITE (https://www.genome.jp/kegg/brite.html, accessed on 14 September 2021) [31] was utilized to explore functional hierarchies of the imputed genes.

2.6. Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 7.01 (GraphPad Software, La Jolla, CA, USA) and SPSS version 26.0 software (IBM Corporation, Armonk, NY, USA). The Mann–Whitney test was used to compare continuous variables, and Fisher's exact or Chi-squared tests were used to compare the categorical variables. To assess the correlations between the expression of SATB1, SMAD3, Ezrin/*EZR* and β-catenin/*CTNNB1*, Spearman's correlation coefficient was employed. Survival outcomes were assessed using the Kaplan–Meier method, and differences were evaluated using the log-rank test. Univariate and multivariate survival analyses were performed using the Cox proportional hazard regression model. Additionally, 95% confidence intervals (95% CIs) and the hazard ratios (HRs) were calculated. Variables that had a significant relation to OS in the univariate analysis were introduced into the multivariate Cox regression model. The p value < 0.05 was considered statistically significant.

3. Results

3.1. Comparison of Protein Expression in Cancer and Normal Tissue and Its Relation to Clinicopathological Characteristics in Our Cohort

The study included the analysis of the expression of four proteins—SATB1, SMAD3, Ezrin and β-catenin. Representative images of IHC staining are presented in Figure 1A–J. Comparison of SATB1^{n} expression between PDAC and healthy tissue margin indicated a statistically significant reduction in PDAC positive samples ($p < 0.0001$; Figure 2A). Interestingly, in the case of cancer tissue, in 21 cases (30.88%), cytoplasmic labeling was also present, which was not observed in any of the control samples ($p < 0.0001$; Figure 2B). Fifty-two samples (76.47%) of tumor tissue were characterized by low and 16 (23.53%) by high SATB1^{n} expression. SATB1^{n} did not correlate with histological type, age, stage, pT and pN status. However, we found its correlation with histological grade. Low SATB1^{n} expression was observed in 100% of G1 tumors ($n = 5$), 78.18% of G2 ($n = 55$) tumors and 50% of G3 tumors ($n = 8$), and these differences were statistically significant ($p = 0.03$). SATB1^{c} did not correlate with histological type, age, histological grade, stage or pT and pN status (Table 1).

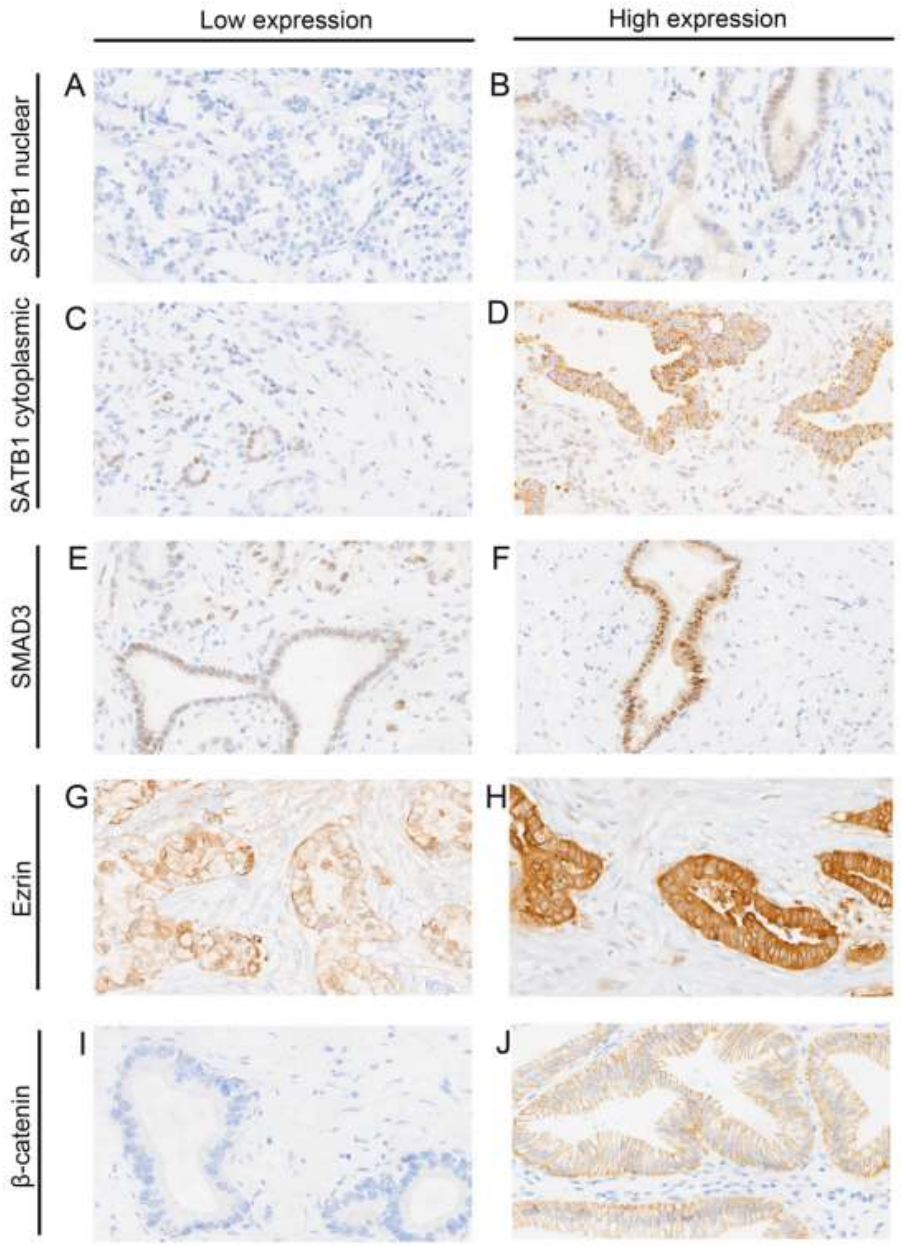

Figure 1. Representative images of immunohistochemical expression of nuclear SATB1 (**A**,**B**), cytoplasmic SATB1 (**C**,**D**), SMAD3 (**E**,**F**), Ezrin (**G**,**H**) and β-catenin (**I**,**J**) in pancreatic ductal adenocarcinoma. Primary magnification ×20.

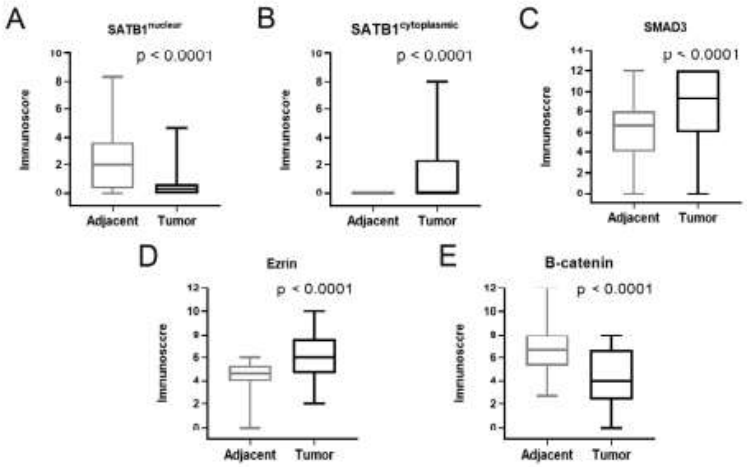

Figure 2. Comparison of the immunohistochemical expression of nuclear SATB1 (**A**), cytoplasmic SATB1(**B**), SMAD3 (**C**), EZR (**D**) and β-catenin (**E**) in tumor and adjacent tissues of PDAC patients.

Considering the available literature data and the obtained results regarding SMAD3, only its nuclear expression pattern was included. For both normal tissue margin and tumor samples, SMAD3 expression was confirmed in most of the cases (96.88% for control and 98.53% for tumor specimens). However, its level was significantly increased in the case of PDAC samples compared to the adjacent tissue ($p < 0.0001$; Figure 2C). The adopted division allowed for the determination of 26 samples (38.24%) with low and 42 (61.77%) with high expression. The expression of SMAD3 did not correlate with histological type, histological grade, age, stage or pT and pN status (Table 1).

In the case of Ezrin, the dominant expression pattern for tumor samples was membranous-cytoplasmic ($n = 55$, 80.88%). Cytoplasmic without membrane labeling was visible for 13 samples (19.12%). In contrast, in normal tissue, the membrane-cytoplasmic pattern was observed only for 28 specimens ($n = 28$, 45.16%), while the most common was the cytoplasmic one (100%). As shown in Figure 2D, compared to the adjacent tissue, tumor samples were characterized by significantly increased expression of Ezrin ($p < 0.0001$). In addition, overexpression of the protein was observed in 15 samples (22.06%), while low expression was for 53 (77.94%). The expression of Ezrin did not correlate with histological type, histological grade, age, stage and pN status. However, we found its correlation with pT status ($p = 0.048$; Table 1). Ezrin overexpression was markedly more frequent in the T3-T4 group ($n = 5$, 50.00%) than T1-T2 ($n = 10$, 18.87%).

For β-catenin, the membrane expression pattern was assessed. In normal tissue, membrane expression was observed in all (100%) samples. In turn, for tumor tissue, membrane expression was present for 61 (89.71%) cases. Comparison between control and tumor tissues showed a statistically significant decrease in the β-catenin expression in PDAC specimens ($p < 0.0001$; Figure 2E). Twenty-five cancer tissue specimens were characterized by low (36.77%) and 43 by high membrane expression (63.24%). The expression of β-catenin did not correlate with histological type, histological grade, age, stage or pT and pN status (Table 1).

Table 1. Immunohistochemical expression of SATB1^{n}, SATB1^{c}, SMAD3, Ezrin and β-catenin proteins and their relationship with clinicopathological features of PDAC patients.

Variables	*n* (%)	SATB1 Nuclear Expression		*p* Value	SATB1 Cytoplasmic Expression		*p* Value	SMAD3		*p* Value	Ezrin		*p* Value	β-Catenin		*p* Value
		Low *n* = 52	High *n* = 16		Absent *n* = 47	Present *n* = 21		Low *n* = 26	High *n* = 42		Low *n* = 16	High *n* = 52		Low *n* = 25	High *n* = 43	
Age (years)																
≤60	29 (42.65)	22 (75.86)	7 (24.14)	>0.99	20 (68.97)	9 (31.03)	>0.99	12 (41.38)	17 (58.62)	0.8	24 (87.76)	5 (17.24)	0.56	11 (37.93)	18 (62.07)	>0.99
>60	39 (57.35)	30 (76.92)	9 (23.08)		27 (69.23)	12 (30.77)		14 (35.9)	25 (64.1)		29 (74.36)	10 (25.64)		14(35.90)	25 (64.10)	
Gender																
Male	34 (50.00)	27 (79.41)	7 (20.59)	0.78	22 (64.71)	12 (35.29)	0.6	13 (38.24)	21 (61.76)	>0.99	25 (80.65)	6 (19.35)	0.77	11 (32.35)	23 (67.65)	0.62
Female	34 (50.00)	25 (73.53)	9 (26.47)		25 (73.53)	9 (26.47)		13 (38.24)	21 (61.76)		28 (75.68)	9 (24.32)		14 (41.18)	20 (58.82)	
Grading																
G1	5 (7.35)	5 (100.0)	0 (0.00)		4 (80.00)	1 (20.00)		2 (40.00)	3 (60.00)		3 (60.00)	2 (40.00)		1 (20.00)	4 (80.00)	
G2	55 (80.88)	43 (78.18)	12 (21.82)	**0.03**	39 (70.91)	16 (29.09)	0.42	18 (32.73)	37 (67.27)	0.1	43 (78.18)	12 (21.82)	0.30	21 (38.18)	34 (61.82)	0.60
G3	8 (11.77)	4 (50.00)	4 (50.00)		4 (50.00)	4 (50.00)		6 (75.00)	2 (25.00)		7 (87.5)	1 (12.5)		3 (37.50)	5 (62.50)	
pT status																
T1-T2	53 (84.13)	39 (73.58)	14 (26.42)	>0.99	22 (62.26)	20 (37.74)	0.09	18 (33.96)	35 (66.04)	0.16	43 (81.13)	10 (18.87)	0.048	17(32.08)	36 (67.92)	>0.72
T3-T4	10 (15.87)	8 (80.00)	2 (20.00)		9 (90.00)	1 (10.00)		6 (60.00)	4 (40.00)		5 (50.00)	5 (50.00)		4(40.00)	6 (60.00)	
pN status																
N0	30 (45.46)	24 (80.00)	6 (20.00)	0.57	23 (76.67)	7 (23.33)	0.42	14 (46.67)	16 (53.33)	0.32	23 (76.67)	7 (23.33)	0.55	12 (40.00)	18 (60.00)	0.80
N1-N2	36 (54.54)	26 (72.22)	10 (27.78)		24 (66.67)	12 (33.33)		12 (33.33)	24 (66.67)		30 (83.33)	6 (16.67)		13 (36.11)	23 (63.89)	
TNM stage																
I	24 (38.71)	20 (83.33)	4 (16.67)		17 (70.83)	7 (29.17)		9 (37.5)	15 (62.5)		20 (83.33)	4 (16.67)		8 (33.33)	16 (66.67)	
II	24 (38.71)	14 (58.33)	10 (41.67)	0.08	19 (79.17)	5 (20.83)	0.17	11 (45.83)	13 (54.17)	0.78	19 (79.17)	5 (20.83)	0.39	11 (45.83)	13 (54.17)	0.30
III-IV	14 (22.58)	12 (85.71)	2 (14.29)		7 (50.00)	7 (50.00)		5 (35.71)	9 (64.29)		10 (74.43)	4 (28.57)		3(21.43)	11 (78.57)	
Location																
head	60 (88.24)	46 (76.67)	14 (23.33)	>0.99	42 (70.00)	18 (30.00)	0.70	23 (38.33)	37 (61.67)	>0.99	48 (80.00)	12 (20.00)	0.36	24 (40.00)	36 (60.00)	0.24
body and tail	8 (11.77)	6 (75.00)	2 (25.00)		5 (62.50)	3 (37.50)		3 (37.50)	5 (62.50)		5 (62.50)	3 (37.50)		1 (12.50)	7 (87.50)	
PNI																
Absent	22 (34.38)	19 (86.36)	3 (13.64)	0.22	17 (77.27)	5 (22.73)	0.57	9 (40.91)	13 (59.1)	0.79	16 (72.73)	6 (27.27)	0.34	7 (31.82)	15 (68.18)	0.43
Present	42 (65.62)	29 (69.05)	13 (30.95)		28 (66.67)	14 (33.33)		15 (35.71)	27 (64.29)		35 (83.33)	7 (16.67)		18 (42.86)	24 (57.14)	
LVI																
Absent	39 (62.90)	28 (71.79)	11 (28.21)	0.77	30 (76.92)	9 (23.08)	0.25	18 (46.15)	21 (53.85)	0.18	28 (71.80)	11 (28.20)	0.11	17 (43.59)	22 (56.41)	0.42
Present	23 (37.10)	18 (78.26)	5 (21.74)		14 (60.87)	9 (39.13)		6 (26.09)	17 (73.91)		21 (91.30)	2 (8.70)		7 (30.43)	16 (69.57)	

Significant *p* values ($p < 0.05$) are marked in bold. Abbreviations: LVI—vascular invasion; PNI—perineural invasion.

3.2. Association between the Protein Expression and Patient Survival in Own Cohort

Next, we constructed Kaplan–Meier curves to determine the impact of individual expression patterns of the studied proteins on the overall survival (OS) of PDAC patients. A suggestive association was observed between SATB1^{n} expression and OS, but without statistical significance (297 days vs. 561 days, $p = 0.118$; Figure 3A). Notably, the presence of SATB1^{c} expression in PDAC was associated with a significantly shorter OS rate (median OS: 117 days) than the absence of SATB1^{c} expression (median OS: 458 days; $p = 0.036$; Figure 3B). Combining these two staining patterns of SATB1 did not improve the survival prediction (Figure S1, Tables S1 and S2). Furthermore, Kaplan–Meier survival analysis indicated that PDAC patients with a high level of SMAD3 expression (median OS: 290 days) had lower OS rates ($p = 0.004$) than those with SMAD3 low expression level (median OS: 531 days, Figure 3C). Analysis of patient survival data also showed that low Ezrin expression correlated with a higher OS rate (median OS: 450 days) than high expression (median OS:118 days). This survival difference was of borderline significance ($p = 0.055$, Figure 3D). Low β-catenin expression tended to be associated with longer survival (median OS: 450 days) than high (median OS: 274 days; $p = 0.088$, Figure 3E).

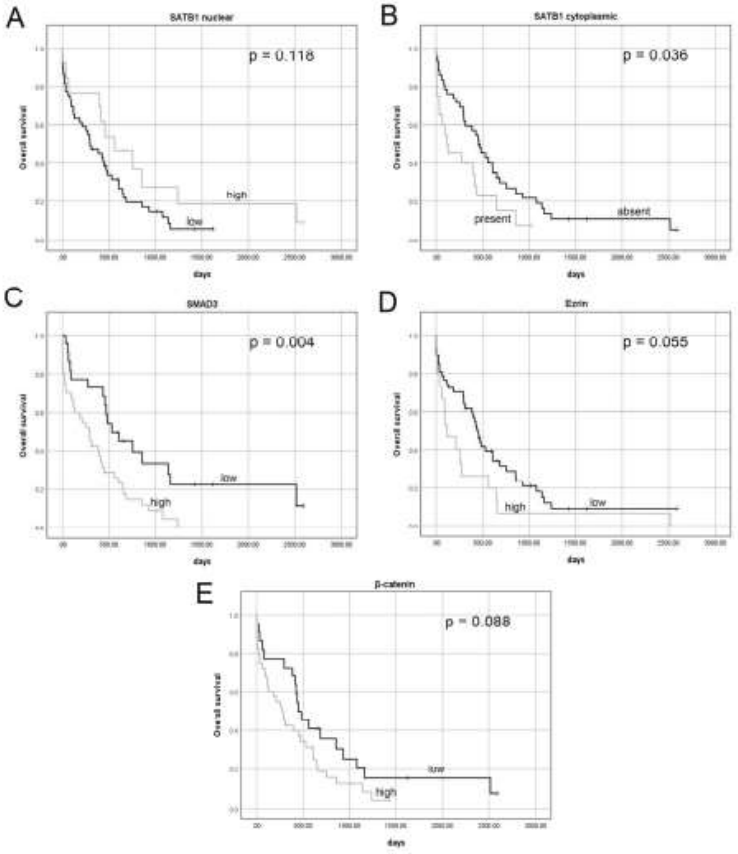

Figure 3. Kaplan–Meier survival curves stratified by SATB1^{n} (**A**), SATB1^{c} (**B**), SMAD3 (**C**), Ezrin (**D**) and β-catenin (**E**) expression in pancreatic adenocarcinoma. Protein expression was measured using immunohistochemistry, and the calculated IRS scores were divided into two categories as specified in the Material and methods.

3.3. Significance of Assessed Characteristics as Prognostic Factors in Own Cohort

To further examine the prognostic value of the studied proteins as single indicators, univariate and multivariate survival analyses were performed using the Cox proportional hazard regression model. The univariate Cox analysis pointed to age ($p = 0.04$), TNM stage ($p = 0.03$), vascular invasion ($p = 0.01$), SATB1^c ($p = 0.039$) and SMAD3 ($p = 0.005$) as significant prognostic factors for OS in PDAC patients. In turn, Ezrin expression reached borderline significance ($p = 0.06$). After adjusting for classical prognostic factors using the multivariate Cox regression analysis, the strength of this association increased and was statistically significant ($p = 0.01$). Furthermore, the multivariate analysis confirmed the significance of high SMAD3 expression (HR 3.08, 95% CI 1.52–6.23, $p = 0.002$) as an independent negative prognostic factor for PDAC patients. Moreover, SATB1^c expression was indicated as an independent poor prognostic factor of near significance (HR 1.86, 95% CI 0.93–3.74, $p = 0.08$) after adjustment for age, TNM stage and LVI. The results of univariate and multivariate Cox analyses are shown in Tables 2 and 3, respectively.

Table 2. Univariate analysis of prognostic factors by Cox proportional hazard model in own cohort.

Univariate Analysis of Own Cohort				
Variable	**HR**	**95% CI**		***p* Value**
		Lower	**Upper**	
SATB1^n	1.73	0.86	3.47	0.12
SATB1^c	1.87	1.03	3.40	**0.039**
SMAD3	2.40	1.31	4.40	**0.005**
Ezrin	1.80	0.98	3.30	0.06
β-catenin	1.64	0.92	2.92	0.09
age	1.03	1.00	1.07	**0.04**
gender	0.96	0.56	1.65	0.88
grading	0.95	0.37	2.41	0.91
pN	1.30	0.74	2.27	0.36
pT	1.09	0.53	2.27	0.81
TNM stage	2.08	1.09	3.98	**0.03**
PNI	1.58	0.89	2.82	0.12
LVI	2.16	1.17	3.96	**0.01**

Significant p values ($p < 0.05$) are marked in bold. Abbreviations: CI, confidence interval; HR, hazard ratio; LVI—vascular invasion; PNI—perineural invasion.

3.4. Correlation between the Expression of SATB1, SMAD3, Ezrin and β-Catenin in Own Cohort

A weak negative and significant association was found between the expression of β-catenin and SATB1^n ($r = -0.291$, $p = 0.015$). In addition, a weak positive and significant association was revealed between the expression of β-catenin and SMAD3 ($r = 0.251$, $p = 0.039$). Furthermore, moderate positive and significant association was confirmed between the expression of β-catenin and SATB1^c ($r = 0.321$, $p = 0.007$). A weak positive association was also noted between the expression of SATB1^c and SMAD3, but this correlation did not reach statistical significance ($r = 0.214$, $p = 0.08$).

Table 3. Multivariate analysis of prognostic factors by Cox proportional hazard model in own cohort.

Multivariate Analysis of Own Cohort													
Variable	**HR**	**95% CI**		***p* Value**	**Variable**	**HR**	**95% CI**		***p* Value**	**Variable**	**HR**	**95% CI**		***p* Value**
		Lower	**Upper**				**Lower**	**Upper**				**Lower**	**Upper**	
SATB1[n]	1.19	0.56	2.55	0.66	SATB1[c]	1.86	0.93	3.74	0.08	SMAD3	3.08	1.52	6.23	**0.002**
age	1.04	1.00	1.08	**0.03**	age	1.05	1.01	1.10	**0.015**	age	1.05	1.01	1.09	**0.024**
TNM stage	2.28	1.09	4.74	**0.03**	TNM stage	2.46	1.22	4.95	**0.012**	TNM stage	2.79	1.38	5.67	**0.005**
LVI	2.59	1.33	5.02	**0.005**	LVI	2.76	1.40	5.43	**0.003**	LVI	2.71	1.38	5.30	**0.004**
Variable	**HR**	**95% CI**		***p* Value**	**Variable**	**HR**	**95% CI**		***p* Value**					
		Lower	**Upper**				**Lower**	**Upper**						
Ezrin	2.70	1.25	5.85	**0.011**	β-catenin	1.69	0.86	3.32	0.13					
age	1.05	1.01	1.09	**0.013**	age	1.05	1.01	1.09	**0.016**					
TNM stage	2.11	1.04	4.27	**0.038**	TNM stage	2.32	1.16	4.64	**0.017**					
LVI	3.55	1.74	7.26	**0.001**	LVI	2.64	1.35	5.15	**0.004**					

Significant *p* values ($p < 0.05$) are marked in bold. Abbreviations: CI, confidence interval; HR, hazard ratio; LVI—vascular invasion. HR: adjusting for age, TNM stage, VI.

3.5. Comparison of mRNA Expression in Cancer and Normal Tissue and Its Relation to Clinicopathological Characteristics in TCGA Cohort

In 177 PAC, based on the established cutpoint, high *SATB1* mRNA expression of *SATB1* mRNA was observed for 106 cases (59.89%). There was no significant difference in the expression levels of SATB1 between PAC and normal pancreatic tissues ($p = 0.9694$; Figure 4A). *SATB1* level was dependent on pN status ($p = 0.026$). In the case of *SMAD3* more samples were characterized by high expression ($n = 103$, 58.19%) than low ($n = 74$, 41.81%). The expression of *SMAD3* mRNA was significantly higher in PAC tissues compared to normal pancreatic tissues ($p < 0.0001$; Figure 4B). *SMAD3* expression correlated with grading ($p = 0.012$), pT status ($p = 0.026$) and TMN stage ($p = 0.008$). High *EZR* mRNA levels were found in 145 (81.92%) PAC cases, whereas the remaining 32 (18.08%) had low levels of this marker. The expression of *EZR* mRNA in PAC tissues was significantly up-regulated as compared with normal pancreatic tissues ($p < 0.0001$; Figure 4C). *EZR* level was dependent on grading ($p = 0.02$), pT status ($p = 0.0157$) and TNM stage ($p = 0.0007$). More of the patients were characterized by high *CTNNB1* expression ($n = 120$, 67.80%) than low ($n = 57$; 32.20%). Additionally, in the case of *CTNNB1*, its mRNA levels were significantly higher in PAC tissues compared to normal pancreatic tissues ($p < 0.0001$; Figure 4D). The analysis showed statistically significant associations between *CTNNB1* expression and grading ($p = 0.001$), pT status ($p = 0.009$), pN status ($p = 0.0002$) and TMN stage ($p = 0.0001$). The described results are presented in Table 4.

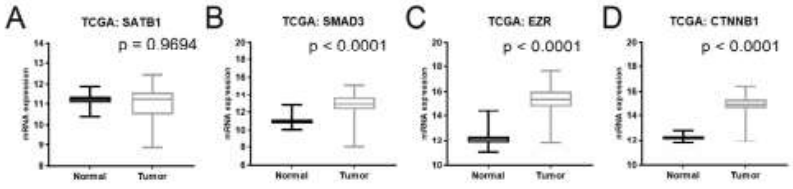

Figure 4. Comparison of mRNA expression levels of SATB1 (**A**), SMAD3 (**B**), Ezrin (**C**) and CTNNB1 (**D**) in tumor and normal tissues in PAC patients of TCGA cohort.

Table 4. mRNA expression and its relationship with clinicopathological features of PAC patients in TCGA cohort.

Variables	*n* (%)	SATB1 Expression		*p* Value	SMAD3 Expression		*p* Value	EZR Expression		*p* Value	CTNNB1 Expression		*p* Value
		Low *n* = 71	High *n* = 106		Low *n* = 74	High *n* = 103		Low *n* = 32	High *n* = 145		Low *n* = 57	High *n* = 120	
Age (years)													
≤60	59 (33.33)	20 (33.90)	39 (66.10)	0.26	22 (37.29)	37 (62.71)	0.42	11 (18.64)	48 (81.36)	>0.99	15 (25.41)	44 (74.58)	0.23
>60	118 (66.67)	51 (43.22)	67 (56.78)		52 (44.07)	66 (55.93)		21 (17.80)	97 (82.20)		42 (35.59)	76 (64.41)	
Gender													
Male	97 (54.80)	40 (41.24)	57 (58.76)	0.76	41 (42.27)	56 (57.73)	>0.99	20 (20.62)	77 (79.38)	0.43	30 (30.93)	67 (69.07)	0.75
Female	80 (45.20)	31 (38.75)	49 (61.25)		33 (41.25)	47 (58.75)		12 (15.00)	68 (85.00)		27 (33.75)	53 (66.25)	
Grading													
G1	31 (17.51)	13 (41.94)	18 (58.06)	0.93	19 (61.29)	12 (38.71)	**0.012**	11 (35.48)	20 (64.52)	**0.020**	18 (58.06)	13 (41.94)	**0.001**
G2	94 (53.12)	38 (40.43)	56 (59.57)		40 (42.55)	54 (57.45)		15 (15.96)	79 (84.04)		27 (28.72)	67 (71.28)	
G3-G4	50 (28.25)	19 (38.00)	31 (62.00)		14 (28.00)	36 (72.00)		6 (12.00)	44 (88.00)		10 (20.00)	40 (80.00)	
pT status													
T1-T2	30 (16.95)	12 (40.00)	18 (60.00)	>0.99	18 (60.00)	12 (40.00)	**0.026**	10 (33.33)	20 (66.67)	**0.0157**	16 (53.33)	14 (46.67)	**0.009**
T3-T4	145 (81.92)	57 (39.31)	88 (60.69)		54 (37.24)	91 (62.76)		20 (13.79)	125 (86.21)		39 (26.90)	106 (73.10)	
pN status													
N0	49 (27.68)	26 (53.06)	42 (85.71)	**0.026**	26 (53.06)	23 (46.94)	0.06	12 (24.49)	37 (75.51)	0.11	26 (53.06)	23 (46.94)	**0.0002**
N1	123 (63.84)	42 (34.15)	81 (65.85)		45 (36.59)	78 (63.41)		17 (13.82)	106 (86.18)		28 (22.76)	95 (77.24)	
TNM stage													
I	21 (11.86)	10 (47.61)	11 (52.38)	0.56	15 (71.43)	6 (28.57)	**0.008**	9 (42.86)	12 (57.14)	**0.0007**	15 (71.43)	6 (28.57)	**0.0001**
II	146 (82.49)	55 (37.67)	91 (62.33)		53 (36.30)	93 (63.70)		18 (12.33)	128 (87.67)		38 (26.03)	108 (73.97)	
III-IV	8 (4.52)	4 (50.00)	4 (50.00)		4 (50.00)	4 (50.00)		3 (37.50)	5 (62.50)		2 (25.00)	6 (75.00)	

Significant *p* values ($p < 0.05$) are marked in bold.

3.6. Association between themRNA Expression and Patient Survival in TCGA Cohort

Survival analysis of the TCGA cohort showed that *SATB1* expression was not significantly correlated with OS (median OS for low vs. high expression = 517 vs. 695, $p = 0.123$). While for *SMAD3*, *EZR* and *CTNNB1*, high expression was connected with a poorer survival rate (median OS for high vs. low expression = 498 vs.1332, 592 vs. undefined and 592 vs. 2182, respectively). Except for *SATB1*, all the results were statistically significant ($p < 0.0001$ for *SMAD3* and *EZR*, and $p = 0.0001$ for *CTNNB1*). Kaplan–Meier survival curves are presented in Figure 5.

3.7. Significance of Assessed Characteristics as Prognostic Factors in TCGA Cohort

The univariate Cox analysis showed that *SMAD3* ($p < 0.0001$), *EZR* ($p < 0.0001$) and *CTNNB1* ($p = 0.002$) expression levels were prognostic factors for OS of statistical significance, as well as age ($p = 0.01$), grading ($p = 0.02$), pN ($p = 0.01$) and pT status ($p = 0.02$). In turn, the multivariate analysis pointed to *SATB1* ($p = 0.002$), *SMAD3* ($p = 0.05$) and *EZR* ($p = 0.033$) expression as independent prognostic factors for OS. The obtained results are presented in Tables 5 and 6, respectively.

3.8. Correlation between the Expression of SATB1, SMAD3, Ezrin and CTNNB1 in TCGA Cohort

Spearman correlation coefficient statistics uncovered strong positive correlations between *SMAD3* and *EZR* ($r = 0.757$; $p < 0.0001$) as well as between *CTNNB1* and *SMAD3* ($r = 0.606$, $p = 0.0049$). Moreover, a moderate positive and significant association was found between the expression of *CTNNB1* and *EZR* ($r = 0.512$, $p < 0.0001$). A weak positive and significant association was also confirmed between the expression of *SATB1* and *CTNNB1* ($r = 0.244$, $p = 0.001$). There were no other connections between studied mRNAs.

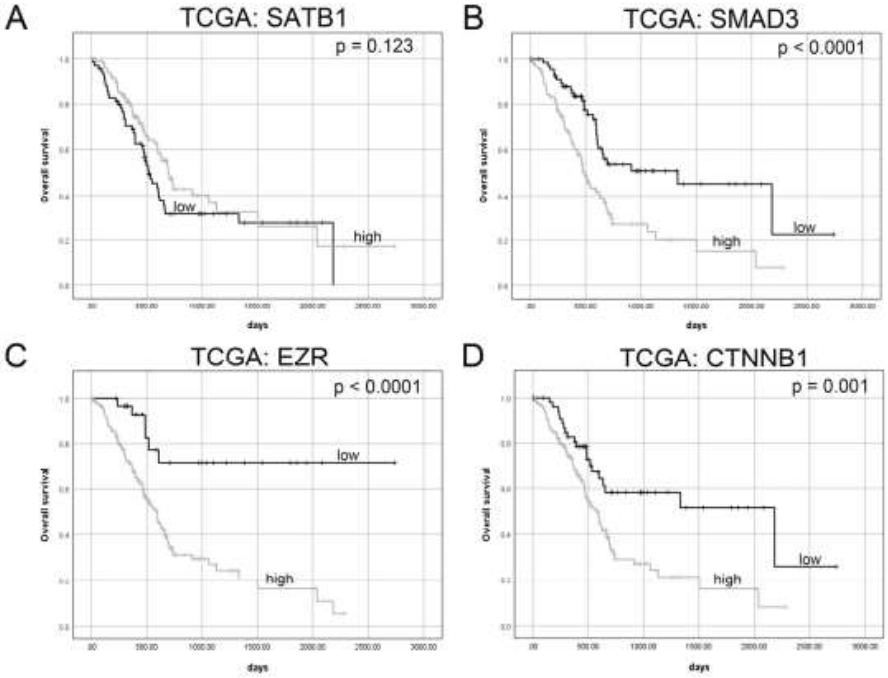

Figure 5. Kaplan–Meier survival curves stratified by SATB1 (**A**), SMAD3 (**B**), EZR (**C**) and CTNNB1 (**D**) mRNA levels in TCGA cohort.

Table 5. Univariate analysis of prognostic factors by Cox proportional hazard model in TCGA cohort.

	Univariate Analysis of TCGA Cohort			
Variable	**HR**	**95% CI**		***p* Value**
		Lower	**Upper**	
SATB1	0.73	0.48	1.09	0.12
SMAD3	2.26	1.45	3.54	**<0.0001**
EZR	4.65	2.02	10.69	**<0.0001**
CTNNB1	2.21	1.34	3.65	**0.002**
age	1.03	1.01	1.05	**0.01**
gender	0.81	0.54	1.23	0.33
grading	2.18	1.15	4.13	**0.02**
pN	2.10	1.25	3.52	**0.01**
pT	2.21	1.14	4.28	**0.02**
TNM stage	0.74	0.23	2.34	0.60

Significant p values ($p < 0.05$) are marked in bold. Abbreviations: CI, confidence interval; HR, hazard ratio; TCGA—The Cancer Genome Atlas.

Table 6. Multivariate analysis of prognostic factors by Cox proportional hazard model in TCGA cohort.

Multivariate Analysis of TCGA Cohort									
Variable	HR	95% CI		*p* Value	Variable	HR	95% CI		*p* Value
		Lower	Upper				Lower	Upper	
SATB1	0.50	0.32	0.78	**0.002**	SMAD3	1.57	0.99	2.49	**0.05**
age	1.02	1.00	1.04	0.09	age	1.02	1.00	1.04	**0.04**
grade	1.76	0.92	3.37	0.09	grade	1.51	0.79	2.89	0.21
pN	2.53	1.42	4.52	**0.0016**	pN	1.77	1.02	3.09	**0.04**
pT	1.32	0.64	2.72	0.46	pT	1.43	0.70	2.92	0.32
Variable	HR	95% CI		*p* Value	Variable	HR	95% CI		*p* Value
		Lower	Upper				Lower	Upper	
EZR	2.50	1.07	5.83	**0.033**	CTNNB1	1.42	0.83	2.45	0.20
age	1.02	1.00	1.04	**0.05**	age	1.02	1.00	1.04	**0.04**
grade	1.43	0.75	2.74	0.28	grade	1.54	0.81	2.94	0.19
pN	1.83	1.06	3.17	**0.030**	pN	1.76	0.98	3.14	0.06
pT	1.32	0.65	2.69	0.45	pT	1.39	0.68	2.86	0.37

Significant *p* values ($p < 0.05$) are marked in bold. Abbreviations: CI, confidence interval; HR, hazard ratio; TCGA—The Cancer Genome Atlas. HR: adjusting for age, grade, pN and pT.

3.9. Overall Survival Analysis According to the Combined Biomarker Expression

Following the establishment of the significance of the investigated factors as single prognostic markers, we also examined the effect of their combined expression on OS in both our cohort and the TCGA cohort. Kaplan–Meier analysis revealed that the worst OS was observed in patients whose PDACs co-expressed SATB1$^{c\text{-present}}$SMAD3^{high}Ezrin^{high}β-catenin^{high}. In turn, patients whose PDACs had opposite expression profiles of these proteins had significantly longer OS (8 vs. 1160 days; $p = 0.001$; Figure 6B). In univariate (HR 3.06, 95% CI 1.27–7.36, $p = 0.01$; Table 7B) and multivariate (HR 7.32, 95% CI 2.05–26.21, $p = 0.002$; Table 8B) analyses, the combined SATB1$^{c\text{-present}}$SMAD3^{high}Ezrin^{high}β-catenin^{high} was a significant poor prognostic factor for OS with a particularly high hazard ratio when compared to each marker as a single indicator. Furthermore, SATB1^{n}/SMAD3/Ezrin/β-catenin expression panel was significantly related to the duration of OS, with patients in the SATB1$^{n\text{-high}}$SMAD3^{low}Ezrin^{low}β-catenin^{low} expression group experiencing the longest survival (median overall survival not reached), whereas the opposite pattern of the expression panel predicted a poor survival (118 days, $p = 0.023$; Figure 6A). The SATB1$^{n\text{-high}}$SMAD3^{low}Ezrin^{low}β-catenin^{low} expression group was associated with a reduced risk of death by 89% after adjusting for age, tumor stage and LVI (HR 0.11, 95% CI 0.01–0.93, $p = 0.004$; Table 8A). In turn, in univariate and multivariate analysis, the crude and adjusted HRs were 2.16 (95% CI 1.03–4.52, $p = 0.04$) and 2.91 (95% CI 1.07–7.91, $p = 0.04$), respectively, for SATB1$^{n\text{-low}}$/SMAD3^{high}/Ezrin^{high}/β-catenin^{high} expression group (Tables 7A and 8A).

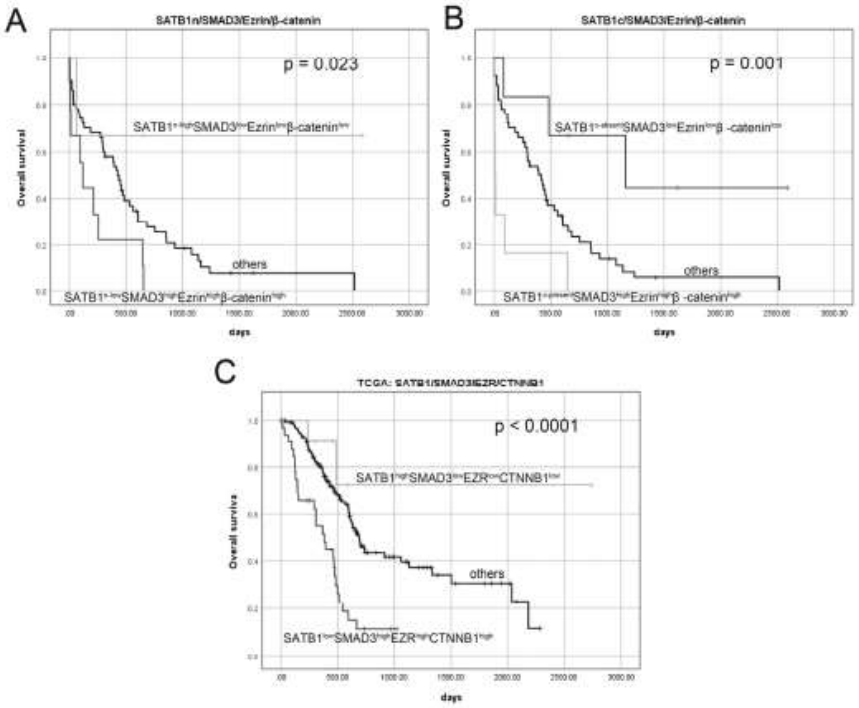

Figure 6. Overall survival analysis according to the combination of the panel (**A**) SATB1^{n}/SMAD3/Ezrin/β-catenin, (**B**) SATB1^{c}/SMAD3/Ezrin/β-catenin and (**C**) TCGA: SATB1/SMAD3/EZR/CTNNB1.

Table 7. Univariate analysis of prognostic factors by the Cox proportional hazard model for combined expression.

(A) Univariate Analysis of Own Cohort: SATB1^{n}/SMAD3/Ezrin/β-Catenin				
Variable	**HR**	**95% CI**		***p* Value**
		Lower	**Upper**	
Others		Ref.		
SATB1$^{n\text{-high}}$SMAD3^{low}Ezrin^{low}β-catenin^{low}	0.21	0.03	1.60	0.13
SATB1$^{n\text{-low}}$SMAD3^{high}Ezrin^{high}β-catenin^{high}	2.16	1.03	4.52	**0.04**
(B) Univariate Analysis of Own Cohort:SATB1^{c}/SMAD3 /Ezrin/β-Catenin				
Variable	**HR**	**95% CI**		***p* Value**
		Lower	**Upper**	
Others		Ref.		
SATB1$^{c\text{-present}}$SMAD3^{high}Ezrin^{high}β-catenin^{high}	3.06	1.27	7.36	**0.01**
SATB1$^{c\text{-absent}}$SMAD3^{low}Ezrin^{low}β-catenin^{low}	0.26	0.08	0.86	**0.03**

Table 7. *Cont.*

(C) Univariate Analysis of TCGA Cohort: SATB1/SMAD3/EZR/CTNNB1				
Variable	**HR**	**95% CI**		***p* Value**
		Lower	**Upper**	
Others		Ref.		
$SATB1^{low}SMAD3^{high}EZR^{high}CTNNB1^{high}$	2.97	1.863	4.745	**<0.0001**
$SATB1^{high}SMAD3^{low}EZR^{low}CTNNB1^{low}$	0.35	0.084	1.420	0.14

Significant *p* values ($p < 0.05$) are marked in bold. Abbreviations: CI, confidence interval; HR, hazard ratio; TCGA—The Cancer Genome Atlas. (A) Univariate analysis of own cohort: $SATB1^{n}$/SMAD3/Ezrin/β-catenin:$SATB1^{n\text{-}high}SMAD3^{low}Ezrin^{low}$β-$catenin^{low}$ ($n = 5$); $SATB1^{n\text{-}low}SMAD3^{high}Ezrin^{high}$β-$catenin^{high}$ ($n = 9$); Others ($n = 54$). (B) Univariate analysis of own cohort: $SATB1^{c}$/SMAD3/Ezrin/β-catenin:$SATB1^{c\text{-}present}SMAD3^{high}Ezrin^{high}$β-$catenin^{high}$ ($n = 6$); $SATB1^{c\text{-}absent}SMAD3^{low}Ezrin^{low}$β-$catenin^{low}$ ($n = 8$); Others ($n = 54$). (C) Univariate analysis of TCGA cohort: SATB1/SMAD3/EZR/CTNNB1:$SATB1^{low}$ $SMAD3^{high}$ $EZR^{high}CTNNB1^{high}$ ($n = 32$); $SATB1^{high}SMAD3^{low}EZR^{low}CTNNB1^{low}$ ($n = 12$); Others ($n = 133$).

Survival analysis of the TCGA cohort showed that *SATB1/SMAD3/EZR/CTNNB1* expression panel was strongly associated with OS (381 days vs. undefined, $p < 0.0001$; Figure 6C). The univariate analysis demonstrated that $SATB1^{low}SMAD3^{high}EZR^{high}CTNNB1^{high}$ expression was significantly associated with a worse survival prognosis (HR 2.97, 95% CI 1.86–4.76, $p < 0.0001$; Table 7C), and it persisted as an independent prognostic factor for poorer OS in the multivariate analysis (HR 3.28, 95% CI 2.02–5.33, $p < 0.0001$; Table 8C).

Table 8. Multivariate analysis of prognostic factors by the Cox proportional hazard model for combined expression.

(A) Multivariate Analysis of Own Cohort: $SATB1^{n}$/SMAD3/Ezrin/β-Catenin				
Variable	**HR**	**95% CI**		***p* Value**
		Lower	**Upper**	
Others		Ref.		
$SATB1^{n\text{-}high}SMAD3^{low}Ezrin^{low}$β-$catenin^{low}$	0.11	0.01	0.93	**0.04**
$SATB1^{n\text{-}low}SMAD3^{high}Ezrin^{high}$β-$catenin^{high}$	2.91	1.07	7.91	**0.04**
age	1.06	1.02	1.11	**0.005**
TNM stage	1.67	0.77	3.62	0.19
LVI	3.81	1.85	7.83	**<0.0001**
(B) Multivariate Analysis of Own Cohort:$SATB1^{c}$/SMAD3 /Ezrin/β-catenin				
Variable	**HR**	**95% CI**		***p* Value**
		Lower	**Upper**	
Others		Ref.		
$SATB1^{c\text{-}present}SMAD3^{high}Ezrin^{high}$β-$catenin^{high}$	7.32	2.05	26.21	**0.002**
$SATB1^{c\text{-}absent}SMAD3^{low}Ezrin^{low}$β-$catenin^{low}$	0.09	0.02	0.46	**0.003**
age	1.07	1.03	1.12	**0.001**
TNM stage	3.08	1.49	6.36	**0.002**
LVI	2.68	1.37	5.25	**0.004**

Table 8. *Cont.*

(C) Multivariate Analysis of TCGA Cohort: SATB1/SMAD3/EZR/CTNNB1				
Variable	**HR**	**95% CI**		***p* Value**
		Lower	**Upper**	
Others		Ref.		
$SATB1^{low}SMAD3^{high}EZR^{high}CTNNB1^{high}$	3.28	2.02	5.33	**<0.0001**
$SATB1^{high}SMAD3^{low}EZR^{low}CTNNB1^{low}$	0.57	0.14	2.35	0.44
age	1.02	1.00	1.05	**0.01**
grade	1.57	0.83	2.98	0.17
pN	2.24	1.27	3.95	**0.01**
pT	1.28	0.61	2.66	0.51

Significant p values ($p < 0.05$) are marked in bold. Abbreviations: CI, confidence interval; HR, hazard ratio; LVI—vascular invasion. HR: adjusting for age, TNM stage, VI. HR from TCGA: adjusting for age, grade, pN, pT. (A) Multivariate analysis of own cohort: $SATB1^{n}$/SMAD3/Ezrin/β-catenin:$SATB1^{n\text{-}high}SMAD3^{low}Ezrin^{low}$β-$catenin^{low}$ (n = 5); $SATB1^{n\text{-}low}SMAD3^{high}Ezrin^{high}$β-$catenin^{high}$ (n = 9); Others (n = 54). (B) Multivariate analysis of own cohort: $SATB1^{c}$/SMAD3 /Ezrin/β-catenin:$SATB1^{c\text{-}present}SMAD3^{high}Ezrin^{high}$β-$catenin^{high}$ (n = 6); $SATB1^{c\text{-}absent}SMAD3^{low}Ezrin^{low}$β-$catenin^{low}$ (n = 8); Others (n = 54). (C) Multivariate analysis of TCGA cohort: SATB1/SMAD3/EZR/CTNNB1: $SATB1^{low}SMAD3^{high}EZR^{high}CTNNB1^{high}$ (n = 32); $SATB1^{high}SMAD3^{low}EZR^{low}CTNNB1^{low}$ (n = 12); Others (n = 133).

3.10. PPI Network Constructionand Functional Enrichment Analysis

To better understand the biological relationship of β-catenin, Ezrin, SATB1 and SMAD3, the PPI network consisting of seed proteins and their interaction partners was constructed using the STRING online database and Cytoscape software (Figure 7A). There were 54 nodes and 538 edges in the PPI network (PPI enrichment p value < 1.0×10^{-16}; local clustering coefficient 0.69). Further details on the network parameters were obtained by the Cytoscape plugin Network Analyzer, and these are presented in Supplementary data (Table S3). The 10 hub genes were then determined based on the degree score of the nodes in the network using the Cytoscape plugin cytoHubba (Figure 7A; colored nodes). Next, the top two cluster subnetworks were identified from the PPI network with the help of the MCODE plugin (cluster 1: 25 nodes, 236 edges, cluster score: 19.67 (Figure 7B); cluster 2:10 nodes, 18 edges, cluster score: 4.0 (Figure 7C)).

Furthermore, the Reactome Pathway, KEGG Pathway, GO and KEGG Brite enrichment analyses were performed to predict signaling pathways, gene ontological features and functional hierarchies of *CTNNB1*, *EZR*, *SATB1*, *SMAD3* and their 50 interaction partners. The Reactome Pathway hierarchy panel is illustrated in Figure 8A. This analysis showed that the queried genes and their neighbors were mainly involved in signaling by WNT/β-catenin, diseases of signal transduction by growth factor receptors and second messengers, transcription and signal transduction (Figure 8B). KEGG pathway analysis demonstrated that the imputed genes were highly associated with adherens junction, endometrial cancer, WNT signaling pathway, Hippo signaling pathway and pathways in cancer (Figure 9A). KEGG BRITE functional hierarchies revealed that there was a preponderance of genes representing transcription factors, chromosome and associated proteins, enzymes and cytoskeleton proteins (Figure 9B). In GO analysis, the most enriched ontology terms were canonical WNT signaling pathway (Figure 9C), cell–cell adherens junction (Figure 9D) and β-catenin binding (Figure 9E).

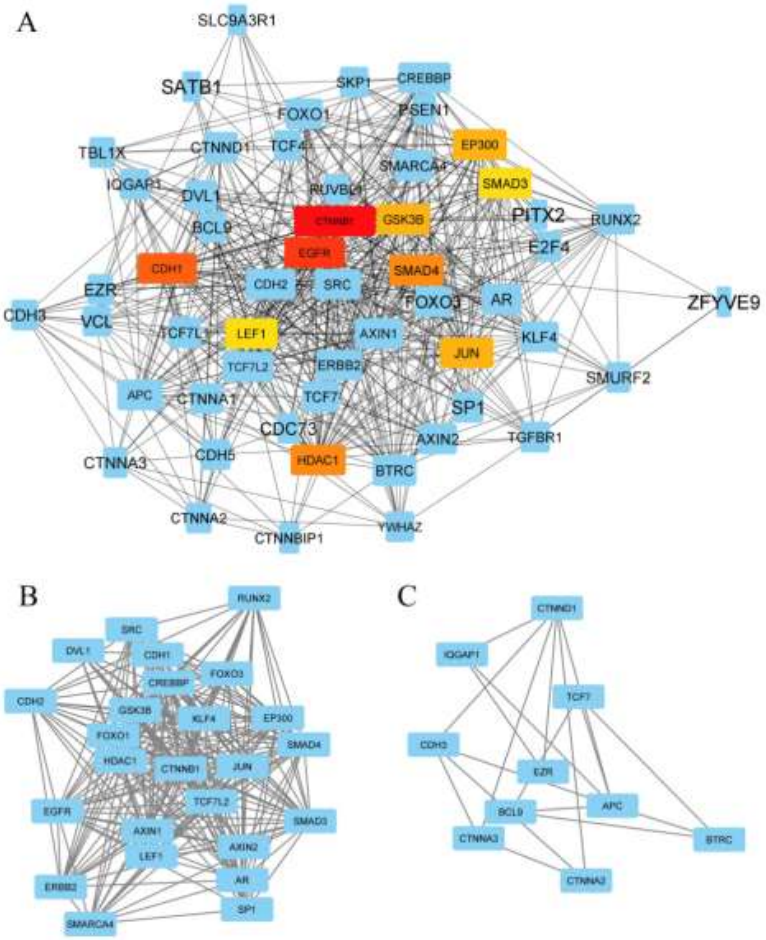

Figure 7. Protein–protein interaction network for β-catenin, Ezrin, SATB1, SMAD3 and their 50 neighbors. PPI network properties, such as node degree and clustering coefficient, are visualized by shape size and label font size, respectively. Top 10 hub genes in the network identified by the CytoHubba Cytoscape plugin are highlighted in a red to yellow gradient. The deeper color, the higher degree of enrichment. Other nodes in the network are highlighted in blue (**A**). The MCODE clustering algorithm was used to identify the clusters in the PPI network. The top two clusters identified by MCODE are displayed (**B**,**C**).

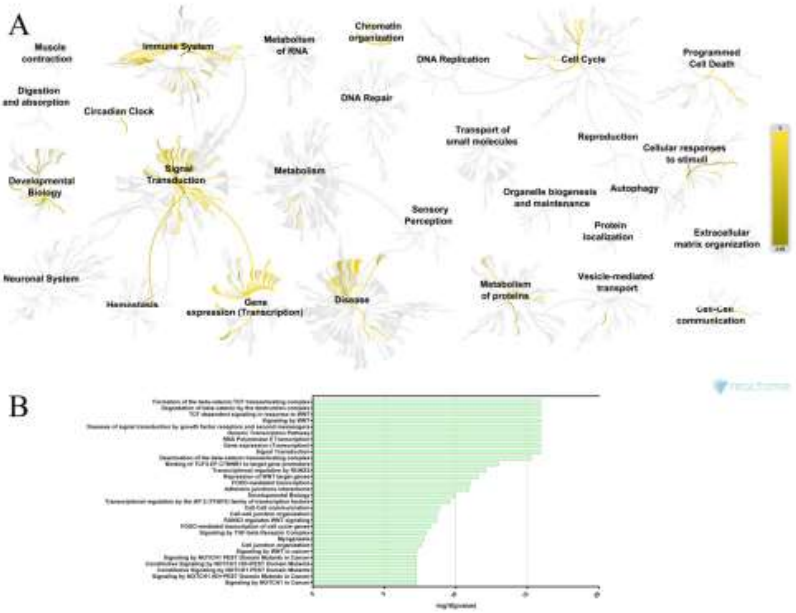

Figure 8. Reactome Pathway functional enrichment analysis for *CTNNB1*, *EZR*, *SATB1*, *SMAD3* and their 50 interaction partners. (**A**) Reactome Pathway hierarchy panel; (**B**) Top 30 Reactome pathways. The *p* value was calculated and sorted with −log10 (*p* value).

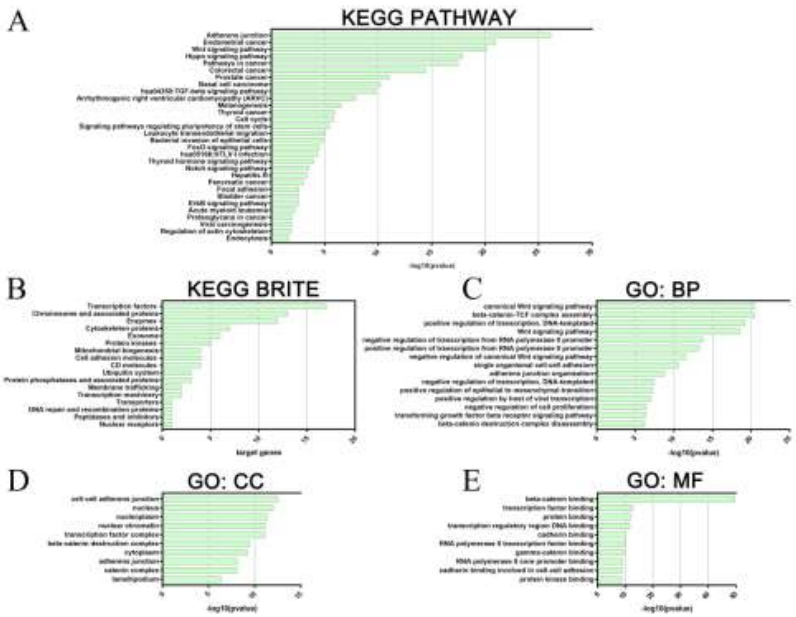

Figure 9. KEGG Pathway, KEGG Brite and GO functional enrichment analysis for *CTNNB1*, *EZR*, *SATB1*, *SMAD3* and their 50 interaction partners. (**A**) Top 15 terms in KEGG pathway analysis;

(**B**) all terms in KEGG Brite analysis; (**C**) Top 15 terms in biological processes (BP) category; (**D**) Top 10 terms incellular components (CC) category; (**E**) Top 10 terms in molecular functions (MF) category. The *p* value was calculated and sorted with −log10 (*p* value).

4. Discussion

In this study, we investigated the associations between the expression status of SATB1, SMAD3, Ezrin, β-catenin and clinicopathological variables, including survival outcome of PDAC patients. Importantly, we evaluated the utility of the examined factors as prognostic markers in pancreatic cancer separately and in combination. To verify the results, we complemented the protein expression data of our cohort with mRNA-seq data of PAC cases obtained from the TCGA. In addition, the protein–protein interaction (PPI) network for β-catenin, Ezrin, SATB1, SMAD3 and their 50 neighbors was constructed and functionally annotated.

In the past few years, several studies have been conducted to evaluate the expression level and role of SATB1 in many human cancers, including PDAC [32–34]. In our investigation, expression of SATB1^{n} was significantly reduced or lost in PDAC specimens as compared to non-cancerous adjacent tissues, while SATB1^{c} was present in the abnormal tissue, but it was not observed in any of the control samples. Guo et al. revealed that SATB1 expression was significantly upregulated in pancreatic cancer specimens compared to in non-cancerous adjacent tissues [32]. However, Guo et al. did not specify what type of SATB1 immunoexpression patterns they assessed, but on the representative micrograph of pancreatic cancer tissue, exclusively cytoplasmic pattern could be seen [32]. Our finding suggests that cells of the pancreatic ducts undergo a significant loss of SATB1^{n} expression during pancreatic carcinogenesis, and this preferentially takes place in well-differentiated and moderately differentiated cancer cells than in the poorly differentiated ones. Notably, loss of SATB1^{n} expression in PDAC specimens was associated with reduced OS (297 days vs. 561 days), but the survival difference was not statistically significant ($p = 0.118$). On the other hand, SATB1^{c} was only present in PDAC (30.88%) but not in the control tissue, leading us to hypothesize that this staining pattern may have a clinical meaning in PDAC. Indeed, patients with SATB1^{c} expression had significantly shorter median OS than patients without cytoplasmic SATB1 staining (458 vs. 117 days). Furthermore, in the multivariate analysis accounting for conventional risk factors, SATB1^{c} expression tended to be an independent prognostic factor for poor overall survival. Our study may be simply underpowered to observe statistically significant effects of SATB1 in our cohort because of the relatively small sample size and the limited number of samples overexpressing SATB1^{n} or those exhibiting cytoplasmic SATB1 staining. Nevertheless, without reaching statistical significance in certain survival analyses, our results do not allow us to infer anything conclusively on the role of SATB1 in PDAC. However, they still raise the possibility that SATB1 may function as a tumor suppressor in at least some PDAC cases, i.e., under specific clinicopathological circumstances. SATB1 functions as a nuclear DNA-binding protein, and herein, it was SATB1's inability to fulfill its nuclear roles due to the loss of nuclear expression and/or cytoplasmic retention that correlated with a poor prognosis of PDAC patients. It is not unexpected, given that SATB1 acts as a global epigenetic and transcriptional regulator of gene expression. Thus, loss of its function may lead to widespread genomic consequences that can contribute to cancer [35]. Furthermore, in our TCGA data, high expression levels of *SATB1* mRNA were found to predict better OS independently of age at diagnosis, tumor grade, as well as pT and pN stage. As an aside, Nakayama et al. reported that cytoplasmic SATB1 localization in T cells could be attributed to a single point mutation at either Lys29 or Arg32, which abrogates its nuclear localization [36]. Whether this stays true in PDAC requires additional studies. Additionally, the fact that cytoplasmic SATB1 labeling was exclusively observed in cancer cells, but not in normal cells, which retained nuclear expression in the majority of cases (95.31%), argues in favor of a potential tumor-suppressive function of SATB1 in PDAC. It is generally known that tumor suppressors possessing

transcriptional functions tend to localize in the nucleus of normal cells but in the cytoplasm of cancer cells [37]. In the context of clinical utility, our study is suggestive of the opposite prognostic meaning for the cytoplasmic and nuclear SATB1 in pancreatic adenocarcinoma. However, this conclusion is hampered by the fact that, in our series, the association between high $SATB1^{n}$ and improved OS did not reach statistical significance. More convincing evidence is therefore required to ascertain whether the clinical impact of SATB1 indeed corresponds to its subcellular distribution in PDAC. From all this, we can more confidently conclude that our research results recommend the individual analysis of cytoplasmic and nuclear SATB1 for a more precise prognostic prediction in pancreatic adenocarcinoma.

As far as we are aware, the present study is the first to distinguish the individual prognostic impact of cytoplasmic and nuclear SATB1 in PDAC. Previous studies, including our own [38], performed similar analyses in colorectal cancer [35,39] and non-small cell lung cancer [38]. We found that in our series of PDACs, the prognostic ability of cytoplasmic SATB1 in OS analysis was even superior to nuclear SATB1. In turn, nuclear SATB1 expression was evaluated by Elebro et al., who demonstrated its association with adverse prognosis in pancreatobiliary-type adenocarcinomas and its ability to predict responses to adjuvant treatment in both intestinal-type and pancreatobiliary-type periampullary adenocarcinomas, including pancreatic cancer [34]. Moreover, Chen et al. have shown that SATB1 expression is associated with pancreatic cancer invasion depth and tumor staging, which confirmed their in vitro results showing that SATB1 promotes pancreatic cancer proliferation and invasion [33]. Nevertheless, the authors, similar to Guo et al., did not specify what type of SATB1 immunoexpression patterns they evaluated, making the results hard to compare [32,33]. Undoubtedly interesting and complex, yet still inconsistent or contradictory picture of SATB1 emerges from pancreatic cancer studies. Therefore, SATB1's role in PDAC merits further examination in additional patient cohorts, as well as in a mechanistic context.

As demonstrated by Lv et al., SATB1 might promote the epithelial to mesenchymal transition by increasing the aberrant expression of β-catenin [40]. SATB1 has also been shown to interact with β-catenin and recruit it into its genomic binding sites, hence mediating Wnt/β-catenin signaling in T-helper type 2 cells. Additionally, in our study, SATB1 expression was found to be correlated with that of β-catenin, both at protein and mRNA levels [41]. Notably, in our investigation, we observed that β-catenin expression in PDAC was membranous and cytoplasmic but without nuclear staining. In accordance with Wang et al., we found no association of cytoplasmic β-catenin expression with PDAC (Figure S2, Tables S4–S6); therefore, our results considered only membrane staining pattern [42]. Consistent with some previous studies, the membranous expression of β-catenin was significantly lower in PDAC than in adjacent normal tissue [43–45]. However, opposite findings have also been made in some other studies. Indeed, up-regulation of β-catenin in PDAC was presented by Wang et al., Zeng et al. and Magliano et al. [42,46,47]. These discrepancies are most probably due to different control tissues and evaluation of different staining patterns. In contrast to our cohort, *CTNNB1* mRNA was significantly up-regulated in PAC tissues of the TCGA cohort compared with normal pancreatic tissues. At mRNA, but not protein level, high expression of *CTNNB1* was associated with features of biological aggressiveness, including high grade, increased T stage and advanced TMN stage. Kaplan–Meier survival analysis of our dataset showed that there was a suggestive association between high β-catenin expression and shorter survival of PDAC patients (274 vs. 450 days; $p = 0.09$). In the TCGA cohort, this association was statistically significant (592 vs. 2182 days; $p = 0.001$). Nevertheless, neither protein nor mRNA expression of β-catenin was an independent prognostic factor when considering confounding factors, including age, tumor stage and LVI. Sano et al. showed that a high IHC score for β-catenin correlated with a poor prognosis, but the researchers evaluated nuclear expression in tissues from PDAC patients and did not perform th multivariate analysis [48]. The opposite relationship regarding β-catenin protein expression and patient survival was presented by Saukkonen et al.,

however, due to the difficulty in evaluating membrane and cytoplasm staining separately, the authors of the cited results assessed only cytoplasmic expression pattern [13].

In our investigation, we also found positive associations between the expression of β-catenin and SMAD3, both in our cohort and TCGA cohort. Furthermore, SMAD3 expression was significantly higher in PDAC specimens as compared to control tissues both at mRNA and protein levels, which is consistent with the report of Yamazaki et al. [49]. Furthermore, our analyses showed that patients with high SMAD3 expression had significantly lower median OS than did patients with SMAD3 underexpression (290 vs. 531 days). Importantly, further analysis using a Cox proportional hazard regression model revealed that SMAD3 expression was an independent prognostic factor predicting poorer survival in PDAC patients. Analysis of *SMAD3* mRNA levels from the TCGA cohort confirmed these results. Similar findings regarding OS have been presented by Yamazaki et al. [49]. Moreover, they demonstrated that the expression of SMAD3 in PDAC correlated with malignant characteristics, including EMT-like features and lymph node metastasis. This is in partial agreement with our study since we observed a significant correlation of *SMAD3* mRNA but not protein expression with the features of aggressive tumor behavior, such as higher grade, positive nodal status, higher pT category and TNM stage. These results confirm that SMAD3 expression may reflect the malignancy potential of PDAC and serve as a biomarker of a poor prognosis.

Spearman's analysis showed strong positive correlations between *SMAD3* and *EZR* in the TCGA cohort. Recently, various studies demonstrated that Ezrin may play an important role in cancer progression, while its overexpression correlates with patient survival and various clinicopathological parameters [50–52]. According to our report, Ezrin expression was elevated in PDAC samples compared to adjacent tissues, which is consistent with the studies by other researchers [50,52,53]. Immunohistochemical analysis of our cohort showed that Ezrin protein expression did not correlate with clinicopathological parameters. However, *EZR* mRNA expression significantly correlated with aggressive phenotypes of PDACs from the TCGA cohort, including differentiation stage, pT status and TNM stage. Previous studies have shown elevated Ezrin protein expression correlated with tumor size, clinical stage and positive lymph node metastasis in PDAC [50]. Our Kaplan–Meier survival analysis demonstrated that high Ezrin expression correlated with a shorter OS rate than a low expression (118 vs. 450 days). Moreover, we revealed that elevated Ezrin expression in our cohort was an independent prognostic factor predicting poorer survival in PDAC patients. The effects of *EZR* expression on the survival of patients from our cohort and TCGA cohort are consistent. Similar findings have also been presented in the studies by other investigators; thus, we confirmed the role of EZR in the prognosis prediction of PDAC [50,51].

Furthermore, given the relationship between SATB1 and β-catenin [40], SATB1 and SMAD3 [54], β-catenin and SMAD3 [55], SMAD3 and Ezrin [56], we next evaluated whether there was any possible added value of combining SATB1, SMAD3, Ezrin and β-catenin to the prognostic value of each of the proteins alone. Kaplan–Meier survival analysis demonstrated that the subset of patients whose tumors co-expressed high levels of $SATB1^{c}$, SMAD3, Ezrin and β-catenin had extremely poor OS, and the combined expression of these markers better predicted patient survival than looking at each marker individually. Moreover, this combined 4-protein panel proved to be a powerful independent prognostic factor associated with worse outcome. In turn, a particularly improved OS was experienced by patients with tumors expressing $SATB1^{n\text{-}high}SMAD3^{low}Ezrin^{low}$ β-$catenin^{low}$, and this expression panel appeared as an independent positive prognostic factor in the multivariate Cox analysis.

Likewise, in the TCGA dataset, a combined 4-gene panel of *SATB1/SMAD3/EZR/CTNNB1* better predicted patient survival than looking at each marker individually, and it emerged as a powerful independent prognostic factor associated with poorer patient outcomes. Thus, our analyses showed that examining the combined expression of SATB1, SMAD3, Ezrin and β-catenin may be more helpful in predicting the prognosis of PAC patients than single

markers. This could be because the predicted biology related to their co-expression included significant interconnectivity with certain master regulators associated with cancer phenotype, as shown by our functional enrichment analysis. Likewise, the biological processes and signaling pathways that emerged from the PPI network are known or are suspected to have a tight cause-and-effect relationship with carcinogenesis. However, the answer to whether these are implicated in the mechanisms linking SATB1/SMAD3/Ezrin/β-catenin co-expression to PDAC will require further study.

As a major limitation, we want to emphasize that the small cohort size may hamper the statistical power of our study. Specifically, when we focused the subsequent analyses on the combined expression of the studied proteins, the sample size in certain subgroups became relatively small, but the number of cases was still within the range accepted in other studies [57,58]. Nevertheless, our findings should be validated with a larger sample size. It is appreciated that a more robust multivariate analysis should include at least 10 events per variable.

5. Conclusions

In conclusion, protein and/or mRNA expression levels of SATB1, SMAD3, Ezrin and β-catenin are significantly altered in PAC and may correlate with some tumor features as well as provide prognostic information as single indicators, and even better when combined.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app12010306/s1, Figure S1. Kaplan–Meier curve for overall survival in patients with pancreatic ductal adenocarcinomas by nuclear/cytoplasmic SATB1 protein expression. *p* value was calculated using the log-rank test. Figure S2. Kaplan–Meier curve for overall survival in patients with pancreatic ductal adenocarcinomas by cytoplasmic β-catenin protein expression. *p* value was calculated using the log-rank test. Table S1. Univariate analysis of prognostic factors by Cox proportional hazard model in own cohort for cytoplasmic β-catenin protein expression. Table S2. Multivariate analysis of prognostic factors by Cox proportional hazard model in own cohort for cytoplasmic β-catenin protein expression. Table S3. Parameters of network determined using the network analyzer Cytoscape plugin. Table S4. Immunohistochemical expression of cytoplasmic β-catenin proteins and their relationship with clinicopathological features of PDAC patients. Table S5. Univariate analysis of prognostic factors by Cox proportional hazard model in own cohort for cytoplasmic β-catenin protein expression. Table S6. Multivariate analysis of prognostic factors by Cox proportional hazard model in own cohort for cytoplasmic β-catenin protein expression.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz (KB 342/2020; 23 June 2020).

Informed Consent Statement: Patient consent was waived due to the retrospective nature of the study. The requirement for informed consent was waived by the Institutional Ethics Committee of Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz.

Data Availability Statement: Publicly available datasets were analyzed in this study. These data can be found here: http://www.cbioportal.org/study/summary?id=paad_tcga_pan_can_atlas_2018 (accessed on 3 July 2020); https://xenabrowser.net (accessed on 3 July 2021). Our own data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions.

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

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7. Podsumowanie

W ciągu ostatnich lat nastąpił znaczny postęp w badaniach nad zidentyfikowaniem nowych markerów nowotworowych, a wyniki tych prac poszerzają wiedzę na temat mechanistycznych aspektów chorób nowotworowych, mając coraz większy wpływ na postępowanie terapeutyczne. W szczególności poszukiwanie biologicznych czynników prognostycznych i predykcyjnych wykorzystywanych w celu określenie stopnia zaawansowania nowotworu i jego potencjalnej złośliwości, a także oceny ryzyka nawrotu choroby i odpowiedzi na planowane leczenie ma niebagatelny wpływ na wybór indywidualnych terapii dla chorych. Szczególnie istotne jest podejmowanie tych działań w odniesieniu do nowotworów o wysokiej zapadalności i śmiertelności, których znaczna heterogenność i skomplikowana patobiologia utrudniają znalezienie swoistych i czułych biomarkerów czy celów dla terapii. Do takich nowotworów należą niewątpliwie NSCLC oraz PAC.

Na przestrzeni ubiegłych lat przeprowadzono wiele badań mających na celu ocenę poziomu ekspresji i roli SATB1 w wielu nowotworach. Szereg doniesień naukowych wskazuje, że SATB1 jest niekorzystnym czynnikiem rokowniczym w wielu nowotworach, w tym raku piersi, jelita grubego, żołądka, jajnika, endometrium i szyjki macicy. Z drugiej strony wiele badań przedstawia odwrotne wyniki nawet w odniesieniu do tych samych typów nowotworów [11, 15-28]. W niektórych podtypach raka płuca podwyższony poziom SATB1 okazał się pozytywnym czynnikiem prognostycznym, a utrata ekspresji SATB1 była związana z krótszym całkowitym przeżyciem pacjentów. Podobny wpływ ekspresji SATB1 na przeżycie pacjentów zaobserwowano analizując próbki raka trzustki. Wobec niewystarczającej liczby oraz braku spójności literatury przedmiotu na temat klinicznej wartości białka SATB1 w NSCLC oraz PAC, w niniejszej rozprawie doktorskiej oceniono zależności pomiędzy tkankową ekspresją SATB1 a wybranymi danymi klinicznymi, histologicznymi oraz biologicznymi w analizowanych grupach badawczych. Interesującym i innowacyjnym aspektem niniejszych badań była ocena subkomórkowej lokalizacji SATB1, która przynajmniej częściowo może wyjaśniać kontrowersje wokół jego wartości prognostycznej, gdyż większość dotychczasowych prac pomijała analizę odczynu cytoplazmatycznego SATB1, co w świetle uzyskanych przez nas wyników wydaje się uzasadnione. Uzyskane wyniki pozwalają przypuszczać, że SATB1 może pełnić funkcję supresorową w niektórych nowotworach. Jak powszechnie wiadomo SATB1 pełni funkcję jądrowego białka wiążącego

DNA, przy czym w niniejszej pracy to właśnie niezdolność SATB1 do pełnienia swoich ról jądrowych z powodu utraty ekspresji jądrowej i/lub retencji cytoplazmatycznej korelowała ze złym rokowaniem pacjentów. Zważywszy, że SATB1 pełni rolę globalnego epigenetycznego i transkrypcyjnego regulatora ekspresji genów, zjawisko to nie jest nieoczekiwane. Utrata jego funkcji może więc prowadzić do rozległych konsekwencji genomowych, które mogą przyczynić się do powstania nowotworu. Ponadto, korelacje pomiędzy SATB1 a funkcjonalnie powiązanymi białkami: TLR2, SMAD3, ezryną oraz β-kateniną potwierdzają znaczenie SATB1 w procesie karcynogenezy. Przedstawione dane wskazują, że SATB1 może być użytecznym biomarkerem prognostycznym w NSCLC oraz PAC przy indywidualnej ocenie jego lokalizacji komórkowej.

Głównym ograniczeniem niniejszej rozprawy doktorskiej jest niewielka liczebność grupy badanej. Niemniej jednak, była ona wystarczająca do wykazania silnych, statystycznie istotnych oddziaływań, z których zdecydowana większość jest zgodna z wynikami uzyskanymi na podstawie danych TCGA oraz danymi opisanymi w literaturze. Warto zaznaczyć, że prezentowane rozważania uwzględniają wyniki uzyskane dla odmiennych populacji pacjentów. Przedstawione obserwacje wymagają weryfikacji w badaniach, wieloośrodkowych, przeprowadzanych na większą skalę.

8. Wnioski

1. Ocena znakowania immunohistochemicznego SATB1 z uwzględnieniem jego subkomórkowej lokalizacji pozwala na stratyfikację pacjentów do grup ryzyka różniących się medianą czasu przeżycia całkowitego w grupie pacjentów z PAC oraz NSCLC w I i II stadium choroby.
2. Ocena ekspresji mRNA *SATB1* pozwala na stratyfikację pacjentów do grup ryzyka różniących się istotnie medianą czasu przeżycia całkowitego w grupie pacjentów z PAC oraz NSCLC w I i II stadium choroby.
3. Ujawniono korelację pomiędzy poziomem SATB1 ocenianym w frakcji jądrowej, a TLR2 oraz SATB1 ocenianym w lokalizacji cytoplazmatycznej, a także SATB1 ocenianym w lokalizacji cytoplazmatycznej, a TLR2 w materiale tkankowym pacjentów z NSCLC w I i II stadium choroby. W grupie pacjentów z PAC wykazano zależności pomiędzy poziomem β-kateniny, a SATB1 ocenianym w frakcji jądrowej oraz SATB1 ocenianym w lokalizacji cytoplazmatycznej.
4. Stratyfikacja chorych w odniesieniu do łącznej ekspresji SATB1 (zlokalizowanego na terenie jądra komórkowego), SMAD3 oraz TLR2 pozwala zidentyfikować podgrupy pacjentów z NSCLC w I i II stadium z istotną różnicą w czasie przeżycia. Skonstruowany panel pozwala lepiej przewidywać przeżycie w porównaniu do rozpatrywania każdego z tych markerów osobno.
5. Poziomy połączonej ekspresji SATB1, SMAD3, ezryny i β-kateniny mogą służyć jako biomarkery prognostyczne w grupie pacjentów z PAC analizując dane dotyczące białka oraz mRNA.
6. Przewidywana strategia biologiczna związana z koekspresją *SATB1*, *SMAD3*, *EZR* oraz *CTNNB1* obejmuje znaczące wzajemne powiązania z klasycznymi regulatorami związanymi z procesem nowotworzenia.

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10. Oświadczenia autorów publikacji włączonych do cyklu

lek. Adam Kowalewski
Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

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oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

- udział w zbieraniu danych pacjentów
- akceptacja manuskryptu i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

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

Bydgoszcz, 28.12.2022

Jako współautor pracy:

1) Prognostic significance of SATB1, SMAD3, Ezrin and β-catenin in patients with pancreatic adenocarcinoma; Durślewicz Justyna, Klimaszewska-Wiśniewska Anna, Domanowska Ewa, Skoczylas-Makowska Natalia, Antosik Paulina, Zielińska Wioletta, Gzil Arkadiusz, Czajkowska Paulina, Mikołajczyk Klaudia, Grzanka Dariusz; Appl. Sci.-Basel 2022 : Vol. 12, nr 1, s. 1-26, 306.

oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

- przygotowanie danych do analizy in silico
- akceptacja manuskryptu i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Arkadiusz Gzil

dr Anna Klimaszewska-Wiśniewska
adiunkt
Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Bydgoszcz, 28.12.2022

Jako współautor prac:

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2) Prognostic significance of SATB1, SMAD3, Ezrin and β-catenin in patients with pancreatic adenocarcinoma; Durślewicz Justyna, Klimaszewska-Wiśniewska Anna, Domanowska Ewa, Skoczylas-Makowska Natalia, Antosik Paulina, Zielińska Wioletta, Gzil Arkadiusz, Czajkowska Paulina, Mikołajczyk Klaudia, Grzanka Dariusz; Appl. Sci.-Basel 2022 : Vol. 12, nr 1, s. 1-26, 306.

oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

- udział w interpretacji wyników
- udział w przygotowaniu manuskryptu
- akceptacja manuskryptów i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

dr Ewa Domanowska
asystent
Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Bydgoszcz, 28.12.2022

Jako współautor pracy:

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oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

- ocena barwienia immunohistochemicznego
- akceptacja manuskryptu i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Ewa Domanowska

dr hab. Maciej Gagat
profesor uniwersytetu
Katedra Histologii i Embriologii
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Bydgoszcz, 28.12.2022

Jako współautor pracy:

1) Prognostic significance of TLR2, SMAD3 and localization-dependent SATB1 in stage I and II non-small-cell lung cancer patients; Durślewicz Justyna, Klimaszewska-Wiśniewska Anna, Jóźwicki Jakub, Antosik Paulina, Smolińska-Świtała Marta, Gagat Maciej, Kowalewski Adam, Grzanka Dariusz; Cancer Control 2021 : Vol. 28, s. 1-14.

oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

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- akceptacja manuskryptu i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

dr Jakub Jóźwicki
asystent
Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Bydgoszcz, 28.12.2022

Jako współautor pracy:

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oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

- ocena barwienia immunohistochemicznego, analiza i interpretacja danych dotyczących barwienia
- akceptacja manuskryptu i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Jóźwicki Jakub

dr Marta Smolińska-Świtała
Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Bydgoszcz, 28.12.2022

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oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

- przygotowane materiału badawczego do barwienia
- akceptacja manuskryptu i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Marta Smolińska-Świtała

dr Natalia Skoczylas-Makowska
asystent
Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Bydgoszcz, 28.12.2022

Jako współautor pracy:

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oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

- ocena barwienia immunohistochemicznego
- akceptacja manuskryptu i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Natalia Skoczylas-Makowska

dr Paulina Antosik
adiunkt
Katedra Patomorfologii Klinicznej
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

Bydgoszcz, 28.12.2022

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oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

- udział w optymalizacji metodologii
- akceptacja manuskryptów i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Paulina Antosik

mgr Klaudia Mikołajczyk
specjalista inżynieryjno-techniczny
Katedra Histologii i Embriologii
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

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oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

- udział w analizie statystycznej wyników
- akceptacja manuskryptu i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Mikołajczyk Klaudia

mgr Paulina Czajkowska
specjalista inżynieryjno-techniczny
Katedra Histologii i Embriologii
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

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Paulina Czajkowska

mgr Wioletta Zielińska
asystent
Katedra Histologii i Embriologii
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu

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oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

- udział w opisie wyników
- akceptacja manuskryptu i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

Zielińska Wioletta

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

Bydgoszcz, 28.12.2022

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2) Prognostic significance of SATB1, SMAD3, Ezrin and β-catenin in patients with pancreatic adenocarcinoma; Durślewicz Justyna, Klimaszewska-Wiśniewska Anna, Domanowska Ewa, Skoczylas-Makowska Natalia, Antosik Paulina, Zielińska Wioletta, Gzil Arkadiusz, Czajkowska Paulina, Mikołajczyk Klaudia, Grzanka Dariusz; Appl. Sci.-Basel 2022 : Vol. 12, nr 1, s. 1-26, 306.

oświadczam że mój własny wkład merytoryczny w powstawanie niniejszej publikacji to:

- udział w zaprojektowaniu i zaplanowaniu badań
- interpretacja wyników
- akceptacja manuskryptów i wniesienie uwag

Jednocześnie wyrażam zgodę na przedłożenie wskazanej pracy przez mgr Justynę Durślewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w recenzowanych czasopismach naukowych.

11. Zgoda Komisji Bioetycznej

Uniwersytet Mikołaja Kopernika w Toruniu

Collegium Medicum im L. Rydygiera w Bydgoszczy

KOMISJA BIOETYCZNA

Ul. M. Skłodowskiej-Curie 9, 85-094 Bydgoszcz, tel.(052) 585-35-63, fax.(052) 585-38-11

KB 336/2018

Bydgoszcz, 24.04.2018 r.

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

Komisja Bioetyczna przy UMK w Toruniu, Collegium Medicum w Bydgoszczy

(skład podano w załączeniu), na posiedzeniu w dniu **24.04.2018 r.** przeanalizowała wniosek, który złożył kierownik badania:

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

z zespołem w składzie:

- dr n. med. Maciej Gagat, dr n. med. Łukasz Szylberg, dr n. med. Anna Klimaszewska-Wiśniewska, dr n. med. Tomasz Szczęsny, lek. Izabela Neska-Długosz, lek. Jakub Jóźwicki, mgr Paulina Antosik, Adam Kowalewski,

w sprawie badania:

„Ocena klinicznego znaczenia wybranych białek o potencjalnej roli w patogenezie niedrobnokomórkowego raka płuca w grupie pacjentów onkologicznych leczonych operacyjnie."

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

Uchwałę o pozytywnym zaopiniowaniu wniosku

w sprawie przeprowadzenia badań w zakresie określonym we wniosku pod warunkiem zachowania tajemnicy wszystkich danych, w tym danych osobowych pacjenta umożliwiających ich identyfikację w ewentualnych publikacjach. Zgoda obejmuje tylko materiał biologiczny pobrany w latach 2010-2017r. od pacjentów, którzy nie wyrazili stosownego sprzeciwu w Centralnym Rejestrze Sprzeciwów.

Zgoda obowiązuje od daty posiedzenia (22.05.2018 r.) do końca 2021 r.

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

Prof. dr hab. med. Karol Śliwka

Przewodniczący Komisji Bioetycznej

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

Lista obecności

na posiedzeniu Komisji Bioetycznej

w dniu 22.05.2018 r.

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

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

KB 336/2018

Bydgoszcz, 25.06.2019 r.

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

Komisja Bioetyczna przy UMK w Toruniu, Collegium Medicum w Bydgoszczy

(której skład podano w załączeniu) na posiedzeniu w dniu **25.06.2019 r.** przeanalizowała prośbę o wyrażenie zgody na:

- poszerzenie zespołu badawczego o mgr Justynę Durślewicz,

którą złożył:

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

w sprawie badania:

„Ocena klinicznego znaczenia wybranych białek o potencjalnej roli w patogenezie niedrobnokomorkowego raka płuca w grupie pacjentów onkologicznych leczonych operacyjnie."

Po zapoznaniu się ze złożonym dokumentem i w wyniku przeprowadzonej dyskusji oraz głosowania jawnego Komisja przyjęła do wiadomości podane informacje i wyraża zgodę na powyższe pod warunkami określonymi w uchwale Komisji podjętej w dniu 22.05.2018 r.

Zgoda na kontynuowanie przedmiotowego badania obowiązuje do końca 2021 roku.

Prof. dr hab. med. Karol Śliwka

Przewodniczący Komisji Bioetycznej

Otrzymuje:

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

Lista obecności

na posiedzeniu Komisji Bioetycznej

w dniu 25.06.2019 r.

Lp.	Imię i nazwisko	Funkcja/ Specjalizacja	Podpis
1.	Prof. dr hab. med. Karol Śliwka	Przewodniczący *medycyna sądowa*	
2.	Mgr prawa Joanna Połetek-Żygas	Z – ca przewodniczącego *prawniczka*	
3.	Prof. dr hab. med. Mieczysława Czerwionka-Szaflarska	*pediatra, alergologia i gastroenterologia dziecięca*	
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5.	Prof. dr hab. med. Marek Grabiec	*położnictwo, ginekologia onkologiczna*	
6.	Prof. dr hab. med. Zbigniew Włodarczyk	*chirurgia ogólna, transplantologia kliniczna*	
7.	Dr hab. n. med. Katarzyna Pawlak-Osińska, prof. UMK	*organizacja ochrony zdrowia, otolaryngologia*	
8.	Dr hab. n med. Maria Kłopocka	*choroby wewnętrzne, gastroenterologia*	
9.	Ks. dr hab. Wojciech Szukalski, prof. UAM	*duchowny*	
10.	Dr n. med. Radosława Staszak-Kowalska	*pediatria, choroby płuc*	
11.	Mgr prawa Patrycja Brzezicka	*prawniczka*	
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13.	Mgr Lidia Iwińska-Tarczykowska	*pielęgniarska*	

Uniwersytet Mikołaja Kopernika w Toruniu

Collegium Medicum im L. Rydygiera w Bydgoszczy

KOMISJA BIOETYCZNA

Ul. M. Skłodowskiej-Curie 9, 85-094 Bydgoszcz, tel.(052) 585-35-63, fax.(052) 585-38-11

KB 342/2020

Bydgoszcz, 23.06.2020 r.

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

Komisja Bioetyczna przy UMK w Toruniu, Collegium Medicum w Bydgoszczy

(skład podano w załączeniu), na posiedzeniu w dniu **23.06.2020 r.** przeanalizowała wniosek, który złożył kierownik badania:

dr hab. n. med. Dariusz Grzanka, prof. UMK
Katedra Patomorfologii Klinicznej
Collegium Medicum w Bydgoszczy

z zespołem w składzie

- dr hab. n. med. Maciej Słupski, dr hab. Łukasz Szylberg, prof. UMK, dr n. med. Anna Klimaszewska-Wiśniewska, dr n. med. Ewa Domanowska, dr n. med. Natalia Skoczylas-Makowska, lek. Izabela Neska-Długosz, lek. Anna Kasperska, mgr Paulina Antosik, mgr Justyna Durślewicz, mgr Joanna Ligmanowska, mgr Joanna Gerc, Izabela Zarębska, Karolina Buchholtz, Arkadiusz Gzil, Damian Jaworski,

w sprawie badania:

„Ocena klinicznego znaczenia wybranych białek o potencjalnej roli w patogenezie nowotworu trzustki w grupie pacjentów onkologicznych leczonych operacyjnie."

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

Uchwałę o pozytywnym zaopiniowaniu wniosku

w sprawie przeprowadzenia badań w zakresie określonym we wniosku pod warunkiem uzyskania zgody osób badanych na wykorzystanie materiału biologicznego do celów naukowych, a w przypadku braku takiej zgody, analizowania jedynie materiału zanonimizowanego tj. pozbawionego danych personalnych (zgodnie z RODO).

Zgoda obowiązuje od daty podjęcia uchwały (23.06.2020 r.) do końca 2025 r.

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

Prof. dr hab. med. Karol Śliwka

Przewodniczący Komisji Bioetycznej

Otrzymuje:
dr hab. n. med. Dariusz Grzanka, prof. UMK
Katedra Patomorfologii Klinicznej
Collegium Medicum w Bydgoszczy

Lista obecności

na posiedzeniu Komisji Bioetycznej

w dniu 23.06.2020 r.

Lp.	Imię i nazwisko	Funkcja/ Specjalizacja	Podpis
1.	Prof. dr hab. med. Karol Śliwka	Przewodniczący *medycyna sądowa*	
2.	Mgr prawa Joanna Połetek-Żygas	Z – ca przewodniczącego *prawniczka*	
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12. Streszczenie

Wobec niewystarczającej liczby oraz braku spójności doniesień na temat klinicznej wartości białka SATB1 w NSCLC oraz PAC, celem niniejszej rozprawy doktorskiej była ocena zależności pomiędzy tkankową manifestacją SATB1, a wybranymi danymi klinicznymi, histologicznymi oraz biologicznymi (TLR2, SMAD3, ezryna oraz β-katenina) analizowanych grup pacjentów. Istotnym założeniem badań było określenie niezależnej wartości rokowniczej ekspresji SATB1 z uwzględnieniem jego subkomórkowej lokalizacji ocenionej immunohistochemicznie w kohorcie własnej oraz publicznie dostępnych danych transkryptomicznych zgromadzonych w ramach projektu TCGA. Biorąc również pod uwagę, że jako globalny czynnik transkrypcyjny SATB1 nie jest izolowanym 'graczem' wpływającym na rokowanie pacjentów, lecz częścią wzajemnie przenikających się ścieżek sygnalizacyjnych regulujących równowagę dynamiczną fundamentalnych procesów komórkowych, postanowiono ocenić korelacje biologiczne pomiędzy ekspresją SATB1 a funkcjonalnie powiązanymi białkami: TLR2, SMAD3, ezryną oraz β-kateniną, jak również ich indywidualny i łączny wpływ na czas przeżycia całkowitego pacjentów.

Wysoki poziom SATB1 oceniony w frakcji jądrowej był niezależnym korzystnym czynnikiem prognostycznym u chorych z NSCLC, podczas gdy wysoki poziom SATB1 zlokalizowany na terenie cytoplazmy komórki okazał się niezależnym predyktorem niekorzystnego rokowania. Bazując na zbiorze danych TCGA wykazano, że nadekspresja mRNA *SATB1* była istotnie związana z dłuższym czasem przeżycia pacjentów. Ponadto, stratyfikacja chorych z NSCLC na podstawie kombinacji poziomów ekspozycji SATB1, SMAD3 oraz TLR2 umożliwiła wyróżnienie podgrup pacjentów z największą różnicą w czasie przeżycia. Przeciwstawne znaczenie prognostyczne SATB1 w zależności od lokalizacji barwienia wykazano również w grupie pacjentów z PAC. Obecność odczynu immunohistochemicznego w frakcji cytoplazmatycznej była niezależnym niekorzystnym czynnikiem prognostycznym w grupie pacjentów z PAC. Analiza związana z manifestacją SATB1 na terenie jądra nie osiągnęła istotności statystycznej, jednak krzywe Kaplana-Meiera wyodrębniły chorych z niską ekspresją i niekorzystnym rokowaniem od chorych z wysoką ekspresją i korzystnym rokowaniem. Wysoka ekspresja mRNA *SATB1* okazała się niezależnym wskaźnikiem prognostycznym dla korzystnego czasu przeżycia pacjentów. Ponadto, kombinacja ekspozycji SATB1, SMAD3, ezryny oraz β-kateniny była związana z rokowaniem pacjentów niezależnie od konwencjonalnych czynników ryzyka, zarówno w kohorcie własnej,

jak i w zbiorze danych TCGA w grupie PAC. Dodatkowo przewidywana strategia biologiczna związana z koekspresją *SATB1*, *SMAD3*, *EZR* oraz *CTNNB1* obejmowała znaczące wzajemne powiązania z klasycznymi regulatorami związanymi z procesem nowotworzenia.

Z punktu widzenia użyteczności klinicznej, niniejsza rozprawa doktorska implikuje przeciwne znaczenie prognostyczne w zależności od subkomórkowej dystrybucji SATB1 w PAC oraz NSCLC w I i II stadium choroby. Przedstawione wyniki badań wskazują na konieczność indywidualnej analizy: cytoplazmatycznej i jądrowej SATB1, w celu dokładniejszego przewidywania rokowania pacjentów z PAC oraz NSCLC w I i II stadium choroby. Przeprowadzone w toku niniejszej pracy doktorskiej analizy pozwalają rozpatrywać SATB1 oraz powiązane z nim funkcjonalnie białka jako potencjalne cele terapeutyczne w PAC oraz NSCLC w I i II stadium choroby.

13. Summary

Considering the lack and inconsistency of reports on the clinical value of SATB1 protein in NSCLC and PAC, this dissertation aimed to evaluate the relationship between tissue manifestation of SATB1 and selected clinical, histological, and biological data (TLR2, SMAD3, ezrin, and β-catenin) of the analyzed patient groups. An essential premise of the study was to determine the independent prognostic value of SATB1 expression, taking into account its subcellular localization assessed immunohistochemically in our cohort and publicly available transcriptomic data collected by TCGA project. Considering also that as a global transcription factor, SATB1 is not an isolated 'player' affecting patient prognosis but part of intertwined signaling pathways regulating the dynamic balance of fundamental cellular processes, we decided to assess the biological correlations between SATB1 expression and functionally related proteins: TLR2, SMAD3, ezrin, and β-catenin, as well as their individual and combined effects on patients' overall survival time.

High levels of SATB1 assessed in the nuclear fraction were an independent favorable prognostic factor in patients with NSCLC, while high levels of SATB1 localized to the cytoplasm of the cell proved to be an independent predictor of adverse prognosis. Based on the TCGA dataset, it was shown that *SATB1* mRNA overexpression was significantly associated with more prolonged patient survival. In addition, the stratification of patients with NSCLC based on combinations of SATB1, SMAD3, and TLR2 exposure levels made it possible to distinguish subgroups of patients with the most significant difference in survival time. The opposing prognostic significance of SATB1 depending on the location of staining was also demonstrated in the group of patients with PAC. The presence of an immunohistochemical reaction in the cytoplasmic fraction was an independent adverse prognostic factor in the group of patients with PAC. Analysis related to SATB1 manifestation in the testicular area did not reach statistical significance, but Kaplan-Meier curves separated patients with low expression and unfavorable prognosis from those with high expression and favorable prognosis. High SATB1 mRNA expression proved an independent prognostic indicator for favorable patient survival time. Moreover, the combination of SATB1, SMAD3, Ezrin, and β-catenin expression was associated with patient prognosis independent of conventional risk factors in both our own cohort and the TCGA dataset in the PAC group. In addition, the predicted biological strategy associated with

the coexpression of SATB1, SMAD3, EZR, and CTNNB1 included significant cross-talk with classical tumor-associated regulators.

Regarding clinical utility, this dissertation implies opposite prognostic significance depending on the subcellular distribution of SATB1 in PAC and NSCLC in stage I and II disease. The results presented here indicate the need for individual analysis: of cytoplasmic and nuclear SATB1 to more accurately predict the prognosis of patients with PAC and NSCLC in stage I and II diseases. The analyses carried out in this dissertation make it possible to consider SATB1 and its functionally related proteins as potential therapeutic targets in PAC and stage I and II NSCLC.